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IL-4 Pretreatment Selectively Enhances Cytokine and Chemokine Production in Lipopolysaccharide-Stimulated Mouse Peritoneal Macrophages

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Although well recognized for its anti-inflammatory effect on gene expression in stimulated monocytes and macrophages, IL-4 is a pleiotropic cytokine that has also been shown to enhance TNF-α and IL-12 production in response to stimulation with LPS. In the present study we expand these prior studies in three areas. First, the potentiating effect of IL-4 pretreatment is both stimulus and gene selective. Pretreatment of mouse macrophages with IL-4 for a minimum of 6 h produces a 2- to 4-fold enhancement of LPS-induced expression of several cytokines and chemokines, including TNF-α, IL-1α, macrophage-inflammatory protein-2, and KC, but inhibits the production of IL-12p40. In addition, the production of TNF-α by macrophages stimulated with IFN-γ and IL-2 is inhibited by IL-4 pretreatment, while responses to both LPS and dsRNA are enhanced. Second, the ability of IL-4 to potentiate LPS-stimulated cytokine production appears to require new IL-4-stimulated gene expression, because it is time-dependent, requires the activation of STAT6, and is blocked by the reversible protein synthesis inhibitor cycloheximide during the IL-4 pretreatment period. Finally, IL-4-mediated potentiation of TNF-α production involves specific enhancement of mRNA translation. Although TNF-α protein is increased in IL-4-pretreated cells, the level of mRNA remains unchanged. Furthermore, LPS-stimulated TNF-α mRNA is selectively enriched in actively translating large polyribosomes in IL-4-pretreated cells compared with cells stimulated with LPS alone. The Journal of Immunology, 2002, 168: 2456–2463.

Interleukin-4 is a multipotent cytokine associated with the modulation of host defense and immunity through action on a variety of cell types (1, 2). Perhaps best appreciated is the pivotal role of IL-4 in regulating T and B lymphocyte differentiation where it is required for the development of type 2 immune responses (1–3). In addition to and as part of this role, IL-4 is well recognized as a modulator of macrophage activation (4–9). In this context it is frequently perceived as an anti-inflammatory cytokine that suppresses the production of proinflammatory gene expression initiated in response to such stimuli as LPS and IFN-γ. As many LPS- and/or IFN-γ-stimulated macrophage genes are important in controlling the adaptive immune response, the anti-inflammatory action of IL-4 on macrophages may be an important component of the effect of IL-4 on Th2 cell differentiation in vivo (1, 2).

There are, however, a number of studies in which IL-4 treatment, either in vitro or in vivo, is associated with proinflammatory or type 1 immune responses (10–13). For example, IL-4 in combination with GM-CSF can promote the differentiation of monocytes into dendritic cells that may exhibit enhanced ability to support type I T cell responses (10, 11, 13). Furthermore, IL-4 treatment in vivo can exacerbate a Th1-dependent model of colitis (12). In such circumstances, IL-4 may be expected to promote proinflammatory gene expression, and indeed, pretreatment of human monocytes or mouse macrophages has been shown to enhance the production of cytokines such as TNF-α and IL-12p70 in response to stimulation with LPS (6, 14, 15). In the present study we wished to expand upon prior findings and determine whether other LPS-induced genes may be subject to IL-4-dependent potentiation and to explore the mechanisms involved. The results demonstrate that the enhancing effects of IL-4 are gene and stimulus selective. Furthermore, it appears to depend upon IL-4-induced, STAT6-mediated new protein synthesis and operates at least in part by modulating the translation of specific mRNAs.

Materials and Methods

Reagents

Brewer’s thioglycolate (TG) broth was purchased from Difco (Detroit, MI). LPS prepared from the Escherichia coli serotype 0111:B4 was purchased from Sigma-Aldrich (St. Louis, MO), rIFN-γ, RPMI 1640, antibiotics, and glutamine were purchased from Life Technologies (Gaithersburg, MD). Recombinant murine IL-2 was obtained from Genzyme (Cambridge, MA). FBS was obtained from HyClone Laboratories (Logan, UT) and was heat-inactivated before use. All cell culture reagents were specified to be endotoxin free. Cesium chloride, guanidine thiocyanate, agarose, SDS, Tris, protease K, RNase-free DNase, and random priming kits were purchased from Roche (Indianapolis, IN). Formamide was obtained from U.S. Biochemical (Cleveland, OH). Dextran sulfate was obtained from Pharmacia Biotech (Uppsala, Sweden). Nylon transfer membrane was purchased from Micron Separations (Westborough, MA). DuPont NEN Research Products (Boston, MA) was the source of [α-32P]dCTP. ELISA kits for TNF-α, IL-1α, IL-12p40, KC, macrophage-inflammatory protein-2 (MIP-2), and monocyte chemoattractant protein-1 (MCP-1) were obtained from R&D Systems (Minneapolis, MN).

Mice

Specific pathogen-free, inbred C57BL/6 mice, 8–12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in microisolator cages with autoclaved food and bedding to minimize exposure to viral and microbial pathogens. Mice deficient in STAT6 were maintained as described previously (16, 17). STAT6−/− heterozygotes were
crossed, and the genotype of STAT6+/− and STAT6−/− littersmates was determined by PCR with primers specific for the neomycin resistance cassette used to disrupt the STAT6 gene (18).

**Cell culture**

TG-elicited macrophages were obtained as reported previously (19). Peritoneal lavage was performed using 10 ml cold HBSS containing 10 U/ml heparin. Macrophages were plated in plastic petri dishes, incubated for 2 h at 37 °C in an atmosphere of 5% CO2, and then washed three times with HBSS to remove nonadherent cells. The macrophages were cultured overnight in RPMI 1640 containing 10% FBS, penicillin, and streptomycin at 37 °C in 5% CO2 and then cultured in the presence or the absence of stimuli for the indicated times.

**Preparation of plasmid DNA**

The plasmids encoding TNF-α and GAPDH were prepared as described previously (20).

**Cytokine ELISA**

TNF-α, IL-1α, KC, MIP-2, and IL-12p40 protein levels were measured in cell culture supernatants using ELISA kits according to the manufacturer’s instructions.

**Preparation of RNA and Northern hybridization analysis**

Total cellular RNA was prepared by the guanidine thiocyanate-cesium chloride method (21). Equal amounts of RNA (10 μg) were loaded in each lane of the gel. The RNA was denatured, separated by electrophoresis in a 1% agarose-formaldehyde gel, and transferred to a nylon membrane as previously described (19). The blots were prehybridized 12–24 h at 42 °C in 50% formamide, 1% SDS, 5× SSC, 1× Denhardt’s solution (0.02% Ficoll, 0.02% BSA, and 0.02% polyvinylpyrrolidone), 0.25 mg/ml denatured herring testis DNA, and 50 mM sodium phosphate buffer, pH 6.5. Hybridization was conducted at 42 °C for 18 h with 1 × 106 cpm denatured plasmid DNA containing appropriate specific cDNA inserts. The filters were rinsed with a solution of 0.1% SDS/0.2% SSC and washed at 42 °C for 1 h and at 65°C for 15 min. The blots were dried and exposed using XAR-5 x-ray film (Eastman Kodak, Rochester, NY) with DuPont (Wilmingon, DE) Cronex Lightening Plus intensifying screens at −70°C. Blots were quantified by phosphorescence analysis using an instrument from Molecular Dynamics (Sunnyvale, CA).

**Polyribosome analysis**

The distribution of mRNA within polyribosomes was assessed as described by Pieczyk et al. (22) with some modifications. Peritoneal macrophages from C57BL/6 mice were cultured as described above at a density of 5 × 106 cells/ml buffer (formamide, formaldehyde, and MOPS), heated to 55°C for 10 min, and then frozen and thawed 3 × in the presence or the absence of IL-4. Cells were washed twice in ice-cold PBS containing 10 μg/ml cycloheximide (CHX) and scraped into 1 ml ice-cold lysis buffer (140 mM KCl, 1 mM DTT, 20 mM Tris (pH 8.5), 5 mM MgCl2, 0.5% Nonident P-40, 0.5 μM RNasin, 10 mM CHX, and protease inhibitor mix (Roche)). Nuclei were removed by centrifugation at 2000 × g for 10 min. The supernatant was layered onto a 10.5 ml 20–60% (v/v) continuous sucrose gradient. Centrifugation was performed at 40,000 rpm for 3 h and 15 min using an SW41 rotor (Beckman Coulter, Fullerton, CA). One-milliliter fractions were collected from the top of the gradient, and UV absorption at 254 nm was monitored continuously. Five hundred microliters of each fraction was digested with 0.5 mg/ml protease K in the presence of SDS (0.2% final concentration) and EDTA (5 mM final concentration) at 37°C for 10 min, extracted in phenol-chloroform, precipitated with ethanol, and resuspended in deionized pyrrocar-bonate-treated H2O. Part (15.4 μl) of each sample was then added to 49.6 μl buffer (formamide, formaldehyde, and MOPS), heated to 55°C for 10 min before application to a nylon membrane using a dot-blott apparatus, and rinsed with 2× SSC. Hybridization was performed with [32P]dCTP-labeled TNF-α cDNA as described above. Blots were subsequently stripped and hybridized with cDNA encoding GAPDH.

**Results**

**Macrophage pretreatment with IL-4 selectively potentiates LPS-induced cytokine and chemokine production**

Several recent studies indicate that IL-4 can, under select circumstances, promote type 1 immune responses both in vivo and in vitro (11, 12, 15, 23). This may reflect the ability of IL-4 to modulate proinflammatory gene expression in cells of the innate im-
mune system that support and/or direct the character of T cell-dependent adaptive immune responses (4, 13, 24). To explore this hypothesis, the effect of IL-4 treatment on the expression of a spectrum of LPS-inducible proinflammatory cytokine and chemokine genes was assessed in cultured primary macrophages. Elicited peritoneal macrophages from C57BL/6 mice were stimulated with LPS in the presence of IL-4 or following pretreatment with IL-4 for 18 h, and the supernatant culture medium was tested for the presence of several cytokines (TNF-α, IL-1α, and IL-12p40) and chemokines (KC and MIP-2; Fig. 1). As expected, LPS treatment alone induced strong expression for all cytokine and chemokine products measured. If IL-4 was added at the same time as LPS, the expression of TNF-α, IL-1α, KC, and MIP-2 was not affected, while the levels of IL-12p40 secreted were reduced by ~40%. In contrast, macrophage cultures that had been exposed to IL-4 for 18 h before stimulation with LPS showed a significant increase in cytokine and chemokine expression. IL-12p40 was selectively inhibited under the same conditions. The enhanced cytokine and chemokine expression induced by IL-4 pretreatment is cooperative with the initiating stimulus LPS, because IL-4 alone did not produce detectable expression of any of the four gene products.

These data indicate that the proinflammatory response to LPS seen in cultures pretreated with IL-4, although selective, impacts on the expression of a diverse group of cytokines and chemokines. In a second experiment we determined whether the proinflammatory effects of IL-4 were dependent upon the nature of the initiating proinflammatory stimulus. Cultured elicited macrophages were pretreated, or not, with IL-4 and then stimulated for 18 h with LPS, a combination of IFN-γ and IL-2, or poly(IC), a model ligand used as a source of dsRNA (25) (Fig. 2). In confirmation of the first experiment, LPS-stimulated TNF-α expression was markedly enhanced in cells pretreated with IL-4, and a comparable response was seen in cells stimulated with poly(IC). This was in contrast to the effects of IL-4 treatment on subsequent response to stimulation with a combination of IFN-γ and IL-2. While IFN-γ/IL-2 is an effective stimulus of TNF-α production (26), IL-4 suppresses the response to LPS regardless of the time of administration. The finding that IL-4 can selectively inhibit IFN-γ/IL-2-stimulated, but not LPS-stimulated, TNF-α expression has been previously reported (20) and suggests that the mechanisms through which these different stimulatory conditions lead to TNF-α production are distinct from one another.

IL-13 shares many properties with IL-4, including use of receptors that contain the IL-4R α-chain, activation of similar signal transduction pathways, and promotion of similar biological functions (27, 28). Pretreatment of macrophages with IL-13 was also able to potentiate the induction of TNF-α by LPS (Fig. 3). The proinflammatory action of IL-4 requires STAT6-dependent new gene expression

The proinflammatory effect of IL-4 is obtained after 18–24 h treatment of macrophages. To more precisely define the time dependence of this effect, macrophage cultures were pretreated for the indicated times with IL-4 before stimulation with LPS for 18 h and measurement of TNF-α in supernatant culture medium (Fig. 4). Within 2 h small increments in LPS-induced TNF-α production were seen, and this increased with longer pretreatment times through a full 24 h. This finding suggests that IL-4 enhances sensitivity to LPS by inducing changes in the macrophage phenotype.

To determine whether this change required the synthesis of new proteins, cultures were pretreated with IL-4 for 16 h in the presence or the absence of the reversible protein synthesis inhibitor CHX. The inhibitor was removed from the culture before stimulation with LPS for an additional 8 h. The inclusion of CHX in the
culture medium during the IL-4 pretreatment period prevented the enhancement of TNF-α production. Cultures pretreated with CHX alone showed normal TNF-α production following exposure to LPS (Fig. 5A). These findings suggest that the enhanced production of cytokines in IL-4-pretreated macrophages may depend upon the expression of new gene products induced via the activation of STAT6 by IL-4. To test this hypothesis, the pretreatment effects of IL-4 were determined in macrophages obtained from mice deficient in STAT6 (Fig. 5B). Elicited peritoneal macrophages obtained from STAT6−/− littermates were pretreated, or not, with IL-4 for 16 h and stimulated with LPS for an additional 8 h before measurement of TNF-α production. The behaviors of STAT6+/+ and wild-type C57BL/6 mice were indistinguishable, and both showed a >2-fold increase in TNF-α. In contrast, STAT6−/− macrophages were normally responsive to LPS but showed no IL-4-dependent enhancement of LPS-stimulated TNF-α production.

**IL-4-enhances translation of TNF-α mRNA**

IL-4 pretreatment may enhance the production of TNF-α through a variety of mechanisms, including increased gene transcription, decreased degradation of cytoplasmic TNF-α mRNA, enhanced TNF-α mRNA translation, or enhanced secretion of TNF-α protein. The ratio of intracellular to secreted TNF-α was comparable in untreated and IL-4-pretreated macrophages after stimulation with LPS (data not shown). Hence, IL-4 pretreatment does not appear to alter the rate of post-translational processing and/or secretion of TNF-α. Furthermore, the time course of LPS-stimulated TNF-α production was also comparable in untreated and IL-4-pretreated macrophages, indicating that the difference does not reflect an alteration in the kinetics of the response (Fig. 6A). When levels of TNF-α mRNA were assessed over the full time course of the response by Northern hybridization, no differences between IL-4-pretreated and untreated cultures were observed, indicating that the enhanced TNF-α production did not result from alterations in the rates of TNF-α gene transcription or mRNA decay (Fig. 6B). These findings strongly suggest that the enhanced production of TNF-α protein reflects changes in the rate of mRNA translation.

To assess this directly, the distribution of TNF-α mRNA within polyribosomes was measured (22). If IL-4 pretreatment alters the rate of translation of TNF-α mRNA, this may be reflected by TNF-α mRNA association with larger polyribosomes. Cytoplasmic
extracts were prepared from untreated or IL-4-pretreated macrophages following stimulation with LPS for 3 h (to achieve peak levels of mRNA). The extracts were sedimented through 20–60% sucrose gradients to separate single ribosomes and small and large polyribosomes, and individual gradient fractions were used to determine the levels of specific mRNA encoding either TNF-α or the housekeeping gene GAPDH (Fig. 7). Fig. 7A illustrates the distribution of single ribosomes and polyribosomes within the gradient as determined by absorbance at 254 nm. In cultures treated with LPS alone, TNF-α mRNA was found in fractions likely to contain free mRNA and smaller polyribosomes (Fig. 7, B and C). The TNF-α mRNA pool was clearly distinguished from that encoding GAPDH, which sedimented in heavier fractions composed of larger polysomes. In contrast, in cells that were pretreated with IL-4 and then stimulated with LPS, the TNF-α mRNA distribution was markedly shifted and cosedimented with the larger polysomes containing GAPDH mRNA. This finding of sequence-specific change in the ribosome association of TNF-α mRNA indicates that the translation of TNF-α mRNA has been selectively altered as a result of IL-4 pretreatment.

**FIGURE 3.** IL-4 and IL-13 can each potentiate LPS-stimulated TNF-α production. TG-elicited macrophages were treated, or not, with IL-4 (10 ng/ml) or IL-13 (10 ng/ml) for 24 h before or at the same time as stimulation with LPS (10 ng/ml) for 18 h. Culture supernatants were used to measure TNF-α protein production by ELISA. Each value is the mean of duplicate determinations, and similar results were obtained in two separate experiments.

**FIGURE 4.** The enhancement by IL-4 of LPS-stimulated TNF-α production requires a minimum of 6 h of pretreatment. TG-elicited peritoneal macrophages were treated with IL-4 (10 ng/ml) or IL-13 (10 ng/ml) for 24 h before or at the same time as stimulation with LPS (10 ng/ml) for 18 h. Culture supernatants were used to measure TNF-α production by ELISA. Each value represents the mean of duplicate determinations, and similar results were obtained in two separate experiments.
Discussion

IL-4 was first recognized as a B cell differentiation factor promoting the production of select classes of Ig and is known to promote expression of class II MHC molecules in some cell types (1, 2, 4). This cytokine is now widely recognized as one of the major determinants of T cell differentiation and is requisite for the development of type 2 T cell-mediated immune responses with the attendant anti-inflammatory potential (1–3). A major component of this activity involves the ability of IL-4 to suppress a broad range of inducible proinflammatory genes in monocytes and macrophages, including TNF-α and IL-12p40 (4, 5, 7–9). In most cases these inhibitory effects have been shown to target the transcription of inducible inflammatory gene products. A number of recent studies, however, have reported that pretreatment of monocytes and macrophages with IL-4 can enhance the production of select proinflammatory gene products (6, 14, 15). Furthermore, multiple findings suggest that IL-4 can promote type I, proinflammatory T cell responses (10–13). The intent of the present study was to assess the spectrum of genes whose expression may be subject to IL-4-dependent enhancement and to explore the molecular mechanisms through which this is accomplished. Our current findings expand the understanding of this behavior by demonstrating 1) that pretreatment, but not simultaneous treatment, of macrophages with IL-4 potentiated the expression of a select set of proinflammatory genes in a stimulus-selective fashion; 2) that this effect of IL-4 is dependent upon IL-4-induced STAT6-mediated new gene expression; and 3) that the IL-4-induced potentiation of proinflammatory gene expression is mediated by enhanced cytokine mRNA translation.

The effects of IL-4 pretreatment on LPS-stimulated cytokine and chemokine gene expression exhibited both gene and stimulus selectivity. Of the individual genes examined in this study, only a subset showed enhanced expression in IL-4-pretreated cells. These included TNF-α, IL-1α, KC, and MIP-2; the expression of the IL-12p40 gene was inhibited by IL-4 pretreatment, and the expression of the chemokine JE/Monocyte chemoattractant protein-1 was
Furthermore, a prior report demonstrated that the effect of IL-4 pretreatment in potentiating IL-12p70 expression in mouse macrophages is mediated by an alteration in expression of IL-12p35, but not IL-12p40 (15).

The ability of IL-4 to enhance cytokine and chemokine gene expression also varied with the nature of the eliciting stimulus. Responses to both LPS and poly(IC) (dsRNA) were enhanced in IL-4-pretreated cultures, while the induction of TNF-α by IFN-γ and IL-2 was suppressed. This differential effect of IL-4 on TNF-α production probably reflects the well-documented ability of IL-4 to suppress IFN-γ-driven transcriptional responses (4, 29–31). IL-4 has also been shown to suppress LPS-induced gene expression in monocytes and macrophages in certain experimental circumstances (5–7, 14). The efficacy of suppression varies with the concentration of IL-4 as well as the anatomical origin and/or species from which the monocyte/macrophage cell populations were obtained. In multiple prior studies, cotreatment of LPS-stimulated mouse macrophages with IL-4 did not suppress TNF-α expression (5, 6, 20).

The ability of IL-4 to enhance select proinflammatory responses in mononuclear phagocytes appears to be mediated by induced expression of one or more new gene products in response to IL-4. While this conclusion must remain tentative until the identity of such a gene(s) is determined, the concept is well supported by three independent observations. First is the significant time dependency of IL-4 pre-exposure necessary to enhance subsequent cytokine expression following secondary stimulation (see Fig. 4). This finding suggests possible requirement for a newly expressed gene product possessing the capacity to alter specific cytokine gene expression. Second is the ability of CHX to block the stimulatory effects of IL-4 when included in the pretreatment period. Because CHX is a reversible inhibitor of protein synthesis, its presence during the pretreatment phase could block the production of this necessary inhibitor but, upon removal, still allow normal response to the subsequent stimulus LPS (see Fig. 5). Finally, the effect of IL-4 is lost in macrophages prepared from mice deficient in the gene for the STAT6 transcription factor, known to mediate many of the actions of IL-4 through transcriptional activation of IL-4-inducible genes (32–36). Collectively, these three findings strongly suggest that IL-4, through the activation of STAT6, induces new gene expression that enables enhanced cytokine production.

Several prior reports demonstrating the ability of IL-4 to enhance cytokine production in human monocytes and mouse macrophages concluded that the effects were mediated by increased rates of target gene transcription (14, 15). Thus, we anticipated such a finding when examining the broader effects of this treatment on mouse macrophage gene expression and were surprised to note little or no change in the pattern of TNF-α mRNA expression.
throughout the time course of response to IL-4 (see Fig. 6). Furthermore, the enhanced production of TNF-α protein following stimulation of IL-4-pretreated cells with LPS was not due to an increase in the secretion of TNF-α, as there was no difference in the relative accumulation of intracellular protein between the different experimental conditions (data not shown). Together these observations suggest that IL-4 pretreatment enhances cytokine production (and specifically that of TNF-α) in mouse macrophages by increasing the rate of mRNA translation. This effect of translation was demonstrated directly by examining the distribution of TNF-α mRNA within polyribosomes. In macrophages treated with LPS alone, TNF-α mRNA was equally distributed between two pools that apparently represent free and polysome-bound mRNA. In contrast, in cells pretreated with IL-4 before stimulation with LPS, TNF-α mRNA was found almost entirely in the fractions containing larger polysomes. This accumulation of specific mRNA within large polysomes could arise either by an increase in the initiation of translation or a decrease in the rate of elongation or termination. Because the production of TNF-α protein was increased, it is most reasonable to conclude that the effect of IL-4-pretreatment on the translational efficiency that of TNF-α (mediated by dendritic cells. J. Immunol. 165:1877).


