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*J Immunol* 2000; 165:1612-1617; doi: 10.4049/jimmunol.165.3.1612

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Role of Stat3 in Lipopolysaccharide-Induced IL-10 Gene Expression

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IL-10 is a unique cytokine because it is anti-inflammatory and immunosuppressive. IL-10 is regulated at the level of transcription, but the critical motifs and the relevant transcription factors controlling this gene have remained elusive to date. We now report that a sequence at ~120 bp in the human IL-10 promoter binds Stat3 but no other Stat proteins. Mutation of this motif abrogates LPS-induced trans-activation. Overexpression of dominant negative Stat3 suppresses promoter activity, while wild-type Stat3 leads to an enhancement of this activity. Our results show that Stat3, by binding to a single motif in the IL-10 promoter, is controlling expression of the human IL-10 gene. The Journal of Immunology, 2000, 165: 1612–1617.

Interleukin-10 was originally discovered as a product of murine Th2 cells (1). It later was found to be expressed by additional leukocytes such as B cells, monocytes and in man by Th1 cells (2). IL-10 can suppress immune response and inflammation by suppressing the production of proinflammatory cytokines such as TNF-α and IL-1 and by reducing the cell surface expression of immunoreceptors such as MHC class II (3). In human cells the suppressive effect of IL-10 also extends to Th2 cells, in that proliferation and cytokine production are reduced (4). The importance of IL-10 is highlighted by the IL-10 knockout mice. These animals show enhanced autoimmunity and increased resistance to infection (5, 6).

IL-10 is regulated at the level of transcription, and knowledge of the transcription factors involved would greatly improve our tools to manipulate expression of this cytokine for therapy of inflammation. Much in contrast to other cytokine genes the transcription factors that control expression of IL-10 are unknown despite the fact that the sequence of the IL-10 promoter has been known for a long time. By combining linker scanning analysis and matrix analysis we now have identified Stat3 as the critical transcription factor.

Materials and Methods

Cell culture

The human RPMI 8226.1 B cell line (7) and the human monocytic cell line Mono Mac 6 (8) were grown in RPMI 1640 culture medium supplemented with 2 mM l-glutamine (043-05030 H, Life Technologies, Gaithersburg, MD), 200 U/ml penicillin/200 μg/ml streptomycin (043-05149 H, Life Technologies), 1–2% nonessential amino acids (043-01140 H, Life Technologies), and 10 ml/O PI supplement (O-5003, Sigma, St. Louis, MO). This medium was passed through a Gambro U-2000 ultrafiltration column (Gambro Medizintechnik, Planegg-Martinsried, Germany) to deplete contaminating LPS, and this was followed by addition of low LPS FCS. RPMI 8226.1 cells were passaged in 75-cm² tissue culture flasks, and Mono Mac 6 cells were cultured in 24-well plates (Costar, Cambridge, MA). The H9 T cell line (9) was cultured in RPMI 1640 with FCS and antibiotics. The LPS used for stimulation was from Salmonella minnesota (L6261, Sigma; protein content, <3%).

Gel shift analysis

Nuclear extracts were isolated according to the method of Dignam et al. (10) in the presence of a protease inhibitor cocktail (10 μg/ml aprotinin (A6279, Sigma), 1 mM PMSF (P7626, Sigma), 40 μg/ml leupeptin-protopiolin (L3402, Sigma), 20 μg/ml leupeptin-acetate (L2023, Sigma), 20 μg/ml antipain (A6191, Sigma), 20 μg/ml pepstatin A (P4265, Sigma), 400 μM N-acetyl-leucinyl-leucinyl-norleucinyl (ALLN; A6185, Sigma), and 2 mM DTT (19474, Merck, Rahway, NJ). Three to 5 μg of nuclear protein was then admixed with 32P-labeled double-stranded LS4 oligonucleotide (ATCCCGTCGGGCGGAAACC) in the presence of 0.5 μg of poly(dI-dC)/20 μl. After 20 min of incubation at 21°C samples were electrophoresed on nondenaturing polyacrylamide gels in 0.25 TBE buffer (22.5 mM Tris borate and 0.5 mM EDTA, pH 8.5). For supershift analysis nuclear extracts were first incubated with a 1/20 dilution of Ab for 30 min, followed by incubation with the oligonucleotides. The following Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): Stat1 p84/p91, sc-346; Stat2, sc-839; Stat3, sc-483; Stat3, sc-7179; Stat4, sc-845; Stat5, sc-1656; and Stat6, sc-621. For competition analysis nuclear extracts were admixed with the 32P-labeled double-stranded oligonucleotides in the presence of 50, 10, or 1 ng of unlabeled double-stranded oligonucleotide (LS4, ATCCGTGTCGGGCGGAAACC, Stat consensus, TCGTTCGATTC GTCTTG) primers, respectively, were synthesized. Human plasmid genomic DNA, isolated from one individual (6550-1, Clontech, Heidelberg, Germany) was admixed with the primers, and PCR was performed for 22 cycles using Pfu DNA polymerase (600103, Stratagene, Heidelberg, Germany). The pBTFATA.luc reporter plasmid was digested with BamHI.
and XhoI, such that the β-globin promoter including the TATA box was removed. Into this the BamHI/XhoI-digested PCR product was inserted. Sequencing confirmed a total length of 1074 bp with −1044 bp upstream of the transcription start site as defined by Vieira et al. (14) (GenBank accession no. M57627). Sequencing also showed identity of the clone with the published sequence (13), except for one base at position −146 that was a G as opposed to a C in the published sequence.

For generation of the 5′ deletion series, the pIL-10 luci construct was modified such that it contained a PvuI site upstream of the BamHI site followed by the IL-10 promoter. To obtain a series of 5′ deletions the plasmid was digested with PvuI and BamHI followed by exonuclease III treatment at 25°C in 35-s intervals using the Erase-a-Base system (E5750, Promega, Madison, WI). After S1 nuclease treatment blunt ends were generated with DNA polymerase I (Klenow fragment), and the plasmids were ligated. Bacteria were transformed, and appropriate clones were selected based on insert size. The exact point of deletion was determined by sequencing.

The trimeric LS4 sequence was obtained by oligonucleotide synthesis and was cloned into pSfTATA.luci. The linker scanning series was obtained by annealing and ligation of synthesized oligonucleotides (MWG Biotech, Ebersberg, Germany) that contained at sequential sites the AACCAGATCGAT linker sequence (15). An 183 bp linker used for this LS2 mutation was slightly different from the above sequences, a new response element with high inducibility. Therefore, the linker for this LS2 mutation was slightly different from the above sequence: AACCAGATCGTA.

The wild-type and the systematic mutants were cloned into pSfTATA luciferase reporter plasmid using BamHI and XhoI. The correct mutation was confirmed by digestion with PvuI and by sequencing.

Wild-type and dominant negative constructs for Stat1 and Stat3 were provided by Drs. T. Hirano (Osaka, Japan) via Drs. P. C. Heinrich and I. Behrmann (Aachen, Germany). The constructs used were pCAGGS, pCAGGS Neo HA STAT3, pCAGGS Neo HA STAT3F, and pCAGGS Neo HA STAT3D (16). Although of murine origin, the STAT3 constructs have been shown previously to be active in human cells (17).

Transfection

RPMI 8226 cells were transfected according to the method of Shakhov et al. (18) with 5 μg of reporter plasmid/105 cells in the presence of DEAE-dextran (62.5 μg/ml) for 90 min, followed by addition of DMSO for 150 s. Cells were then cultured for 3 days in six-well plates and were stimulated with LPS (S. minnesota, L-6261, Sigma) at 100 ng/ml. Six hours later cells were harvested and lysed, and luciferase activity in cell lysates was determined using a model LB9501 luminometer (Berthold, Wildbad, Germany) and the Luciferase Assay System (E1500) from Promega. Cotransfection studies were performed with admixture of reporter plasmids (5 μg) with control plasmid and Stat3 wild-type and dominant negative expression plasmids (2 μg).

Western blotting

Nuclear extracts were separated, 20 μg/lane, on 4–12% Tris-glycine gels (EC60385, Novex, Frankfurt/Main, Germany) followed by electroblotting. Blots were reacted with an Ab specific for phospho-Tyr105 of Stat3 (9131, NEB, Schwalbach, Germany) or with an Ab specific for phospho-Sev122 of Stat3 (9134, NEB) followed by goat anti-rabbit peroxidase Ab (A6667, Sigma), development with enhanced chemiluminescence reagent (RPN2106, Amersham, Braunschweig, Germany), and exposure to Hyperfilm (RPN3103, Amersham).

Results

Definition of IL-10 promoter elements

When the RPMI 8226 B cell line is transfected with a deletion series of the human IL-10 promoter, LPS stimulation will result in an efficient transe-activation all the way down to the −150 construct (Fig. 1). This short fragment directs a lower level of basal activity, indicating that elements required for constitutive activity are located further upstream. The trans-activation induced by LPS is, however, 12-fold for the −150 construct, whereas the longer constructs show only a 7-fold trans-activation in these experiments. This then indicates that elements critical to LPS induction of the IL-10 gene are located within this short promoter fragment.

In earlier studies no obvious transcription factor binding sites were reported for this short fragment (13). We therefore cloned a linker scanning series that systematically replaces decameric portions by an irrelevant linker sequence. After transfection into RPMI 8226 cells the LPS-inducible trans-activation was ablated only in the LS4p construct, which carries the mutation at −120 bp, while the other mutations did not lead to any loss in function (Fig. 2). As a complementary approach, a trimeric LS4 construct was transfected, and this demonstrated a strong (on the average, 13-fold) trans-activation after stimulation with LPS (Fig. 3).

Analysis of DNA binding proteins

For demonstration of an NF binding to this LS4 element, we performed gel shift analyses with nuclear extracts prepared from RPMI 8226 cells. As shown in Fig. 4a, there was little constitutive
activity, but after LPS stimulation a strong DNA binding activity was detected (lane 2). Addition of an excess of unlabeled LS4 motif specifically competed this band (Fig. 4a, lanes 3–5).

To identify potential transcription factor binding sites located in or around the LS-4 element the sequence was analyzed using MatInspector Professional software. This search revealed a potential Stat3 binding site (score, 0.768). We therefore performed competition experiments with a Stat3 consensus motif. An excess of this unlabeled DNA fragment also specifically inhibited binding of the LPS-inducible factor (Fig. 4a, lanes 7–9), while an NF-κB motif at the highest concentration of competitor had no effect (lane 11). These data suggest that the protein binding to this motif might be Stat3.

The competition data were therefore complemented by supershift analyses with specific anti-Stat-Abs. Abs to Stat1 and -2 and Stat3. These data suggest that the protein binding to this motif might be Stat3.

The DNA binding protein was directly visible in LPS-stimulated Mono Mac 6, the H9 T cell line, and blood mononuclear cells (Fig. 4c). The DNA binding protein was directly visible in LPS-stimulated Mono Mac 6 cells and in PBMC, but in the stimulated H9 T cells it became clearly visible only after the Stat3N supershift (Fig. 4c, lane 2). By contrast, the TNF-stimulated K562 stem cell line, which does not express IL-10, exhibited no Stat3 protein in supershift analysis (data not shown).

To determine the sequence requirements for binding of Stat3 we tested a series of mutants (Fig. 5a) for their inhibitory capacity in gel shift analysis. The most efficient loss of inhibition was seen with mutant 2 and with mutants 1 and 3 (Fig. 5b), indicating that the nucleotides GGA at the 3′ end of the core motif are most important for binding of Stat3. On the other hand, mutations of the nucleotides TGC at the 5′ end were still capable of inhibiting, albeit less than the wild-type motif, indicating that they are involved but are less important for binding of Stat3.

Next we have asked whether LPS-induced Stat3 is phosphorylated. For this, nuclear extracts were subjected to Western blotting, and blots were reacted with an Ab specific to phospho-Tyr705 or phospho-Ser727 of Stat3. As shown in Fig. 6 LPS induced a strong tyrosine phosphorylation and serine phosphorylation of Stat3 at 4 h.

**Overexpression of wild-type and dominant negative Stat3**

The functional importance of Stat3 in the expression of the IL-10 gene was then analyzed by cotransfection studies using dominant negative Stat3 mutants (16). For this, IL-10 reporter gene constructs were transfected together with different expression plasmids into RPMI 8226 cells, which were left untreated or were stimulated with LPS. These studies revealed a clear-cut blockade of the LPS-induced trans-activation of the −150 bp IL-10 promoter construct by the dominant negative forms of Stat3 (Stat3D and Stat3F in Fig. 7a) such that the LPS-induced trans-activation was reduced to 20% compared with that in the control transfection.

**FIGURE 2.** Functional analysis of the −150 IL-10 promoter by linker scanning. A 10-bp linker was consecutively introduced into the −150 construct, which was then cloned into pβTATA.luci. The scanning series was transfected into RPMI 8226 cells, cells were then stimulated without and with LPS at 100 ng/ml, and luciferase activity was measured. ○, TATA box; ■, Pvu II linker. Results are expressed as the fold trans-activation by LPS and are the average of four independent experiments ± SD.

**FIGURE 3.** Functional analysis of the LS4 trimer. The trimer was generated by oligonucleotide synthesis and cloned into pβTATA.luci. The trimer construct and the control construct were transfected into RPMI 8226 cells, cells were stimulated without (■) and with LPS at 100 ng/ml (○), and luciferase activity was measured. ○, TATA box; ■, LS4 sequence. Results are expressed as relative light units and are the average of four independent experiments ± SD.
match to the Stat3 weight matrix in the entire region of Stat3 in regulation of the IL-10 promoter. LS4 was the only motif over the control value. These data suggest an important function of Stat3 in the regulation of the human IL-10 gene. Hence, we next asked whether this single motif would be sufficient for Stat3 to control trans-activation of the −1044 bp IL-10 promoter-reporter-plasmid. The data shown in Fig. 7b demonstrate that dominant negative Stat3 does, in fact, also suppress promoter activity of the −1044 bp reporter plasmid to about 30%. Furthermore, wild-type Stat3 does enhance trans-activation by a factor of 2. These data provide strong evidence for a major role of Stat3 in the regulation of the human IL-10 gene.

**Discussion**

The classical consensus sequence for a Stat site (TT(N)₅ AA) (19) is not obvious in the LS4 sequence, while the matrix analysis revealed a significant match (ATCCTGTGCGGGAAACCTTG; score, 0.768). This site would have escaped detection with the 0.800 default threshold and could only be found with the optimized threshold used by MatInspector Professional. The prediction of the matrix match could be confirmed by the competition and supershift analyses that clearly demonstrated Stat3 binding to this site. Our studies show comparable competition by LS4 itself and by the Stat consensus motif, indicating that LS4 contains a high affinity Stat3 site. The competition analysis with a series of mutants revealed that the nucleotides GGA at the 3′ end of the core motif are crucial to Stat3 binding. Screening of other promoters with the unusual Stat3 sequence of LS4 may lead to the detection of similar sites in additional genes previously thought not to be regulated by Stat proteins.

Tissue-specific disruption of the Stat3 gene has resulted in enhanced IL-10 levels after LPS stimulation in vivo (20), suggesting a negative, rather than a positive, function for Stat3 in IL-10 regulation. With the in vivo setting, more complex regulatory loops involving different types of cells come into play. Therefore, analysis of the role of Stat3 in the murine IL-10 promoter (containing a potential Stat3 site 33 nucleotides upstream of the TATA box) in defined cell populations is required.

Other transcription factors besides Stat3 may participate in regulation of the IL-10 gene. In a T lymphoma line, gel shift studies revealed DNA binding activity for NF-kB (21). The motifs implicated were rejected by MatInspector Professional with optimized thresholds, although they were recognizable at lower thresholds (KB3 score, 0.806), and in unpublished studies we did not observe trans-activation of the IL-10 gene by p50 and p65 cotransfection. Furthermore, in a recent paper it was demonstrated that blockade of NF-kB by adenoviral Ik-Bα blocked TNF gene expression, while the IL-10 gene expression was unaffected (22). Hence, it appears that LPS-stimulated NF-kB does not contribute to IL-10 gene expression, but additional transcription factors deserve further study.

Another unexpected finding in our studies is the efficient induction of Stat3 by LPS. Although there has been evidence that LPS can induce Stat3 in mouse liver in vivo (23), Stat3 has not been implicated in the LPS signaling cascade in cells of the immune system as yet. Our data show that LPS can efficiently mobilize Stat3 and that the Stat3 protein found in nuclear extracts of LPS-stimulated cells is phosphorylated at both serine and tyrosine, implicating the respective kinases in signal transduction. Thus, activation of Stat3 is another signaling mechanism for LPS, occurring late after stimulation.

Induction of Stat3 appears to be a genuine effect of LPS, since protein contamination of our LPS material was <3%, and protein denaturation by boiling did not ablate the induction of Stat3 (data not shown).

We have tested different types of cells for induction of Stat3 by appropriate stimuli and found induction by PHA plus phorbol ester in the H9 T cell line and induction by LPS in PBMC. In the latter
cell population B cells and monocytes may contribute to the appearance of Stat3 in nuclear extracts. It may be difficult to analyze Stat3 activation in purified monocytes, since purification of these cells could lead to activation. When using intracellular staining for Stat3 in multicolor flow cytometry without purification of cells, we could, however, demonstrate a strong Stat3 signal in CD14-positive monocytes (M. Siedlar, unpublished observations). Finally, LPS stimulation of the human monocytic cell line Mono Mac 6 leads to a clear-cut increase in Stat3 in gel shift assays. Hence, our data indicate that Stat3 can also be activated by LPS in human monocytes.

In addition to LPS, several other stimuli can regulate the expression of the IL-10 gene, including IFN-α, IFN-γ, IL-10 itself, TNF, and cAMP-inducing agents. It will be of interest to analyze whether these reagents also act via Stat3. In preliminary experiments we have studied whether autocrine IL-6 might be involved in LPS-induced IL-10 expression. Neutralization of IL-6 with Ab added to the cultures, however, did not reduce LPS-induced trans-activation. Nevertheless, it is possible that other cytokines might be activated by LPS, and these, then, might lead to activation of Stat3.

In our studies we have focussed on the regulatory elements important for induction of IL-10, elements that are located in the short 3'150 construct. The deletion series demonstrates that important constitutive factors are located further upstream in the region between -304 and -150 (Fig. 1). A MatInspector analysis of this region revealed an ets-2 site at position -300. This site might be involved in the constitutive expression of the IL-10 gene, but...
the significance of this site needs to be addressed by functional studies.

IL-10 is an important immunoregulatory cytokine (2) that may be beneficial in inflammatory disease, autoimmunity, and transplantation, while it may be detrimental in immunodeficiency. Our finding that Stat3 is an important regulator of the IL-10 gene will open new possibilities to control the expression of the gene in various diseases.

Acknowledgments
We acknowledge the expert technical assistance of G. Sulski; the assistance of M. Lötzerich with the LPS quality control; the helpful discussions with M. Lo¨tzerich with the LPS quality control; the helpful discussions with K. Kube, D., C. Platzer, A. von Knethen, H. Straub, H. Bohlen, M. Hafner, and K. W. Moore. 1991. MatInd and MatIndNew: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. 11:2487-2488.


