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Interaction of vascular smooth muscle cells and monocytes by soluble factors synergistically enhances IL-6 and MCP-1 production

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Chen L, Frister A, Wang S, Ludwig A, Behr H, Pippig S, Li B, Simm A, Hofmann B, Pilowski C, Koch S, Buerke M, Rose-John S, Werdan K, Loppnow H. Interaction of vascular smooth muscle cells and monocytes by soluble factors synergistically enhances IL-6 and MCP-1 production. *Am J Physiol Heart Circ Physiol* 296: H987–H996, 2009. First published January 23, 2009; doi:10.1152/ajpheart.01158.2008.—Inflammatory mechanisms contribute to atherogenesis. Monocyte chemoattractant protein (MCP)-1 and IL-6 are potent mediators of inflammation. Both contribute to early atherogenesis by luring monocytes and regulating cell functions in the vessel wall. MCP-1 and IL-6 production resulting from the interaction of invading monocytes with local vessel wall cells may accelerate atherosclerosis. We investigated the influence of the interaction of human vascular smooth muscle cells (SMCs) with human mononuclear cells (MNCs) or monocytes on IL-6 and MCP-1 production in a coculture model. Interaction synergistically enhanced IL-6 and MCP-1 production (up to 30- and 10-fold, respectively) compared with separately cultured cells. This enhancement was mediated by CD14-positive monocytes. It was dependent on the SMC-to-MNC/monocyte ratio, and as few as 0.2 monocytes/SMC induced the synergism. Synergistic IL-6 production was observed at the protein, mRNA, and functional level. It was mediated by soluble factors, and simultaneous inhibition of IL-1, TNF-α, and IL-6 completely blocked the synergism. IL-1, TNF-α, and IL-6 were present in the cultures. Blockade of the synergism by soluble glycoprotein 130Fc/soluble IL-6 receptor, as well as the induction of synergistic IL-6 production by costimulation of SMCs with IL-1, TNF-α, and hyper-IL-6, suggested the involvement of IL-6 *trans*-signaling. The contribution of IL-6 was consistent with enhanced STAT3 phosphorylation. The present data suggest that SMC/monocyte interactions may augment the proinflammatory status in the tissue, contributing to the acceleration of early atherogenesis.

inflammation; monocytes/macrophages; chemokines; cytokines; lipopolysaccharide; interleukin-6; monocyte chemoattractant protein-1

ATHEROSCLEROSIS is among the gravest causes of mortality in the western world. A central role of inflammatory pathways in the initiation and progression of atherosclerosis has been suggested (9, 25, 36). Cytokines are vital regulators of inflammatory pathways relevant for atherosclerosis (23, 43). Cardiovascular cells, including endothelial cells, smooth muscle cells (SMCs), or heart muscle cells, have the capacity to produce cytokines, such as IL-1, TNF-α, monocyte chemoattractant protein (MCP)-1, or IL-6 (13, 21, 22, 30, 42, 47). In the cardiovascular system, these mediators can influence a number of functions important for inflammation and/or vascular dysfunction, such as proliferation, contraction, activation of adhesion molecules, or production of further mediators, including chemokines.

Evidence for a contribution of inflammatory cytokines to atherogenesis has been derived from investigations determining plasma and tissue levels in patient samples, in vitro experiments, and establishment of genetically modified animals (16, 23, 43). Chemokines, potently contributing as lymphocyte and monocyte attractants in inflammatory processes (24), are a group of cytokines potently involved in the development of atherosclerosis. Among them, MCP-1 is of great interest, since it is a major monocyte chemokine. MCP-1 is expressed in atherosclerotic tissues by SMCs in the media and intimal lesion (50). It has also been shown to exert beneficial effects in the prevention of apoptosis of heart cells (27). Double deficiency for this chemokine and the LDL receptor (8), or the MCP-1 receptor and apolipoprotein E (apoE), resulted in decreased lesion areas (6).

A number of investigations have provided evidence for a role of IL-6 in atherosclerosis. However, the role of IL-6 appears to be ambiguous, a point we have discussed in more detail in a recent summary (23). Thus, atheroprotective (26, 37) as well as atherogenic roles of IL-6 (12) and a two-sided and/or biphasic effect have been suggested (7). In the following, we mention just a few reports in more detail. Schieffer et al. (37) showed reduced matrix metalloproteinase-9 expression, reduced monocyte recruitment, and increased lesion size in apoE$^{-/-}$/IL-6$^{-/-}$ mice fed on a standard diet (37). Also, female ovariectomized apoE$^{-/-}$/IL-6$^{-/-}$ mice, fed a normal diet for 1 yr, developed larger lesions than IL-6$^{+/+}$ mice (7). Furthermore, Madan et al. (26) described that apoE$^{+/-}$/IL-6$^{-/-}$ mice had larger lesions and increased macrophage infiltration. However, IL-6 may also play a perilous role in atherogenesis by increasing oxidized LDL degradation and uptake (15) or impairing chemotaxis (10). IL-6 is produced by various cell types, including endothelial cells or SMCs (19, 22, 30, 45). In atherogenesis, it may regulate the functions of invading leukocytes and local cells, with the latter possibly by autocrine mechanisms involving an enhancement of glycoprotein (gp)130 expression (17). Very relevant for atherosclerosis,

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IL-6 induces monocyte differentiation into macrophages (5), it can activate mononuclear cells or SMCs to produce MCP-1 (3, 48), and it has been shown that IL-6 induces the proliferation and migration of SMCs (46).

In the vessel wall, the interaction of invading cells, such as monocytes or T cells, with local cells may take place by means of the cytokines summarized above. Thus, we investigated the role of SMC and monocyte interaction on IL-6 and MCP-1 production. We show that the interaction of SMCs and monocytes in coculture synergistically enhanced LPS-stimulated IL-6 and MCP-1 production. The soluble mediators IL-1, TNF-α, and IL-6 accounted for this tremendous activation. Inhibition experiments suggested a role of endogenous IL-6 mediated by *trans*-signaling. The synergism was dependent on the SMC-to-monocyte ratio and was activated already at a ratio of 0.2 monocytes to 1 SMC. CD14-positive but not CD3-positive cells were essential for the induction of the synergism. These results indicate a role of endogenous-cytokine-mediated activation of invading monocytes and local SMCs in atherogenesis.

METHODS

Reagents. LPS of *Salmonella enterica* serovar Friedenau was generously provided by Prof. Dr. H. Brade (Forschungszentrum Borstel, Borstel, Germany). Recombinant soluble gp130Fc protein was prepared as previously described (39). Recombinant human IL-1 receptor antagonist (IL-1ra), IL-1α, IL-1β, IL-6, and rabbit anti-human TNF-α antibody were from PeproTech (London, UK). AG-490 (cat. no. 658401), SB-203580 (cat. no. 559398), and NF-kB inhibitor (cat. no. 481407) were purchased from Calbiochem (Merck Biosciences, Schwalbach, Germany). Cell culture media and substituents were from Biochrom (Berlin, Germany). IL-6 and MCP-1 ELISAs were from BD Biosciences (Heidelberg, Germany). Human CD14-FITC was from Chemicon Europe (Hofheim, Germany). Mouse anti-human STAT3 and mouse anti-human phospho-STAT3 (Tyr^{705}) antibodies were from Cell Signaling Technology (New England Biolabs, Frankfurt a.M., Germany). The ECL detection system was from Pierce (Thermo Fisher Scientific, Bonn, Germany). The Omniscript reverse transcription kit and Hot Star Taq PCR kit were from Qiagen (Hilden, Germany).

Isolation and coculture of SMCs and mononuclear cells. Human vascular SMCs were isolated from unused portions of saphenous veins obtained after bypass surgery as previously described (22). The use of the otherwise discarded specimens was approved by the local ethics committee. DMEM (1 g/l D-glucose) containing 10% FCS, 1% antibiotics, and 1% L-glutamine (DMEM-medium) was used for cell culture. Upon confluency, cells were subcultured after trypsin-EDTA treatment (0.05%-0.02%, 10 min, 37°C, Biochrom). We used SMCs of *passages 4–7*. Mononuclear cells (MNCs) were isolated from heparinized whole blood by Biocoll (Biochrom) gradient centrifugation (40 min, 500 *g*). Cells were washed three times (200 *g*, 15 min, 21°C) in DMEM-medium. MNCs were added to the dishes at defined cell concentrations in DMEM-medium and incubated for 24 h either alone or in coculture with SMCs (37°C, 7.5% CO_2). Monocytes ($CD14^+$) or lymphocytes ($CD3^+$) were isolated from MNCs using magnetic beads (Milteny Biotec, Bergisch Gladbach, Germany) according to the instructions of the manufacturer. For the isolation of these subpopulations, three aliquots of a MNC preparation were used. One aliquot was directly applied (MNC). From the second aliquot, $CD14^+$ monocytes were isolated, and, from the third aliquot, $CD3^+$ cells were isolated and used for the coculture. Run-throughs of the CD14 and CD3 isolation columns, i.e., $CD14^-$ and $CD3^-$ cells, respectively, were also tested. The purity of monocytes was ≥97% as determined by CD14 staining in FACS analysis.

For the coculture, SMCs were plated at a density of 20,000 cells/cm^2. After 24 h, the medium was replaced with fresh DMEM-medium. Subsequently, MNCs or monocytes were added at the respective ratios in the absence or presence of the stimulus for further 24 h. If insert cocultures were performed, SMCs were cultured in the wells under standard conditions (20,000 SMCs/cm^2) overnight. MNCs were cultured in the inserts. A standard coculture was always performed in parallel. On the other hand, fixation experiments were performed. In these experiments, isolated MNCs were first cultured in 25-cm^2 cell culture flasks (20,000 MNCs/cm^2) with or without the addition of LPS (100 ng/ml) for 4 h. After this incubation, the cells were collected after cultures had been treated with a cell scraper. Subsequently, cells were washed and either treated with paraformaldehyde (1% in PBS, 30 min) or immediately incubated with SMCs. After 24 h, supernatants were harvested, and cytokines were determined with the respective ELISA following the instructions of the manufacturer.

RT-PCR of IL-6 mRNA. Total mRNA was isolated using the RNeasy Mini kit (Qiagen), including DNAse treatment (RNase-free DNase I, Qiagen, room temperature, 15 min). RNA of SMCs, MNCs, or coculture cells was adjusted to 500 ng/reaction and reverse transcribed (Omniscript reverse transcription kit, Qiagen) in a volume of 20 μl and subsequently diluted in 100 μl diethylpyrocarbonate (DEPC)-treated water. PCR was performed in a volume of 100 μl consisting of 20 μl cDNA, 10 μl PCR buffer (10×), 2 μl dNTP mix (10 mM), 2 μl sense primer, and 2 μl antisense primer (100 μM each, MWG-Biotech, Ebersberg, Germany), 0.5 μl Hot Star Taq Plus DNA polymerase, and 65.5 μl DEPC-treated water. The conditions for the IL-6 PCR were as follows: initial denaturation for 5 min at 95°C, 24 cycles of denaturation for 40 s at 95°C, annealing for 1 min at 60°C, extension for 3 min at 72°C, and final extension for 5 min at 72°C. For the 18S PCR, the conditions were as follows: initial denaturation for 5 min at 95°C, 20 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, extension for 30 s at 72°C, and final extension for 5 min at 72°C. The primers used for human IL-6 were sense 5′-

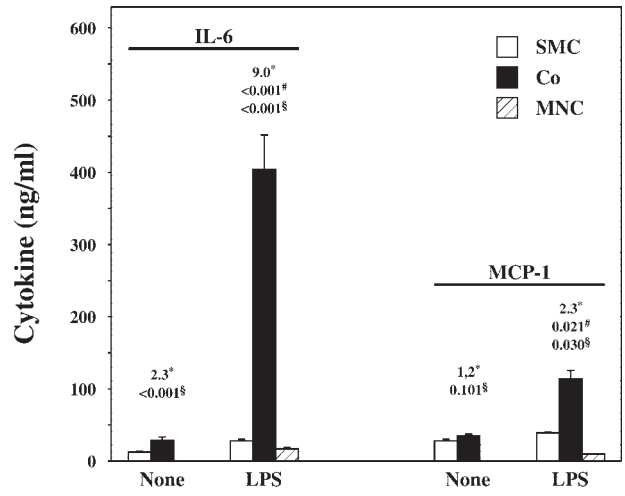

Fig. 1. Cocultured mononuclear cells (MNCs) and smooth muscle cells (SMCs) produce IL-6 and monocyte chemoattractant protein (MCP)-1 synergistically. Human SMCs were cultured in 48-well plates (20,000 cells/cm^2). MNCs were added after 24 h at the ratio of 1 MNC to 1 SMC in medium with or without endotoxin (LPS, 100 ng/ml; none). MNCs and SMCs were either cultured separately or in coculture (Co). Supernatants were harvested after 24 h, and IL-6 or MCP-1 were measured by ELISA. The numbers and symbols above the columns reflect the following: *synergism [*x*-fold increase = coculture/(SMCs + MNCs)]; #significance of unstimulated versus stimulated cocultures (black bars); §significance of cytokine actually measured in the cocultures versus cytokine measured in the separate MNC + SMC supernatants (Calc). Similar results were obtained for IL-6 in >50 experiments applying ratios from 1:1 to 1:30 and for MCP-1 in 16 experiments.

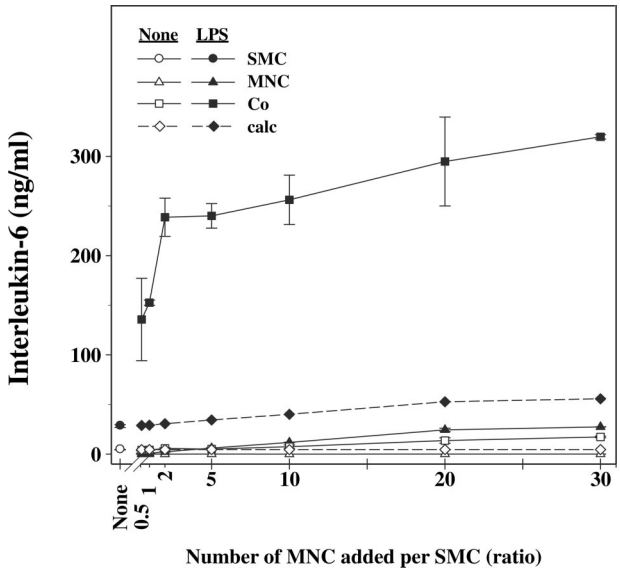

Fig. 2. Synergistic IL-6 production in cocultures of SMC and MNC is dependent on the MNC-to-SMC ratio. SMCs were cultured in 48-well plates (20,000 cells/cm^2). MNCs were added after 24 h at different ratios (none, 0.5, 1, 2, 5, 10, 20, and 30 MNCs per SMC) to the wells. The supernatants were harvested, and IL-6 in the supernatants was measured by ELISA. Four additional experiments showed comparable results.

ATGAACTCCTTCTCCACAAGCGC-3′ and antisense 3′-GAAGAGCCCTCAGGCTGGACTG-5′. The primers used for human 18S rRNA were sense 5′-GTTGGTGGAGCGATTTGTCTGG-3′ and antisense 3′-AGGGCAGGGACTTAATCAACGC-5′. PCR products were analyzed in 1.25% agarose gels using 1× Tris-borate-EDTA buffer, stained with ethidium bromide, and visualized by UV transillumination.

Measurement of IL-6 bioactivity. Biological IL-6 activity was measured using 7TD1 cells (44). Serial dilutions of samples and the IL-6 standard were carried out in dilution medium (50 μl/well; RPMI-1640, including 2% FCS, 1% antibiotics, and 1% L-glutamine) in 96-well flat-bottom microtiter plates. Subsequently, 7TD1 cells (1,500 cells/well; RPMI-1640, including 18% FCS, 1% antibiotics, and 1% L-glutamine) were added and cultured at 37°C and 7.5% CO_2. After 72 h, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (10 μl/well; 5 mg/ml in PBS) was added (4 h). Finally, stop solution was added (100 μl/well; 5% SDS in 50% dimethylformamide), and the plates were agitated in the dark for 1 h and read at 570 nm. The dilution curves were blotted, the ED_{50} values were determined, and the IL-6 activity was calculated with respect to the recombinant IL-6 standard (10 ng/ml).

Detection of STAT3 phosphorylation. For STAT3 measurements, cultures were treated with lysis buffer (2% SDS in PBS), scraped with a rubber policeman, collected in a tube, incubated for 30 min on ice, and centrifuged for 10 min at 10,000 *g*. Proteins were separated using standard SDS-PAGE and transferred to nitrocellulose membranes in Towbin buffer at 1 mA/cm^2 (90 min). Two parallel blots were incubated with antibodies to unphosphorylated STAT3 or phosphorylated STAT3, respectively. Peroxidase-conjugated goat anti-mouse IgG (1:2,000 dilution, Dianova, Hamburg, Germany) was added for 1 h, and staining was visualized using the ECL system (Pierce, Thermo Fisher Scientific).

Statistical analysis. Numbers of experiments are given in the respective figure legends. Calculations of means, SDs, and significance (*t*-test) as well as the correlation analysis (Spearman and Pearson) were performed using SPSS.

RESULTS

Interaction of human vascular SMCs and MNCs or monocytes in cocultures synergistically enhances the production of IL-6 and MCP-1. Interaction of human vascular SMC and invading leukocytes in the vessel wall by soluble mediators may contribute to regulation of local vascular inflammation in early atherosclerosis. Thus, we investigated the interaction of SMCs and human MNCs or monocytes in coculture experiments. In the supernatants of LPS-stimulated SMC-MNC cocultures, IL-6 and MCP-1 concentrations were profoundly enhanced compared with the totalled amount of either cytokine produced by SMCs or MNCs cultured separately (Fig. 1). In the presented experiment, IL-6 and MCP-1 production increased 9- and 2.3-fold, respectively, in LPS-stimulated cocultures. As a rule, we found that the synergistic IL-6 production was more pronounced (5- to 30-fold) than the synergistic MCP-1 production (2- to 10-fold), as observed in many experiments. Similar data were obtained in experiments using lower LPS concentrations (100 pg LPS/ml). In ratio-response experiments, maximal synergism was obtained at the ratios of 2–30 MNCs per SMC (Fig. 2). The synergism at the ratios of 1 and 0.5 MNC/SMC was lower; however, cells at this ratio still produced a half-maximal synergistic effect.

CD14-positive monocytes are essential for the synergistic cytokine production. During atherosclerosis, monocytes, T cells, and other leukocytes invade the vessel wall. In order to identify the cell type of MNC responsible for the synergism,

Table 1. *CD14-positive cells are the MNC subpopulation responsible for the synergism*

	No Subpopulations	MNCs	CD14$^+$	CD14$^-$	CD3$^+$	CD3$^-$
SMCs alone						
Unstimulated	19,219±2,662					
LPS stimulated	28,206±2,796					
Coculture						
Unstimulated		23,781±7,671	23,944±10,482‡	17,026±6,382‡	15,777±5,5796*	26,685±12,044‡
LPS stimulated		142,681±15,073	131,150±52,723‡	34,249±11,808†	34,746±4,953†	141,189±53,067‡
MNCs alone						
Unstimulated		3±3	4±1	1±1	2±1	2±1
LPS stimulated		1,100±80	3,223±34	61±5	4±1	2,185±112

Values are means ± SE of IL-6 (in pg/ml) present in cocultures of smooth muscle cells (SMCs) with various subpopulations of mononuclear cells (MNCs) (ratio of 2 subpopulation cells per 1 SMC) or the respective SMC and MNC cultures. The cultures were incubated overnight and subsequently incubated with MNCs or subpopulation cells for a further 24 h in the absence or presence of LPS (100 pg/ml). IL-6 was measured by ELISA. Significances of differences are shown for SMC + MNC cocultures vs. SMC + subpopulation (CD14$^+$, CD14$^-$, CD3$^+$, and CD3$^-$) cocultures as follows: *$P < 0.05$ and †$P < 0.001$. ‡Not significant.

we isolated CD14$^+$ monocytes and CD3$^+$ T cells. The IL-6 production in cocultures of highly enriched monocytes (CD14$^+$, 97% purity) and SMCs was identical to cocultures of MNCs and SMCs (Table 1). In contrast, cocultures with CD14$^-$ MNCs did not produce significantly more IL-6 than SMCs cultured alone. On the other hand, T cell analysis showed that cocultures of CD3$^+$ cells with SMCs did not produce more IL-6 than SMCs cultured alone. Consequently, cocultures with the CD3$^-$ cells, containing CD14$^+$ monocytes, produced the same amount of IL-6 as the SMC-MNC cocultures. In addition to the MNC ratio-response data mentioned above, CD14$^+$ monocytes at a ratio of as low as 0.2 monocytes per SMC still induced a synergistic IL-6 production (Table 2).

Synergistic IL-6 production correlates with enhanced IL-6 mRNA and increased biological IL-6 activity. In order to corroborate the above data obtained by ELISA, we performed RT-PCR and functional IL-6 measurements with the IL-6-dependent cell line 7TD1. In the PCR analysis of 18S rRNA, used as a control, we detected similar expression in all samples (Fig. 3*A*). On the other hand, we did not detect IL-6 mRNA in unstimulated (−) MNCs at both time points, whereas LPS-stimulated (+) MNCs expressed measurable IL-6 mRNA levels. Already unstimulated SMCs expressed IL-6 mRNA, and LPS stimulation enhanced this expression. On the other hand, cocultured cells always contained higher IL-6 mRNA levels than separately cultured SMCs or MNCs. The mRNA data ("PCR") were then quantified by densitometric methods and correlated with IL-6 activity (Fig. 3*B*, "Biological") determined with 7TD1 cells. For this purpose, supernatants of the cells cultured for mRNA isolation were harvested and stored. The protein measurement in ELISA (Fig. 3*B*, green bars) and the measurement for biological IL-6 activity (Fig. 3*B*, red bars) correlated very well (correlation coefficient = 0.99). The mRNA data (Fig. 3*B*, black bars) also correlated well with the biological activity (correlation coefficient = 0.87).

Soluble factors mediate synergistic IL-6 and MCP-1 production. In order to investigate the involvement of direct cell-cell-contact in the synergistic cytokine production, we performed experiments using fixed MNCs. In these experiments, only surface-associated molecules, rather than released substances, would induce IL-6 or MCP-1 production. However, fixation of MNCs completely abolished the synergism

Table 2. *The IL-6 synergism is detectable in cocultures of 0.2 CD14$^+$ monocytes per 1 SMC*

	2 MNCs per 1 SMC		0.2 CD14$^+$ Monocytes per 1 SMC	
	Unstimulated	LPS stimulated	Unstimulated	LPS stimulated
SMCs alone	7,081±342	11,998±1,713	5,078±342	8,900±606
MNCs/monocytes alone	2±1	1,068±37	1±1	55±2
Coculture	7,460±219	97,575±18,470	4,793±246	23,058±4,306
MNCs + SMCs	7,083	13,066	5,079	8,955
Synergism	1.1	7.5	0.9	2.6

Values are means ± SE of IL-6 (in pg/ml) in cocultures of MNCs or CD14$^+$ monocytes with SMCs at a low ratio. MNCs were isolated from heparinized blood and used in the coculture. CD14+ cells were isolated from the remaining MNCs and used in parallel cultures with the same SMCs (10,000 SMCs/cm2) used for cocultures with MNCs. LPS (100 ng/ml) was used as a stimulus. IL-6 was measured in 24-h supernatants by ELISA. Synergism = coculture/MNCs + SMCs.

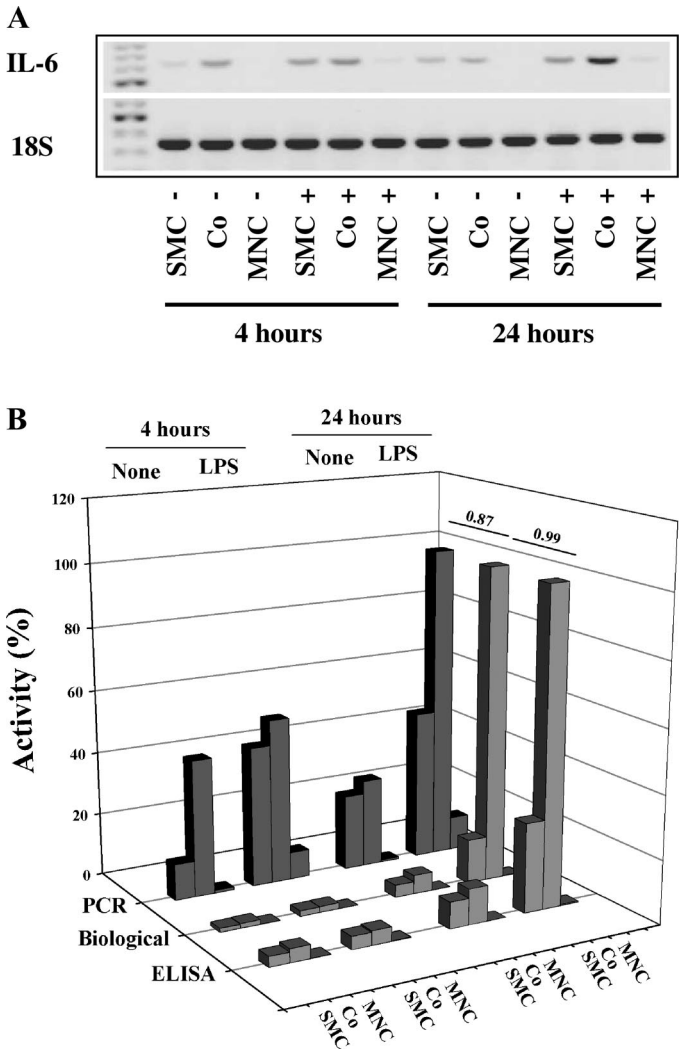

Fig. 3. Synergistic IL-6 production is present at the protein, mRNA, and functional level. SMCs were cultured in 6-well plates (20,000 cells/cm^2) for 24 h. MNCs (ratio of 1 MNC to 1 SMC) were added in the absence or presence of LPS (100 ng/ml). *A*: RT-PCR. After 4 and 24 h, RNA was isolated, reverse transcribed, and amplified (20 cycles for 18S rRNA an 24 cycles for IL-6 mRNA). PCR products were run on an agarose gel, visualized by UV transillumination, and analyzed by the Gene Snap program. *B*: comparison of RT-PCR, IL-6 bioassay, and ELISA. At the same time points (4 and 24 h), before the mRNA preparation, supernatants of the cultures were harvested and stored at −20°C until measurements. The density of the bands of the PCR products was analyzed with the Image Quant program. IL-6 protein was measured by ELISA. Biological IL-6 activity was measured using the IL-6-dependent cell line 7TD1. Each set of data was normalized to the maximal result ("Co, 24 h, LPS") and presented as the percentage of maximal activity. The 100% values for the PCR, biological, and ELISA data are 11,125 units, 296,331 pg/ml, and 250,082 pg/ml, respectively. Correlation of the three sets of data was analyzed by correlation analysis according to Spearman and Pearson. Spearman correlation coefficients for biological activity versus ELISA and biological activity versus PCR are as shown; Pearson correlation coefficients were 0.99 ($P = 8 \times 10^{-10}$) and 0.80 ($P = 0.002$), respectively. Two additional PCR experiments showed similar results.

(Fig. 4*A*, "Fixed"). The pretreatment procedure (scrapping and washing the monocytes) reduced the level of synergism to some degree (Fig. 4*A*, "Unfixed") compared with the standard coculture (Fig. 4*A*, "Normal"). However, in the fixed cultures, the cytokine production under unstimulated conditions was reduced by 89.9% and under stimulated conditions by 91.3%

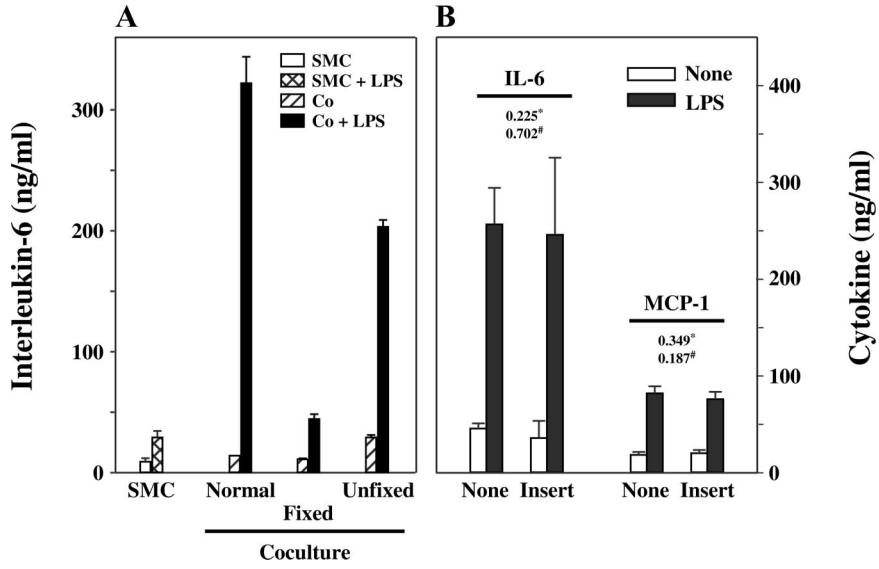

Fig. 4. Soluble factors mediate the synergistic IL-6- and MCP-1 production observed in cocultures of SMCs and MNCs. *A*: cell fixation. Human SMCs were cultured in 24-well plates (20,000 cells/cm^2). MNCs were isolated and treated in the following three different ways: *1*) "Normal," application of MNCs and coculture at a ratio of 1:30; *2*) "Fixed," MNCs were stimulated for 4 h in the absence or presence of LPS (100 ng/ml) in 25-cm^2 flasks, and cells were removed by scraping, washed, fixed for 30 min in 1% paraformaldehyde, subsequently washed, and applied to SMCs at the same ratio as the normal MNCs, and *3*) "Unfixed," MNCs were treated like fixed cells; however, no paraformaldehyde was added. *B*: insert cultures. SMCs were incubated in 24-well plates (20,000 SMCs/cm^2) for 24 h. MNCs were added after 24 h at the ratio of 1:30 into the wells (None) or into the culture inserts separating the MNCs from the SMCs (Insert). Subsequently, cultures were incubated in the absence or presence of endotoxin (LPS, 100 ng/ml) for 24 h. The supernatants were harvested, and IL-6 or MCP-1 was measured by ELISA. Numbers and symbols over the bars indicate the significance of normal cocultures versus insert cocultures under unstimulated (*) or LPS-stimulated (#) conditions, respectively. Two additional experiments showed comparable results.

(both after background, i.e., SMC, subtraction) compared with unfixed cultures. However, the difference of the unstimulated cultures was not significant ($P = 0.169$), whereas the difference of the stimulated fixed and unfixed cultures was significant ($P = 0.037$). These data indicate that cell-associated factors do not have an important role in the induction of synergism. The resultant hypothesis that soluble factors are essential for the synergism was proven by transwell experi-

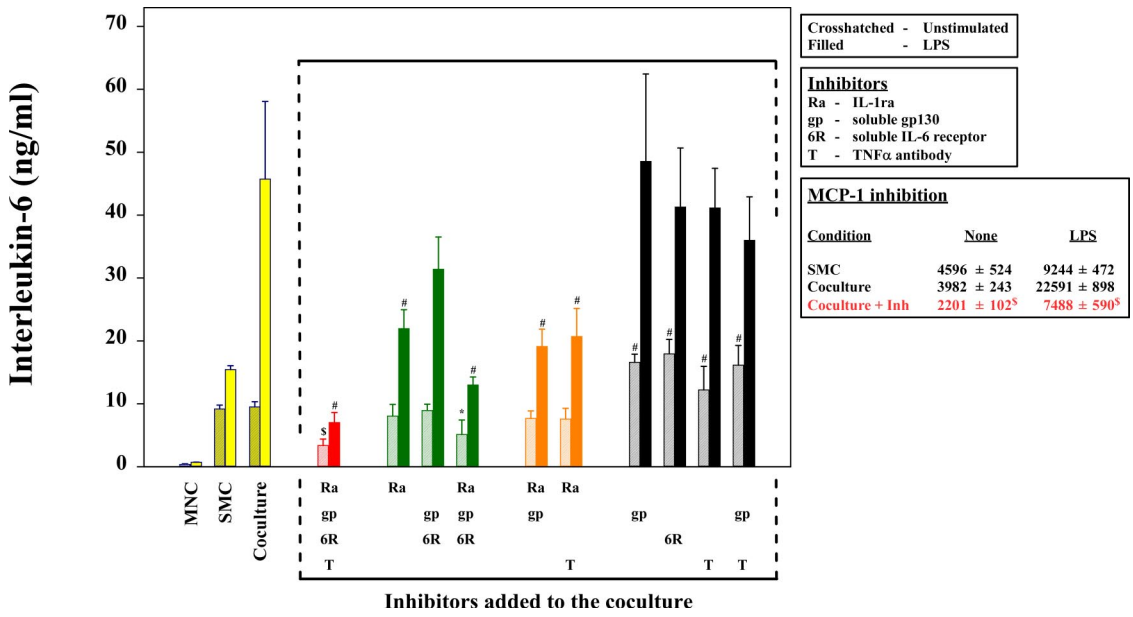

Fig. 5. Simultaneous inhibition of IL-1, TNF-α, and IL-6 completely blocks synergistic IL-6 and MCP-1 production. SMCs were cultured in 48-well plates (20,000 cells/cm^2) for 24 h. MNCs (ratio of 1 MNC to 1 SMC) were added in the absence or presence of LPS (100 ng/ml). Before the addition of MNCs and LPS, inhibitors or combinations thereof were applied: IL-1 receptor antagonist (IL-1ra; 200 ng/ml); soluble glycoprotein (gp)130 fusion protein (gp; 500 ng/ml); soluble IL-6 receptor (6R; 50 ng/ml); and anti-TNF-α antiserum (T; 500 ng/ml). After 24 h, supernatants were harvested, and IL-6 and MCP-1 measured by ELISA. All differences between unstimulated and LPS-stimulated cultures were significant. Significances of the differences between unstimulated cocultures versus unstimulated cocultures + inhibitors, and stimulated cocultures versus stimulated cocultures + inhibitors, respectively, are shown above the bars (*$P < 0.05$; #$P < 0.01$; $$P < 0.001$). The MCP-1 data (in pg/ml) of the inhibition with the inhibitor cocktail (Inh) are shown as a table (*inset*).

Table 3. *IL-1β and TNF-α are present in supernatants and reduced upon the addition of inhibitory cocktail*

	IL-1β, pg/ml		TNF-α, pg/ml	
	No inhibitory cocktail	With inhibitory cocktail	No inhibitory cocktail	With inhibitory cocktail
SMCs				
Unstimulated	5.1±0.7	12.9±5.2	10.6±2.2	16.0±8.5
LPS stimulated	33.6±19.5	6.6±1.4	16.6±7.5	9.2±5.8
MNCs				
Unstimulated	1.1±7.0	7.0±0.4	2.2±0.9	2.4±1.1
LPS stimulated	569.3±10.3	299.1±14.0	261.6±3.7	2.2±0.5
Coculture				
Unstimulated	7.1±2.2	22.5±3.6	11.4±3.9	14.8±8.1
LPS stimulated	200.7±4.7	183.9±2.6	15.6±3.8	7.9±4.6

Values are means ± SE. SMCs and MNCs were cultured separately and under standard coculture conditions at a ratio of 1 SMC/1 MNC. After 24 h, supernatants were harvested, and IL-1β and TNF-α levels were measured by ELISA. LPS (100 ng/ml) was used as a stimulus. The inhibitory cocktail consisted of a mixture of IL-1β, TNF-α, and IL-6 inhibitors [IL-1 receptor antagonist (200 ng/ml), soluble glycoprotein 130 (500 ng/ml), soluble IL-6 receptor (50 ng/ml), and anti-TNF-α antiserum (500 ng/ml)].

ments separating the two cell types by cell culture inserts. Synergistic IL-6 and MCP-1 production induced by LPS was not reduced by the inserts, indicating that soluble factors mediate the majority of the synergism (Fig. 4*B*).

IL-1, TNF-α, and IL-6 are essential for the induction of synergistic IL-6 production. Next, we analyzed which soluble factors are important for the induction of synergistic cytokine production. In order to investigate the role of IL-1, TNF-α, and IL-6 in the synergism, we used IL-1ra, TNF-α antibodies, and a combination of soluble gp130Fc and soluble IL-6 receptor, which can block IL-1, TNF-α, and IL-6 responses, respectively. The standard coculture (Fig. 5, yellow bars) showed the expected synergistic IL-6 production. The addition of the inhibitory cocktail (Fig. 5, red bars), consisting off all inhibitors, resulted in the complete reduction of the synergism. The table (*inset*) in Fig. 5 shows that MCP-1 production was also completely reduced by the inhibitory cocktail. Among the inhibitors tested, IL-1ra was the only substance that potently inhibited synergistic IL-6 production when applied alone (Fig. 5, green bars, "Ra"). The application of soluble gp130Fc together with soluble IL-6 receptor also reduced synergism (Fig. 5, green bars, "gp, 6R"), but the reduction was not significant. However, the combination of soluble gp130Fc and IL-6 receptor with IL-1ra further blocked the synergism (Fig. 5, green bars, "Ra, gp, 6R"), compared with incubation with IL-1ra alone. Combination of soluble gp130Fc or TNF-α antibody with IL-1ra did not augment the inhibitory effect of IL-1ra (Fig. 5, orange bars). Soluble gp130, soluble IL-6 receptor, and TNF-α antibody applied separately, as well as the combination of soluble gp130 and TNF-α antibody, did not inhibit the synergism significantly (Fig. 5, black bars). The data show that, besides IL-1 and TNF-α, IL-6 may contribute to synergistic IL-6 production by *trans*-signaling.

The evidence for a role of IL-1 and TNF-α in the activation of the synergism was substantiated by direct measurements of the presence of IL-1 and TNF-α in the cultures (Table 3). The data show that in the activated SMC cultures, IL-1 and TNF-α levels were enhanced to some degree, but in the MNC cultures, they were potently enhanced. In the cocultures, the presence of

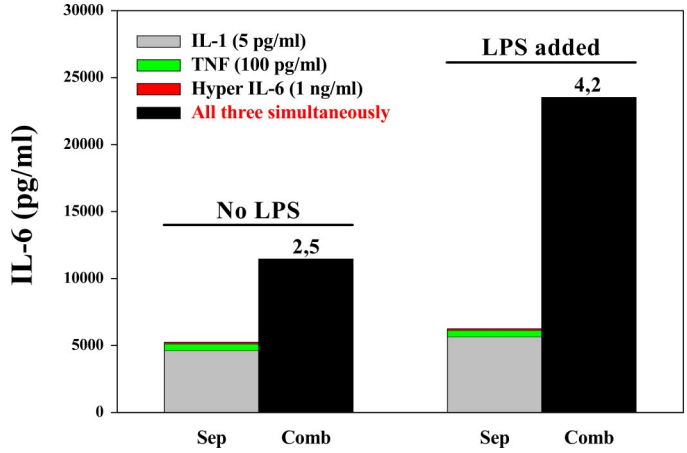

Fig. 6. Costimulation of SMCs with IL-1, TNF-α, and hyper-IL-6 results in synergistic IL-6 production. SMCs were cultured overnight in 96-well plates (20,000 SMCs/cm^2). The medium was changed, and the cytokines were added separately (Sep) or simultaneously (Comb) to the cultures. To parallel cultures, LPS (100 ng/ml) was added. The stacked bars show IL-6 production induced by the separate stimulation. The black bars show IL-6 produced in SMCs stimulated simultaneously by the three cytokines (Comb). The numbers above the bars indicate the synergism (Comb/Sep).

IL-1 was partially reduced and the presence of TNF-α was completely reduced. These data indicate a consumption of the respective cytokine. In the cultures with the inhibitor cocktail, both cytokines were much less abundant than in the cultures without the inhibitor cocktail, pointing to a direct effect of the tested cytokines on their own production. We also analyzed the role of the three cytokines directely in stimulation assays with exogenous recombinant stimuli. Hyper-IL-6 was used instead of IL-6, since hyper-IL-6 activates by *trans*-signaling and not by classical signaling. Figure 6 shows that a synergistic enhancement of IL-6 production was measured in both, the cultures with and without additional LPS. Numerous additional combinations of IL-1 (5, 50, and 500 pg/ml), hyper-IL-6 (1 and 10 ng/ml), and TNF-α (100 and 1,000 pg/ml) also showed a synergism, which was always higher in the presence of LPS.

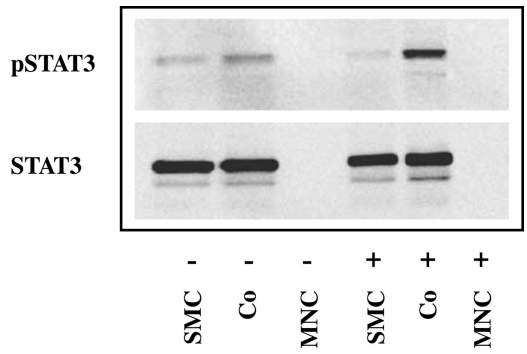

Fig. 7. STAT3 phosphorylation is enhanced in the coculture. SMCs were cultured in 6-well plates (20,000 SMCs/cm^2) for 24 h. MNCs (ratio of 1 MNC to 1 SMC) were added in the absence or presence of LPS (100 ng/ml). The supernatants were harvested and measured by IL-6 ELISA (compare with Table 2). The remaining cells were lysed in lysis buffer (2% SDS in PBS), and proteins were separated using standard SDS-PAGE (7.5%) and transferred to nitrocellulose membranes. Two parallel blots were incubated overnight with antibodies to unphosphorylated STAT3 or phosphorylated STAT3 (pSTAT3), respectively. Peroxidase-conjugated goat anti-mouse IgG was added for 1 h, and staining was visualized using the ECL system.

Table 4. *Synergistic IL-6 production correlates well with the phosphorylation of STAT3*

	IL-6 Determination		STAT3 Determination		
	IL-6, pg/ml	Normalized IL-6	Phospho-STAT3	STAT3	Phospho-STAT3/STAT3
Unstimulated					
MNCs	121±20	0.005	ND	ND	0
SMCs	25,3.9±2,710	1	1	1	1
Coculture	33,175±428	1.311	1.900	1.055	1.800
LPS stimulated					
MNCs	825±130	0.033	ND	ND	0
SMCs	28,435±856	1.124	0.600	0.750	0.800
Coculture	150,847±14,545	5.960	2.400	0.989	2.700

Values are means ± SE. For IL-6 determination, supernatants of the cultures used for the results shown in Fig. 6 were measured by ELISA. LPS-stimulated MNCs ($P = 0.017$) and cocultures ($P = 0.008$) produced significantly more IL-6 than the respective unstimulated cultures. IL-6 concentrations were normalized to unstimulated SMCs. For STAT3 determination, the bands shown in Fig. 6 were quantified using the AIDA program and normalized to unstimulated SMCs. Finally, the determined values were used to calculate the phospho-STAT3-to-STAT3 ratio (phospho-STAT3/STAT3). ND, not detectable. The Pearson correlation coefficient of normalized IL-6 with phospho-STAT3/STAT3 was 0.89 ($P = 0.017$); the Spearman correlation coefficient was 0.93 ($P = 0.008$).

Enhanced STAT3 phosphorylation provides further evidence for the contribution of IL-6 to the synergism in the cocultures. The above-mentioned inhibition by the combination of IL-1ra, gp130Fc, IL-6 receptor, and TNF-α ("Ra, gp, 6R, T") indicated that, in addition to IL-1 and TNF-α IL-6 may be involved in synergistic IL-6 and MCP-1 production. This suggestion would be reflected by enhanced STAT3 phosphorylation in the cocultures. Investigation of unphosphorylated STAT3 using Western blot analysis (Fig. 7) showed comparable expression in SMCs and cocultures, whereas it was not detectable in the low number of MNCs present under the coculture conditions at the ratio of 1 MNC per 1 SMC. At a ratio of 10 MNCs per 1 SMC, a faint band became visible, which was less prominent than the SMC band (data not shown). On the other hand, phosphorylation of STAT3 was considerably enhanced in the cocultures compared with separately cultured SMCs. In LPS-stimulated cocultures, STAT3 phosphorylation was higher than in unstimulated cocultures. As expected from the lack of STAT3 detection in MNCs, no phosphorylated STAT3 was detectable in MNCs. Comparable results were obtained at the time points of 1, 3, 9, and 24 h (data not shown). The above data indicate a role for a STAT3 inducer, such as IL-6, in the synergism observed in the SMC-MNC cocultures. This suggestion was further supported by the finding that the IL-6 levels (Table 4, "IL-6 normalized") present in the cocultures and STAT3 phosphorylation (Table 4, "phospho-STAT3/STAT3") correlated well (correlation coefficient = 0.89, $P = 0.017$). The use of AG-490, an inhibitor of the JAK/STAT pathway, in combination with NF-κB and a kinase inhibitor resulted in complete inhibition of the synergism (Fig. 8), providing further evidence for the contribution and interaction of IL-1, TNF-α, and IL-6 in the synergism.

DISCUSSION

During early atherogenesis, chemokines and cytokines may contribute to the local regulation of inflammation in the vessel wall (9, 23, 25, 36, 43). Interaction of local vessel wall cells and invading leukocytes may trigger this inflammatory process. Thus, we investigated the activation of MCP-1 and IL-6 production upon the interaction of vascular SMCs and MNCs or isolated monocytes in a coculture system. The data showed

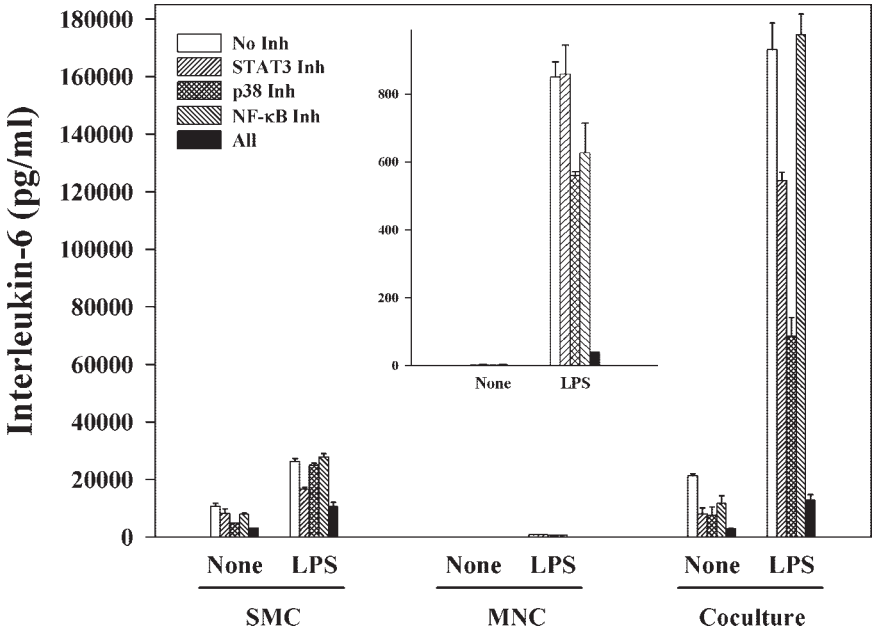

Fig. 8. A combination of inhibitors of IL-6, IL-1, and TNF-α signaling pathways completely abolishes synergistic IL-6 production. Human SMCs were cultured in 24-well plates (20,000 SMCs/cm^2) for 24 h. MNCs (ratio of 1 MNC to 1 SMC) were added in the absence or presence of LPS (100 ng/ml). STAT3 inhibitor (AG-490, 50 μM), MAPK inhibitor (SB-203580, 5 μM), and NF-kB inhibitor (a quinazoline compound, 10 μM) were added separately as well as in combination of all three inhibitors. Cultures were incubated for 24 h. IL-6 was measured by ELISA. The *inset* shows a magnification of the low IL-6 values obtained in the MNC cultures.

a very strong synergistic enhancement of IL-6 and MCP-1 production in the cocultures. This synergistic production was mediated by the soluble factors IL-1, TNF-α, and IL-6, acting together. CD14-positive monocytes were essential for the induction of the synergism. The data suggest that resident vessel wall SMCs and invading monocytes may interact by cytokines, resulting in a potent enhancement of local inflammatory pathways. These sensitive and potent mechanisms may contribute to local activation of inflammatory pathways during early atherogenesis.

A role for cytokines and chemokines in atherosclerosis has been substantiated in a variety of animal experiments using gene-deficient or -overexpressing mice, as summarized recently (16, 23, 43). In the early steps of atherosclerosis, the cytokine/inflammatory network, possibly activated by a variety of independent and/or subsequent triggers, may initiate the modification of cell function(s) in the vessel wall upon interaction of resident and invading cells. This interaction may cause enhanced chemokine and cytokine production, probably resulting in perpetuated attraction of invading cells and alteration of vessel wall functions, including further intensified production and deposition of chemokines, cytokines, and the extracellular matrix. These steps may further enhance cell recruitment, cell activation, and matrix deposition, resulting in an "immunovascular-memory effect," which may lead to an evergrowing response to subsequent infiltration, as suggested previously (23).

Interaction of vessel wall cells and other cells can be studied in coculture systems. We (18) have previously shown that in a coculture of platelets with SMCs, platelet-derived IL-1 activated SMC IL-6 production. We did not find informations about MCP-1 or IL-6 production in SMC-monocyte cocultures. However, it has been reported that in cocultures of monocytes and endothelial cells, IL-6 production was enhanced (1). The importance of MCP-1 and IL-6 for atherosclerosis is evident from a variety of publications, as summarized previously (16, 23, 43). We (20, 22) have shown that SMCs produce IL-6 at a very high level. IL-6 regulates a number of atherosclerosis-relevant cell functions, such as expression of enzymes (41), cell migration (2), cell proliferation (31), cell attraction (33), or even MCP-1 production (13, 42). MCP-1, in addition to its accustomed chemokine function, may then activate the proliferation of SMCs (40), possibly by the induction of further cytokines (32).

The present findings indicate that soluble mediators are essential for the activation of the synergistic IL-6 and MCP-1 production. Although it has been previously reported that VEGF production upon interaction of SMCs and monocytes was mediated by both, cell-to-cell contact and soluble mediators (11), the fixation experiments and the cell culture insert experiments in the present work did not provide evidence that cell-to-cell contact was neccessary for the synergistic IL-6 or MCP-1 production. Rather, soluble factors appeared to be essential for the induction of the synergism. This directed us to study the role of the cytokines IL-1, TNF-α, and IL-6 in the coculture. IL-1 and TNF-α are readily produced by monocytes, and IL-6 is released from monocytes and even more potently from SMCs, suggesting the possibility of an autocrine pathway involving IL-6. On the other hand, IL-1 is released from SMCs or heart cells only to a low degree (30, 38, 49). In agreement with this information, we found IL-1 and TNF-α in the supernatants of MNCs. In the cocultures, the levels of both were lower, pointing to a consumption of these cytokines. Taken together, the present results showed that IL-1, TNF-α, and IL-6 are essential for the synergistic IL-6 and MCP-1 production, based on our observation that an IL-1, TNF-α, and IL-6 inhibitory cocktail completely reduced the stimulation.

The inhibition data, besides the involvement of IL-1 and TNF, suggested an autocrine contribution of IL-6 in the synergistic enhancement of the IL-6 and MCP-1 production. This suggestion was further verified by the observation that hyper-IL-6, IL-1, and TNF-α synergistically enhanced the IL-6 production and that STAT3 phosphorylation was enhanced. The use of soluble gp130Fc and soluble IL-6 receptor further proved the involvement of IL-6. Briefly, IL-6 induces an intracellular signal by "classic" signaling through the cell surface IL-6 receptor, in combination with the membrane-associated signal mediator gp130. The IL-6 receptor can be shed from the cell surface, and this process has been recently shown to occur in apoptotic neutrophils in an acute infection model (4). The soluble IL-6 receptor binds IL-6 with the same affinity as the membrane-associated receptor (28). The IL-6/soluble IL-6 receptor complex can bind to membrane gp130, thereby activating cells. This mechanism is called *trans*-signaling (34). *Trans*-signaling can be blocked by soluble gp130 (35), and the soluble IL-6 receptor may potentiate the antagonistic activity of soluble gp130 (14, 29). In our hands, the combination of soluble gp130Fc and soluble IL-6 receptor elevated the inhibition of the synergism by IL-1ra. This finding indicates that *trans*-signaling is involved in the synergistic IL-6 and MCP-1 production. Furthermore, it has been shown that a construct of IL-6 and soluble IL-6 receptor (hyper-IL-6), which is thought to mimic *trans*-signaling, potently induced the proliferation of SMCs (17). As mentioned above (Fig. 6), it also contributed to the synergistic IL-6 production observed after stimulation with the recombinant cytokines.

Taken together, the present data show that the SMC-monocyte interaction may result in an enhanced inflammatory potential, as indicated by the synergistic IL-6 and MCP-1 production. In the vessel wall, synergistic cytokine production resulting from the interaction of monocytes and SMCs may provide a trigger for the accumulation of cells and activation of resident and invading cells in the vessel wall. These finding may be of importance for the comprehension of the role of inflammatory pathways in early atherosclerosis.

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