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IL-9 Inhibits Oxidative Burst and TNF-α Release in Lipopolysaccharide-Stimulated Human Monocytes Through TGF-β1

Charles Pilette, 2* Youssef Ouadrhiri,* Jacques Van Snick,*† Jean-Christophe Renauld,*† Philippe Staquet, ‡ Jean-Pierre Vaerman,* and Yves Sibille*

IL-9 is a Th2 cytokine that exerts pleiotropic activities on T cells, B cells, mast cells, hematopoietic progenitors, and lung epithelial cells, but no effect of this cytokine has been reported so far on mononuclear phagocytes. Human blood monocytes preincubated with IL-9 for 24 h before LPS or PMA stimulation exhibited a decreased oxidative burst, even in the presence of IFN-γ. The inhibitory effect of IL-9 was specifically abolished by anti-hIL-9R mAb, and the presence of IL-9 receptors was demonstrated on human blood monocytes by FACS. IL-9 also down-regulated TNF-α and IL-10 release by LPS-stimulated monocytes. In addition, IL-9 strongly up-regulated the production of TGF-β1 by LPS-stimulated monocytes. The suppressive effect of IL-9 on the respiratory burst and TNF-α production in LPS-stimulated monocytes was significantly inhibited by anti-TGF-β1, but not by anti-IL-10R mAb. Furthermore, IL-9 inhibited LPS-induced activation of extracellular signal-regulated kinase 1/2 nitrogen-activated protein kinases in monocytes through a TGF-β-mediated induction of protein phosphatase activity. In contrast, IL-4, which exerts a similar inhibitory effect on the oxidative burst and TNF-α release by monocytes, acts primarily through a down-regulation of LPS receptors. Thus, IL-9 deactivates LPS-stimulated blood mononuclear phagocytes, and the mechanism of inhibition involves the potentiation of TGF-β1 production and extracellular signal-regulated kinase inhibition. These findings highlight a new target cell for IL-9 and may account for the beneficial activity of IL-9 in animal models of exaggerated inflammatory response. The Journal of Immunology, 2002, 168: 4103–4111.

Interleukin-9 is a four α-helix bundle cytokine initially identified as a factor produced by activated CD4+ T cells and promoting the growth of some Th clones (1, 2). B lymphocytes, mast cells, and some hematopoietic progenitors (3), as well as lung epithelial cells (4, 5), have been shown to represent additional target cells for IL-9. Stimulation of the proliferation and/or activation of these cells is thought to support both the beneficial activity of IL-9 in some parasitic infection such as by Trichuris muris (6), and its deleterious effect in asthma (7–11). Thus, mice overexpressing IL-9 display airway infiltration by lymphocytes, mast cells, eosinophils, and possibly macrophages, as well as bronchial remodeling and hyperresponsiveness (3, 7, 10). However, studies of IL-9-deficient mice indicated that IL-9 is not mandatory for the induction of the Th2 asthma-related phenotype (12).

Although IL-9 is implicated in Th2 responses and humoral immunity, at least two mouse models also suggest an important role of IL-9 in the inflammatory response. Firstly, prophylactic administration of IL-9 protects mice from death in a model of sepsis induced by i.v. injection of Pseudomonas aeruginosa (13). This protective effect, also observed with IL-4, was associated with a strong reduction of serum levels of TNF-α, IL-12/p40, and IFN-γ induced by the bacteria or LPS injection, and with a dramatic increase of IL-10. Secondly, in a silica-induced lung fibrosis model, IL-9 had a beneficial antifibrotic effect associated with an inhibition of the silica-induced up-regulation of IL-4 expression (14). Interestingly, in a rat model of lung fibrosis induced by irradiation, IL-4 has been shown to be mostly produced by alveolar macrophages (15). Taken together, these data raise the hypothesis that IL-9 might modulate monocyte activation, which plays a key role in these inflammatory disorders. However, whether in these models IL-9 modulates cytokine production through direct or indirect mechanisms remains to be determined. To objectivate a direct regulation by IL-9 of mononuclear phagocyte activation, we assessed the effect of IL-9, in comparison with IL-4 and IFN-γ, on the respiratory burst and cytokine release by human peripheral blood monocytes in response to LPS. Because surface and/or intracellular regulatory events have to mediate the observed effects of IL-9, we further evaluated the activation of extracellular signal-regulated kinases (ERK) 1/2 of the mitogen-activated protein kinase (MAPK) family, and surface expression of LPS receptors by human monocytes exposed to IL-9.

Materials and Methods

Reagents and Abs

HBSS without phenol red was purchased from BioWhittaker (Walkersville, MD), as well as RPMI 1640 culture medium which was supplemented with

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Philippe Staquet, ‡ Jean-Pierre Vaerman,* and Yves Sibille*

3 Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; GAM3, goat anti-mouse IgG3; MAPK, mitogen-activated protein kinase; mAb, mouse IgG; OA, okadaic acid; OV, orthovanadate; ROI, reactive oxygen intermediate; SAM, sheep anti-mouse IgG; SOD, superoxide dismutase; TLR, Toll-like receptor; cRPMI, complete RPMI; hIL-9, human IL-9; DCFH, dichlorofluorescein.
2 mM 1-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% decomplemented (56°C, 30 min) FBS, and referred as complete RPMI (cRPMI). Recombinant human (h) IFN-γ and TGF-β1 were purchased from Genzyme (Cambridge, U.K.). IL-9 and IL-4 as well as anti-IL-9R α-chain mAbs were produced at the Ludwig Institute (Brussels, Belgium). Anti-IL-9 clone AH9R2 mouse mAb (mouse IgG1mG2a) was used for indirect immunofluorescence staining, and blocking mAb against hIL-9R (clone AH9R7, mG2Gb) was used to specifically neutralize IL-9 activity. hIL-9 was purified from SF9 insect cell cultures infected with recombinant baculovirus by passage on Butyl Sepharose (Pharmacia Biotech, Upsala, Sweden). The material eluted with 20 mM Tris-HCl (pH 7.4) containing 1:10,000 v/v Tween 20 (Sigma-Aldrich, St. Louis, MO) was further processed on Yellow3 Sepharose (Sigma-Aldrich) in PBS, and eluted with 1 mM NaCl in the same buffer. After dialysis against 50 mM PBS, hIL-9 was adsorbed onto a Resource S cation exchange fast protein liquid chromatography column and eluted with a NaCl gradient in the same buffer. Final polishing was performed by reverse-phase chromatography on a Vydac C4 column (Hesperia, CA) equilibrated in 0.05% TCA and processed with a gradient of acetonitrile. Purity of this material was checked by silver-stained SDS-PAGE. An hIL-9 1g fusion protein (hIL-9-mG3) was produced as follows: hIL-9 cDNA was amplified by PCR using a modified antisense primer that introduced a BclI restriction site just before the stop codon. 5’-TCTTTGATCTACGGCTTCAATCTC-3’ (sense) and 5’-AATTCTTGGATCTGCTTCAATCTC-3’ (antisense) containing BclI and XhoI sites for cloning. After amplification, both PCR products were digested with appropriate restriction enzymes and cloned into BlueScript II SK(+) pCDNA3/Amp plasmid (Invitrogen, San Diego, CA). Clones with the correct insert were transfected into COS7 cells, and supernatants were collected from 3 days. This hIL-9 cimERIC was found to be functionally active in the bioassay using IL-9-dependent TS1 cells previously described (6). LPS from Escherichia coli (serotype O55:B5) was purchased from Difco Laboratories (Detroit, MI). Neutralizing mAb against IL-10Rø (clone TB21, mG1) was from R&D Systems (Minneapolis, MN), and that against TGF-β1 (clone TB21, mG1) was from BioSource International (Camarillo, CA).

Cell isolation

Human monocytes were obtained from peripheral blood of healthy blood donors by a density gradient method using Polymorphprep (Nycomed, Oslo, Norway). Whole heparinized blood was layered on the gradient, and centrifuged at 450 × g for 30 min at 20°C. Mononuclear leukocytes were collected at the interface, washed twice with PBS, and resuspended in cRPMI. Monocytes were then purified by adherence to plastic (30 min, 37°C). These mononuclear cells were identified by immunofluorescence, and monocytes represent >95% of total adherent cells at flow cytometry and microscopic examination of Giemsa-stained cytopsin. Cell viability assessed by the trypan blue exclusion test was at least of 90% for the different experimental conditions, including with inhibitors of ERK pathway and of protein phosphatases.

Oxidative burst assay

Monocytes (0.2 × 10⁶/well) were distributed in 96-well plates with flat bottoms (BD Labware-Falcon, Franklin Lakes, NJ), and preincubated for 24 h at 37°C, 5% CO₂ with cytokines (20 ng/ml for IL-9 or IL-4, and 200 U/ml for IFN-γ) in cRPMI before being stimulated for 20 h by LPS (1 μg/ml) without removing the cytokines. Intracellular oxidative activity was assessed as described by Bass et al. (16). Briefly, at the end of incubation with cytokines and/or LPS, cells were loaded for 15 min with 15 μM 2’,7’-dichlorofluorescein (DCFH)-diacetate (Sigma-Aldrich) in cRPMI which after passive penetration into cells is hydrolyzed into nonfluorescent polar DCFH trapped inside the cells. DCFH is then oxidized into highly fluorescent dichlorofluorescein according to the intracellular amount of hydrogen peroxide produced by the respiratory burst. After these washings with PBS (pH 7.4), cells were lysed in 0.1% v/v Triton X-100 (Sigma Aldrich) in PBS, and fluorescence was quantified in a fluorescent microplate spectrophotometer (Packard Instruments, Dowers Grove, IL) at 485 nm excitation/530 nm emission wavelengths. DCF concentrations were deduced from a standard curve of known concentrations of fluorescent standard. Results were corrected for total protein concentration determined in cell lysates by the bicinchoninic acid-based method (Pierce, Rockford, IL), and expressed as nanomoles of DCF per milligram of cell protein.

Extracellular release of O₂-derived radicals was evaluated by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c, as previously described (17). Briefly, after incubation with cytokines and/or LPS, cells were washed three times in HBSS to remove phenol red-containing medium and incubated at 37°C with HBSS containing 160 μM ferricytochrome c (Sigma-Aldrich) plus 300 IU/ml SOD (Roche Diagnostics, Bale, Germany) as control for each condition, and 100 ng/ml PMA (Sigma Aldrich) when indicated. OD₅₅₀ was then recorded in a plate spectrometer (Tritertek Multiscan Plus MKII, Labsystems, Finland) after 10 min. The reduced amount of superoxide anion (O₂⁻) was deduced from the OD₅₅₀ (after subtraction of control values with SOD) using the ferricytochrome c extinction coefficient of 21 10⁴ M⁻¹ cm⁻¹. Results were expressed as nanomoles O₂⁻/10⁶ cells/h.

Cytokine release assay

Monocytes (1 × 10⁶/well) were distributed in 24-well plates (Falcon), and incubated in the same conditions as for the oxidative burst assay. Supernatants were harvested and frozen at −20°C until cytokine titration. Release of TNF-α was quantified by a cytotoxicity bioassay using WEHI 164 cells clone 13, as previously described (18), and rhTNF-α from Boehringer (Mannheim, Germany) as standard. IL-10 and TGF-β1 concentrations were determined by ELISA. A kit from CLB (Amsterdam, The Netherlands) was used for IL-10 quantitation, following the manufacturer’s protocol. A kit from Biosource International allowed us to determine TGF-β1 after the release from its latent complexes by acid treatment of supernatants; TGF-β1 was also assessed in crude supernatants. The sensitivity of TNF-α bioassay was 0.2 pg/ml, and that of IL-10 and TGF-β1 immunoassays was 2 pg/ml for both. All supernatants were assayed in duplicate.

Immunofluorescence staining

For FACS analysis, IL-9 expression on monocytes was assayed by indirect immunofluorescence. Adherent mononuclear cells (0.2 × 10⁶/well) were incubated at 4°C for 1 h with anti-hIL-9-R mAb A92R2 or A92R7 diluted at 10 μg/ml in RPMI containing 3% FBS. After three washings with RPMI-3%-FBS, cells were incubated at 4°C for 1 h with 10 μg/ml FITC-conjugated F(ab')₂ of sheep anti-mouse IgG (SAM-FITC; Sigma-Aldrich) in the same medium. Cells incubated with mG2La or mG2Gb and thereafter with SAM-FITC represented negative controls. After three washings, monocytes were fixed in 2% v/v formaldehyde in PBS-3% FBS for 15 min at room temperature, gently scraped with a rubber policeman, and kept in the dark at 4°C until FACS analysis performed on a FACScan from BD Biosciences (Mountain View, CA). Additional stainings for CD14 and Toll-like receptor (TLR)4 were performed on monocytes preincubated for 24 h with cytokines, using FITC-conjugated anti-CD14 Ab (clone Mep9, mG2Lb; BD Biosciences) and anti-TLR4 rabbit Ab (Santa Cruz Biotechnology; Santa Cruz, CA), respectively; followed by F(ab')₂ of mouse anti-rabbit IgG-FITC.

Binding of IL-9 to the surface of monocytes was assayed by incubating these cells (0.2 × 10⁶) at 4°C for 1 h with hIL-9-mG3 chimeric molecule (10% COS cell supernatant). IL-9 binding was revealed after washings by incubation for 1 h at 4°C with FITC-conjugated goat anti-mouse IgG3 (GAM3-FITC; Southern Biotechnology Associates, (Birmingham, AL). Cells incubated with mG3-FITC before GAM3-FITC represented the negative control. FACS analysis of the cell-associated fluorescence was then performed as for the assessment of IL-9 expression. For confocal microscopy, monocytes (0.2 × 10⁶/coverslip) were cultured for 2 h in 24-well plates, washed with cRPMI, and immunostained for IL-9 as for FACS analysis with A92R2 mAb. After washings with PBS-3% FBS and fixation by 2% v/v formaldehyde in the same buffer, cells were permeabilized on slides with 1% v/v Triton X-14, 1,4-diacetylmethanone (Sigma-Aldrich) in Mowiol (Calbiochem-Novabiochem, Darmstadt, Germany), and analyzed by a MRC-1024 confocal microscope (Bio-Rad Laboratories, Richmond, CA) using a ×63 objective under oil immersion. Images were digitally recorded and reproduced with a phototprinter. Both for FACS and confocal microscopy, IL-9 negative and positive control cells consisted in wild-type and hIL-9-transfected BaF-3 cells, respectively (19).

ERK1/2 MAP kinase phosphorylation assay

Monocytes (1 × 10⁶) were preincubated for 24 h with cytokines (20 ng/ml) and stimulated from 5 min to 20 h by 1 μg/ml LPS. When indicated, monocytes were pretreated for 1 h with 100 μM PD98059 (a specific inhibitor of ERK1/2 phosphorylation; New England Biolabs, Beverly, MA), or for 15 min with 1 μM okadaic acid (OA) or 2.5 mM sodium orthovanadate (OV) as inhibitors of serine/threonine and tyrosine phosphatases, respectively (Sigma-Aldrich). Monocytes were lysed in ice-cold lysis
buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% Na deoxycholate, and 0.2% SDS) containing protease inhibitors (Roche Diagnostics) including freshly added 1 mM PMSF, and protein phosphatase inhibitors (25 mM NaF, 1 mM Na3VO4) from Sigma-Aldrich. Cell extracts (10 μg, as determined by the bicinchoninic acid-based assay) were subjected to SDS-12% PAGE, and electropherograms onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) immunblotted for both phosphorylated (on Thr202/Tyr204 residues) and total ERK1/2, using specific Abs and ECL (New England Biolabs).

Statistical analysis

Data were obtained from experiments performed in triplicates and repeated at least three times, and results are expressed as mean ± SEM, except when indicated. The differences observed between the different groups were analyzed by the Student t test using GraphPad InStat, San Diego, CA. Values of p < 0.05 were considered significant.

Results

Preincubation with IL-9 for 24 h inhibits the oxidative burst in activated monocytes

In preliminary experiments, PMA was shown to exert only a minor effect on DCFH oxidation (data not shown), but strongly stimulated cytochrome c reduction by monocytes (Fig. 1). Although incubation for 24 h with IL-9 had little effect on the basal oxidative burst, as also observed with IL-4 (data not shown), preincubation for 24 h with IL-9 significantly down-regulated the O2− release by PMA-stimulated monocytes (23.2 ± 0.8 vs 40.1 ± 2.5 nmol O2−/106 cells/h, p < 0.001; Fig. 1). The effect of IL-9 was not significant for shorter preincubation periods (1 and 4 h), and was not further increased for 96 h of preincubation (Fig. 1). The same inhibitory effect was observed with IL-9 which also started at 24 h of preincubation, but was significantly increased after 96 h. Moreover, an additive effect was observed between IL-9 and IL-4 after 96 h of preincubation. In contrast to IL-9 and IL-4, IFN-γ significantly up-regulated the O2− release by PMA-stimulated monocytes after 24 h of preincubation (Fig. 1).

Stimulation by LPS for 20 h increased DCFH oxidation (and cytochrome c reduction, data not shown) about 2-fold as compared with unstimulated monocytes (Fig. 2). Preincubation for 24 h with IL-9 down-regulated the LPS-stimulated oxidative burst in monocytes (10.7 ± 0.5 vs 17.0 ± 1.3 nmol DCF/mg protein, p < 0.001; Fig. 2) to its baseline level. A similar inhibitory effect was observed with IL-4. In contrast, preincubation with IFN-γ slightly increased the oxidative burst in monocytes, although this effect was not statistically significant (Fig. 2).

IL-9 inhibitory effect on the oxidative burst in LPS-stimulated monocytes is not abrogated by coincubation with IFN-γ

The influence of IFN-γ on the inhibition mediated by IL-9, as well as by IL-4, on the oxidative burst in LPS-stimulated mononuclear phagocytes was evaluated by coincubating monocytes with IL-9 or IL-4 and IFN-γ. Inhibition of the respiratory burst by IL-9 in LPS-stimulated monocytes was maintained in the presence of IFN-γ (Fig. 3). In contrast, IFN-γ completely abrogated the inhibitory effect of IL-4 on the oxidative burst in LPS-stimulated monocytes (Fig. 3).

IL-9 inhibitory effect on the oxidative burst in LPS-stimulated monocytes is specifically blocked by anti-IL-9R mAb

Preincubation of monocytes with neutralizing anti-hIL-9R mAb (AH9R7, 10 μg/ml) 1 h before addition of IL-9 abolished 90% ± 5 (mean ± SEM) of the IL-9 effect on LPS-stimulated DCFH oxidation, in comparison with the absence of blockade by control mlgG2b (Table I). Moreover, using the same mAb (as well as AH9R2 mAb), specific surface receptors for IL-9 were identified on human monocytes by FACs (Fig. 4A). A significant shift of the fluorescence histogram was observed when adherent monocytes were incubated with AH9R7 (or AH9R2) mAb, as compared with cells incubated with control mlgG, and revealed by SAM-FITC (Fig. 4A). The same pattern of staining, more intense with AH9R2 than with AH9R7 mAb, was observed on hIL-9R-transfected (and not on wild-type) Baf-3 cells (data not shown). Expression of IL-9R by human monocytes was also confirmed by confocal microscopy after staining with AH9R2 mAb (Fig. 4A, inset). In addition, a significant binding of IL-9 on the surface of monocytes was observed when these cells were incubated with chimeric IL-9-mlgG3 protein revealed by GAM3-FITC, as compared with control (Fig. 4B).
IL-9 down-regulates the production of TNF-α by LPS-stimulated monocytes

The release of TNF-α by monocytes, which was constitutively very low, was strongly increased by LPS stimulation (Fig. 5). Monocytes preincubated for 24 h with IL-9 before LPS stimulation released much less TNF-α than monocytes preincubated with medium alone (84.2 ± 17.2 vs 212.4 ± 34.1 pg/ml, p < 0.01). A similar effect was observed for monocytes preincubated with IL-4 (72.5 ± 21.5 vs 212.4 ± 34.1 pg/ml, p < 0.01). The combination of IL-9 and IL-4 did not induce a significant increase of the inhibitory effect observed with each cytokine alone. In contrast with IL-9 and IL-4, IFN-γ significantly potentiated the TNF-α release by LPS-stimulated monocytes (Fig. 5).

IL-9 does not modulate surface expression of LPS receptors on monocytes

CD14 and TLR4 expression at the surface of monocytes preincubated for 24 h with IL-9 was not significantly different from that on monocytes preincubated with medium alone (Table II). In contrast, monocyte expression of both CD14 and TLR4 was down-regulated by LPS-stimulated monocytes, which was constitutively very low, was strongly increased by LPS stimulation (Fig. 5). Monocytes preincubated for 24 h with IL-9 before LPS stimulation released much less TNF-α than monocytes preincubated with medium alone (84.2 ± 17.2 vs 212.4 ± 34.1 pg/ml, p < 0.01). A similar effect was observed for monocytes preincubated with IL-4 (72.5 ± 21.5 vs 212.4 ± 34.1 pg/ml, p < 0.01). The combination of IL-9 and IL-4 did not induce a significant increase of the inhibitory effect observed with each cytokine alone. In contrast with IL-9 and IL-4, IFN-γ significantly potentiated the TNF-α release by LPS-stimulated monocytes (Fig. 5).

### Table I. Specific blockade of the IL-9 inhibitory effect on oxidative burst in LPS-stimulated monocytes by anti-hIL-9R mAb

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Oxidative Burst (nmol DCF/mg protein)</th>
<th>Blockade of the IL-9 Inhibitory Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>10.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>17.0 ± 0.3</td>
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<tr>
<td>IL-9/LPS</td>
<td>10.7 ± 0.5</td>
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<tr>
<td>IL-9 + mlgG2b/LPS</td>
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<tr>
<td>IL-9 + anti-IL-9R/LPS</td>
<td>16.4 ± 0.3*</td>
<td>90 ± 5</td>
</tr>
</tbody>
</table>

*Results are shown as mean ± SEM obtained from three experiments, triplicate conditions being performed in each experiment.

#p < 0.001 compared with monocytes preincubated with IL-9 (with or without control mlgG2b).

Inhibition by IL-9 of the oxidative burst and TNF-α release in LPS-stimulated monocytes depends on TGF-β1

To investigate the mechanism of inhibition used by IL-9 in LPS-stimulated monocytes, we examined the potential requirement of known monocyte/macrophage deactivating factors, namely IL-10 and TGF-β. Although anti-IL-10R neutralizing mAb failed to suppress the inhibitory effect of IL-9, preincubation of monocytes with anti-TGF-β1 mAb significantly inhibited the IL-9 effect on both LPS-stimulated respiratory burst (Table III) and TNF-α release (Fig. 6). Moreover, exogenous TGF-β1 (20 ng/ml) inhibited the respiratory burst in LPS-stimulated monocytes (10.2 ± 0.5 vs...
16.2 ± 0.4 nmol DCF/mg protein, \( p < 0.001 \) to the same extent than IL-9, and this effect was suppressed at 76% by the anti-TGF-β1 mAb (15.4 ± 0.6 vs 10.2 ± 0.5 nmol DCF/mg protein, \( p < 0.001 \)), but not by anti-IL-9 mAb (10.4 ± 0.5 vs 10.2 ± 0.5 nmol DCF/mg protein, NS). Exogenous TGF-β was also shown to inhibit the TNF-α release by LPS-activated monocytes (112.8 ± 28.4 vs 214.5 ± 30.9, \( p < 0.001 \)). In addition, a blockade of endogenous TGF-β by specific mAb enhanced the TNF-α response of monocytes to LPS (Fig. 6). In contrast with IL-9, the inhibition by IL-4 of the production of oxygen metabolites and TNF-α was not significantly suppressed by anti-TGF-β1 mAb (data not shown).

**IL-9 down-regulates the IL-10 release by LPS-stimulated monocytes, but strongly potentiates the production of TGF-β1**

IL-10 was not detectable in supernatants from unstimulated monocytes, but IL-10 release was strongly induced by LPS (Fig. 7A). IL-9 down-regulated the LPS-induced IL-10 release by monocytes (119.7 ± 8.7 vs 217.8 ± 25.8 pg/ml, \( p < 0.01 \)). The IL-10 release by LPS-stimulated monocytes was also inhibited by both IL-4 and IFN-γ (Fig. 7A).

Although no modulation of TGF-β1 was observed in unstimulated monocytes pretreated with IL-9 (data not shown), the production of TGF-β1 by LPS-stimulated monocytes was strongly potentiated by IL-9 (1687 ± 94 vs 586 ± 64 pg/ml in acid-treated supernatants, \( p < 0.001 \); Fig. 7B). This effect was not observed with IL-4 nor with IFN-γ. Moreover, IL-9-mediated TGF-β1 up-regulation in monocytes was specifically inhibited by the neutralizing anti-IL-9R mAb (857 ± 89 vs 1687 ± 94 pg/ml, \( p < 0.001 \); Fig. 7B), and not by control mlgG2b.

**PD98059 inhibits ERK1/2 activation and oxidative burst in LPS-stimulated monocytes**

LPS was shown to induce ERK1/2 phosphorylation in monocytes, which increased after 15 min of stimulation, peaked at 30 min, and returned to its baseline level after 20 h. Pretreatment of monocytes with 100 μM PD98059, a specific inhibitor of ERK kinase (MAPK/ERK kinase, MEK), completely suppressed LPS-induced ERK phosphorylation as shown at 30 min (Fig. 8A). Interestingly, PD98059 blocked the LPS effect on the oxidative burst in monocytes as compared with the absence of effect of DMSO control (Fig. 8B). In addition, no significant inhibition was observed on the oxidative burst in LPS-stimulated monocytes pretreated with p38 MAPK inhibitor SB203580 (data not shown). Therefore, ERK pathway was evaluated as a potential target for IL-9-induced monocyte deactivation.

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**FIGURE 5.** Effect of IL-9, IL-4, and IFN-γ on the release of TNF-α by LPS-stimulated monocytes. Cells were preincubated for 24 h with medium alone or cytokines (20 ng/ml for IL-9 or IL-4 and 200 U/ml for IFN-γ) before the stimulation by LPS (1 μg/ml) for 20 h. TNF-α was determined in supernatants by a cytotoxic bioassay using WEHI 164 clone 13 target cells, as described in Materials and Methods. Data are mean ± SD (n = 3). *, \( p < 0.001 \) compared with unstimulated cells; **, \( p < 0.05 \) compared with cells preincubated with medium.

**FIGURE 6.** Effect of anti-TGF-β1 and anti-IL-10Rβ mAbs on the IL-9-mediated inhibition of TNF-α production in LPS-stimulated monocytes. Monocytes were pretreated with medium alone (control) or with neutralizing mAb, either against TGF-β1 or IL-10Rβ (30 μg/ml), or with control mlgG1 (30 μg/ml), 2 h before incubation with medium or IL-9 (20 ng/ml) for 24 h, and stimulated by 1 μg/ml LPS for 20 h. Biologically active TNF-α was determined in supernatants, as described in Materials and Methods. Data are mean ± SD (n = 3), and are representative of two experiments. *, \( p < 0.001 \) compared with monocytes pretreated with medium alone or control mlgG1.

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<table>
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\* Monocytes (0.2 × 10⁶) were preincubated with neutralizing mAb (30 μg/ml), either against TGF-β1 or against IL-10Rβ, or with control mlgG1 (30 μg/ml), 2 h before incubation with IL-9 (20 ng/ml) for 24 h without removing the mAb, and stimulated by LPS (1 μg/ml) for 20 h. Intracellular oxidative capacity was evaluated by the DCFH oxidation assay. Data are mean ± SD (n = 3), and are representative of two experiments.

\* *, \( p < 0.001 \) compared with monocytes pretreated with IL-9 without anti-TGF-β1 mAb.

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<table>
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<tr>
<th>Cell Treatment</th>
<th>CD14 Expression (MFI)</th>
<th>TLR4 Expression (MFI)</th>
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<td>107 ± 4</td>
<td>73 ± 2</td>
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<tr>
<td>IL-9</td>
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<td>71 ± 4</td>
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<td>IL-4</td>
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<td>58 ± 1*</td>
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<tr>
<td>IFN-γ</td>
<td>121 ± 6*</td>
<td>82 ± 2*</td>
</tr>
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\* Monocytes (0.2 × 10⁶) were preincubated for 24 h with cytokines (20 ng/ml for IL-9 or IL-4, and 200 U/ml for IFN-γ), before the assessment of LPS receptor expression by immunostaining using anti-human CD14 mAb conjugated to FITC and anti-human TLR4 rabbit Ab followed by MAR-FITC. Cell-associated fluorescence was evaluated by FACS and expressed as mean fluorescence intensity (MFI). Autofluorescence of monocytes was negligible (MFI, 3 ± 1). Data are mean ± SD (n = 3), and are representative of two experiments.

\* *, \( p < 0.05 \) compared with monocytes preincubated with medium.
IL-9 inhibits ERK1/2 activation in LPS-stimulated monocytes

Although the induction of ERK phosphorylation by LPS was maintained in monocytes preincubated for 24 h with IL-9 as compared with medium alone (Fig. 9, 30 min), the subsequent level of phosphorylated ERK was strongly down-regulated by IL-9 (Fig. 9, 60–240 min). A similar pattern of late ERK inhibition was observed with TGF-β1, whereas IL-4 suppressed the induction of ERK activation as observed from 30 to 240 min (Fig. 9).

In contrast with IL-4, the inhibitory effect of IL-9 on ERK phosphorylation in monocytes was suppressed by anti-TGF-β1 mAb (Fig. 10), as already observed for the oxidative burst and TNF-α release. Moreover, the kinetics of ERK inhibition by IL-9 and TGF-β1 suggested an “accelerated dephosphorylation” of ERK rather than an inhibition of its phosphorylation. In addition, treatment of monocytes with the phosphatase inhibitor OA reversed the effect of IL-9 and TGF-β1 on ERK activation, while no significant effect was observed in monocytes preincubated with IL-4 or medium alone, nor with the tyrosine phosphatase inhibitor OV (Fig. 10).

Discussion

This study identifies human monocytes as new target cells for IL-9. Effects of IL-9 have been described so far on mast cells, T and B lymphocytes, hematopoietic progenitors, and lung epithelial cells. Although IL-9 was initially observed to bind to some mouse macrophage cell lines (20), no biological effect has previously been described on mononuclear phagocytes. In this study, we demonstrate that IL-9 exhibits inhibitory properties on several important monocyte functions, such as the respiratory burst and cytokine release. Hence, our data show that IL-9 inhibits the production of reactive oxygen intermediates (ROI) by activated human blood monocytes, such as H$_2$O$_2$ and O$_2^-$ after LPS and PMA stimulation, respectively. This inhibitory effect is due to the specific interaction of IL-9 with its receptor since it was almost completely abrogated by a mAb against hIL-9Rα-chain (AH9R7 mAb). Moreover, we demonstrated the presence of specific receptors for IL-9 on human monocytes using anti-hIL-9Rα mAbs and chimeric IL-9 protein. The inhibition by IL-9 of the oxidative burst in monocytes was dependent on a preincubation period of minimum 24 h before stimulation with LPS or PMA, as also observed with IL-4 (21). The monocyte deactivating property of IL-9 mimics that previously described with IL-4 (21, 22). However, the inhibition mediated by IL-4 is abolished by IFN-γ (23), which is known to prime monocytes notably for the production of ROI. The absence of antagonistic effect on IL-9 by IFN-γ was not due to a down-regulation by IL-9 of the expression of IFN-γ receptor on monocytes (data not

FIGURE 7. Effect of IL-9, IL-4, and IFN-γ on the release of IL-10 (A) and TGF-β1 (B) by LPS-stimulated monocytes cultured as described in Fig. 5. TGF-β1 was determined by ELISA in “untreated” supernatants as IL-10, and in “acid-treated” supernatants, as described in Materials and Methods. A specific blockade of the IL-9 effect on TGF-β1 production was performed by preincubating monocytes 2 h before IL-9 with anti-IL9R mAb (AH9R7, 10 µg/ml). Data are mean ± SEM (n = 3). *, p < 0.05 compared with unstimulated monocytes; **, p < 0.05 compared with monocytes preincubated with medium.

FIGURE 8. LPS-induced ERK phosphorylation (A) and effect of PD98059 on LPS-stimulated oxidative burst (B) in monocytes. Cells were treated with 100 µM PD98059 (or with the same volume of DMSO as control) 1 h before preincubation for 24 h with medium or IL-9 (20 ng/ml). A, For the phosphoERK immunoblot assay, monocytes were then stimulated by LPS (1 µg/ml) for the indicated periods of time (5 min to 20 h) as compared with unstimulated monocytes (med), and processed for the detection of phosphorylated and total ERK1/2 as described in Materials and Methods. B, For the oxidative burst, monocytes were stimulated by LPS (1 µg/ml) for 20 h and their oxidative capacity was evaluated through DCFH oxidation. Data are mean ± SD (n = 3), and are representative of two experiments. *, p < 0.001 compared with cells treated with DMSO alone, without PD98059.
shown). This major difference between deactivation by IL-9 and by IL-4 suggested that IL-9 uses a different mechanism to mediate its effect on monocytes, a possibility supported by the additive effects of IL-9 and IL-4 observed on the PMA-stimulated oxidative burst.

Cytokine release is a second monocyte function modulated by IL-9. Monocytes incubated with IL-9 showed a decreased production of TNF-α in response to LPS, as also observed with IL-4 in the present study and previously by others (24, 25). Inhibition of the release of inflammatory mediators including TNF-α by LPS-stimulated monocytes has also been described for other Th2 cytokines such as IL-10 and IL-13 (26, 27), as well as in mouse macrophages treated with TGF-β (28), whereas IFN-γ potentiates the TNF-α release by LPS-activated monocytes (29).

Modulation of surface LPS receptors was evaluated as a potential mechanism of cytokine-mediated regulation of the oxidative burst and cytokine release in LPS-stimulated monocytes. IL-4 down-regulated CD14 expression as previously shown on blood monocytes (30), and also significantly inhibited surface expression of TLR4. In contrast, IL-9 did not modulate surface expression of CD14 nor TLR4 on monocytes. Because IL-10 was identified as a major monocyte-suppressing factor (26), inhibiting the production of inflammatory mediators such as TNF-α and ROI by monocytes, we then evaluated the regulation of its release by IL-9. A down-regulation of the LPS-induced production of IL-10 was observed in monocytes preincubated with IL-9. Moreover, neutralization of IL-10 activity by anti-hIL-10R mAb failed to abrogate the IL-9 effect, as well as that of IL-4, on the respiratory burst in LPS-stimulated monocytes. Thus, IL-9 and IL-4 deactivate human monocytes through (an) IL-10-independent mechanism(s). In addition, both IL-4 and IFN-γ were also found to suppress LPS-induced IL-10 release by monocytes, supporting previous studies (31, 32). Interestingly, deactivation by IL-9 of the respiratory burst and TNF-α release in LPS-activated monocytes was significantly inhibited by a mAb neutralizing TGF-β. This is in striking contrast with the inhibitory effect of IL-4 which appeared independent of TGF-β, as previously reported (21). Moreover, IL-9 (and not IL-4) strongly potentiated the production of TGF-β by LPS-stimulated monocytes. We also confirmed that TGF-β, described as another important macrophage-deactivating cytokine (28), was able (in our experimental conditions) to down-regulate the oxidative burst and TNF-α release in LPS-stimulated monocytes. In addition, experiments with anti-TGF-β mAb showed that endogenous TGF-β limits the LPS-induced stimulation of the oxidative burst and TNF-α production in monocytes. Taken together, these results indicate that TGF-β is induced by IL-9 in LPS-activated monocytes and mediates, at least partly, the inhibitory effect of IL-9 on the production of ROI and TNF-α release. Similarly, in mouse mast cells, it was previously suggested that TGF-β is required for the IL-9-potentiated expression and secretion of mast cell protease-1 (33).

The ERK MAPK pathway plays a key role in the control of monocyte/macrophage activation by LPS, as demonstrated for TNF-α release (34). Although ERK may regulate the phosphorylation of p47phox, a subunit of NADPH oxidase (35), induction by LPS of the oxidative burst in neutrophils depends only partly on this MAPK pathway (36). In monocytes, we demonstrate that ERK activation is necessary for the stimulation by LPS of the oxidative

![FIGURE 9. Effect of IL-9, IL-4, and TGF-β1 on ERK phosphorylation in monocytes. Cells were preincubated for 24 h with medium alone (med) or IL-9, IL-4, or TGF-β1 (20 ng/ml), and stimulated by LPS (1 μg/ml) for the indicated periods of time (30–240 min). When indicated, monocytes were pretreated with 100 μM PD98059 1 h before LPS stimulation. Cell lysates were processed for the detection of phosphorylated and total ERK1/2 as described in Materials and Methods.](http://www.jimmunol.org/content/journals/162/6/4108/F9.large.jpg)

![FIGURE 10. Effect of anti-TGF-β1 mAb and protein phosphatase inhibitors on cytokine-mediated ERK inhibition in monocytes. Cells were pretreated with 30 μg/ml anti-TGF-β1 mAb (Ab) for 2 h (without being removed), or with 1 μM OA or 2.5 mM OV for 15 min (and removed), before preincubation with medium alone, IL-9, IL-4, or TGF-β1 (20 ng/ml) for 24 h. Monocytes were then stimulated by 1 μg/ml LPS for 240 min and lysed to assess phosphorylated and total ERK as described in Materials and Methods.](http://www.jimmunol.org/content/journals/162/6/4108/F10.large.jpg)
burst, since PD98059, a specific inhibitor of ERK phosphorylation, completely suppressed the LPS effect on ROI production. Interestingly, we found that IL-9 pretreatment inhibits ERK activation in LPS-stimulated monocytes, as shown with IL-4 (37), and with TGF-β in murine macrophages (38). Moreover, and in contrast with IL-4, the mechanism of ERK inactivation by IL-9 appeared dependent both on TGF-β and on a protein phosphatase activity. Similarly, it has been reported that ERK inhibition by TGF-β in pancreatic carcinoma cells was abrogated by the protein phosphatase inhibitor OA (39). Interestingly, it has been shown that OA-sensitive protein phosphatase 2A can dephosphorylate and deactivate ERK in vitro (40). Thus, in contrast with IL-4, which affects LPS binding to monocytes, our results indicate that IL-9 deactivates LPS-stimulated human monocytes through a TGF-β-mediated dephosphorylation of ERK1/2 MAP kinases.

Stimulation of the growth and/or activation state of Th2 lymphocytes and mast cells, as well as induction of hypereosinophilia, are thought to explain both beneficial and deleterious activities of IL-9 in Th2-related disorders, such as parasitic infections or asthma. The present finding that mononuclear phagocytes are regulated by IL-9 may be more specifically relevant to inflammatory disorders, such as sepsis, in which monocyte/macrophage activation plays a central role. Interestingly, it was recently shown that administration of IL-9 prevented mortality in mice challenged with *P. aeruginosa* (13). Moreover, this beneficial effect was dependent on a prophylactic administration of IL-9 because no improvement in survival was observed when rIL-9 was injected concomitantly or after the infectious challenge. In this model, IL-9 treatment was associated with the suppression of serum TNF-α, as well as IL-12/P40 and IFN-γ. However, in contrast with TNF-α, which is reduced by IL-9 both in this model and in our study, IL-10 was up-regulated in serum from IL-9-treated mice challenged with *P. aeruginosa*. This apparent discrepancy between the endotheliosis in vivo model and our results might be due to alternative regulatory pathways. In additional experiments, we showed that in contrast with the down-regulation of IL-10 release in monocytes, IL-10 is up-regulated in supernatants from unseparated blood mononuclear cells treated with IL-9, and not in lymphocytes (data not shown), suggesting an interplay between monocytes and lymphocytes in culatures. A protection of mice from lethal endotheliosis was shown with other Th2 cytokines, namely IL-4, IL-10, and IL-13 (13, 41–44); and was associated with a reduction of TNF-α production. Th2 cytokine-mediated protection in vivo models of exaggerated inflammatory response is thought to be related to the capacity observed in vitro of these cytokines to deactivate mononuclear phagocytes. Our finding that IL-9 prevents in vitro the release of toxic ROI and TNF-α by monocytes stimulated by LPS might thus explain the beneficial in vivo anti-inflammatory activity of IL-9 observed in LPS-induced systemic inflammation.

In conclusion, we have shown that IL-9 pretreatment inhibits the oxidative burst and TNF-α release in LPS-activated human monocytes. Moreover, we suggest that the mechanism of this deactivation involves the induction by IL-9 of TGF-β secretion by activated monocytes which, in turn, inhibits their production of ROI and TNF-α through ERK inactivation. These findings highlight a new target cell for IL-9, and support the concept of monocyte deactivation by Th2 cytokines which may be of crucial importance to maintain host tissue integrity during inflammatory processes.

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