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STAT1 Regulates Lipopolysaccharide- and TNF-α-Dependent Expression of Transporter Associated with Antigen Processing 1 and Low Molecular Mass Polypeptide 2 Genes in Macrophages by Distinct Mechanisms

Laura Marqués,1 Marina Bruçet,2 Jorge Lloberas,3 and Antonio Celada3,4

Transporter associated with Ag processing 1 and low molecular mass polypeptide 2 (LMP2) are essential for class I MHC function and share a common bidirectional promoter. In murine bone marrow-derived macrophages, LPS and TNF-α induced Tap1 and up-regulated Lmp2, which is constitutively expressed at low levels. These two genes are induced by LPS and TNF-α with distinct kinetics, at 6 and 12–24 h, respectively. Using macrophages derived from the TNF-α receptors of knockout mice, we found that induction by LPS is not due to the autocrine production of TNF-α. In macrophages from STAT-1 knockout mice, neither LPS nor TNF-α induced the expression of Tap1 or Lmp2. The shared promoter contains several areas that can be controlled by STAT-1, such as the proximal and distal IFN-γ activation site (GAS) boxes in the direction of the Tap1 gene. By making deletions of the promoter, we determined that only the proximal GAS box is required for LPS induction of Tap1 and Lmp2. In contrast, TNF-α induction of these two genes is dependent on the IFN regulatory factor-1 and NF-κB boxes, and not on the GAS box. Our experiments using gel shift analysis and Abs indicated that STAT1 binds to the GAS box in nuclear extracts from LPS-treated macrophages. The nuclear extracts obtained from macrophages treated with TNF-α bound to the IFN regulatory factor-1 and NF-κB boxes. These results show that LPS and TNF-α regulate the induction of Tap1 and Lmp2 through STAT1, but use distinct areas of the promoter. The Journal of Immunology, 2004, 173: 1103–1110.

Cytotoxic T lymphocytes recognize and attack virus-infected cells and cells with tumor Ags on their surface through interaction between the TCR and the MHC class I molecules loaded with nonself peptides (1, 2). Surface expression of HLA molecules requires the coordinated expression of several genes, such as Tap1/2, low molecular mass polypeptide (Lmp)-2,7 tapasin, and HLA class I H chain and β2-microglobulin (3). LMP2 and LMP7 are involved in breaking down intracellular proteins into antigenic peptides. Tap1 and Tap2 are involved in the transport of these peptides from the cytosol to the endoplasmic reticulum, where they bind to the assembled MHC class I molecules. The MHC-peptide complex is then transported to the cell surface. Studies of Tap-deficient cell lines, mice with targeted disruptions of Tap genes, and humans with Tap mutations have established that this gene is essential for the expression of MHC class I molecules on the cell surface (4, 5).

Tap1 and Lmp2 are embedded in the class II region of the MHC locus (6) and are transcribed from a shared bidirectional promoter. Their initiation codons for ATG translation are separated by only 596 bp (7). The expression of these two genes is coordinated, and reduced expression and function have been found in several tumor types (8), as well as reduced inducibility by IFN-γ (9) or rapid degradation of mRNA (10). In addition, reduced expression has been reported in animal models of autoimmune diabetes (11, 12). A full functional analysis of the transcriptional regulation of the human bidirectional Tap1/Lmp2 promoter has not been done to date (7, 13, 14).

Macrophages play a key role in immune response. These phagocytic cells are produced in the bone marrow and transported in blood to distinct tissues. Most macrophages die through apoptosis; however, in the presence of certain cytokines or growth factors, they proliferate, differentiate into several cell types (e.g., Kupffer cells, Langerhans cells, microglia), or become activated to develop their functions. At the inflammatory loci, the macrophage phagocytizes bacteria, removes cell debris, releases several mediators, presents Ags to T lymphocytes, and contributes to the resolution of inflammation (15). IFN-γ, which is released by activated T lymphocytes or NK cells, is the most potent activator of macrophages, and induces the expression of >300 genes (16), including Tap1 and Lmp2 (17). IFN-γ induces the phosphorylation of STAT1, which then homodimerizes, allowing it to interact with the IFN-γ activation site (GAS) (5, 18) or, in combination with p48 (IFN-stimulated gene factor 3 γ), with the IFN-stimulated gene factor, thereby trans activating genes that bear one of these sites in their promoter (19). This route induces transcription factors of the IFN regulatory factor (IRF) family, such as IRF-1, IRF-2, and IFN consensus sequence-binding proteins. The IFN-stimulated gene factor binds factors of the IRF family, thereby mediating both constitutive expression and cytokine induction of Tap1 and Lmp2.

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5 Abbreviations used in this paper: Lmp, low molecular mass polypeptide; GAS, IFN-γ activation site; RCS, IFN consensus sequence; IRF, IFN regulatory factor; KO, knockout; WT, wild type.

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(20). Despite the coordinated regulation of human Tap1 and Lmp2 observed in several cell lines (21, 22), the binding of IRF-1 or STAT1 to IFN consensus sequence 2 (ICS2)/GAS is sufficient to regulate the transcription of Tap1; however, the transcription of Lmp2 requires the binding of both factors (23).

LPS, a component of bacterial cell walls, is a potent macrophage-activating stimulus, which induces the expression of many genes necessary for host defense function (24). The cytokine TNF-α affects the growth, differentiation, and function of many cell types, and it is a major mediator of inflammatory immune responses (25). Interestingly, like IFN-γ, when LPS or TNF-α interacts with their respective receptors, STAT1 is involved in the signal transduction (26, 27). Because of the interest in these macrophage activators and the crucial role of Tap1/Lmp2 genes in Ag processing, in this study we investigated the regulation of these two genes in the murine system. Our results show that STAT1 is required for the induction of Tap1 and Lmp2 by LPS and TNF-α. However, different areas of the promoter of Tap1 and Lmp2 are used by LPS and TNF-α: for LPS, a GAS sequence is necessary, while IRF1 and NF-κB are required for TNF-α.

Materials and Methods

Reagents

LPS and recombinant TNF-α and IFN-γ were purchased from Sigma-Aldrich (St. Louis, MO). In several experiments, the results obtained with commercial LPS were compared with purified LPS, which was kindly donated by C. Galanos (Max Planck Institute, Freiburg, Germany) (28), and no differences were found. Anti-STAT1α and anti-IRF1 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and nonspecific rabbit IgG was obtained from Sigma-Aldrich. All other chemicals were of the highest purity grade available and were purchased from Sigma-Aldrich. Deionized water that had been further purified with a Millipore Milli-Q system (Bedford, MA) was used.

Cell culture

Bone marrow-derived macrophages were isolated from 6-wk-old BALB/c mice (Charles River Laboratories, Wilmington, MA), as previously described (29). Macrophages were cultured in plastic tissue culture dishes (50 mm) in 40 ml of DMEM containing 10% FBS (Sigma-Aldrich) and 50% L-cell-conditioned medium as a source of M-CSF. Penicillin/Streptomycin were added. Cells were incubated at 37°C in a humidified 5% CO2 atmosphere. After 7 days of culture, macrophages were obtained as a homogeneous population of adherent cells (>99% Mac-1+). To render cells quiescent, when macrophages were 80% confluent they were deprived of L cell-conditioned medium for 16–18 h before treatments. Macrophages from STAT1−/− or TLR4−/− double knockout (KO) mice and the corresponding wild-type (WT) controls were isolated under the same conditions (30, 31). STAT1 KO mice were kindly donated by R. Schreiber from the Washington University School of Medicine (St. Louis, MO). TNF-α/RI/II KO mice were kindly donated by M. Modolell from the Max Planck Institute, Freiburg, Germany. The RAW 264.7 murine macrophage cell line was cultured in DMEM medium with 10% FBS without antibiotics at 37°C with 5% CO2.

Reporter plasmids

The Tap1/Lmp2 bidirectional promoter was cloned from murine genomic DNA isolated from L929 cells, following the method described by Murusina et al. (32), using restriction with EcoRI and PCR, as previously described (33). The full-length promoter fragment was cloned into the pCR2.1 vector (TA cloning kit; Invitrogen Life Technologies, Carlsbad, CA). The promoter was subcloned directionally into the luciferase reporter plasmid pGL3-basic (Promega, Madison, WI), thereby obtaining the full-length promoter with the mutation. The oligonucleotides used to introduce the mutations contained the same mutated nucleotides as those described for the probes used in the DNA-binding assays.

Transient transfections and luciferase assays

To transfect RAW 264.7 cells, liposomal transfection reagent Fugene 6 (Roche, Indianapolis, IN) was used, following the manufacturer’s instructions. Twelve hours after transfection, cells were treated with LPS or TNF-α. Luciferase activity was measured with the luciferase dual system assay (Promega), using standard procedures, on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

RNA extraction and Northern blot analysis

Cells were washed twice with cold PBS, and total RNA was extracted with the acidic guanidinium thiocyanate-phenol-chloroform method, as described (34). Total cellular RNA samples (15 μg) were separated on 1.2% agarose gels with 5 mM MOPS, pH 7.0, and 1 M formaldehyde buffer. The RNA was transferred overnight onto a nylon membrane (Genescreen; NEN Life Science Products, Boston, MA) and fixed with UV irradiation (150 mJ). After an 18-h incubation at 65°C in hybridization solution (20% formamide, 5× Denhart’s, 0.5% SSC, 10 mM EDTA, 1% SDS, 25 mM Na2HPO4, 0.25 mM NaH2PO4, 0.1% NP-40, 10% dextran sulfate, 0.5 μg/ml hybridization probe), the membranes were exposed to Kodak X-AR films (Kodak, Rochester, NY). The bands of interest were quantified with a Molecular Analyst System (Bio-Rad, Richmond, CA). cDNA probes for mouse Tap1 and Lmp2 were prepared by RT-PCR. To prepare the RNA 18S probe, the plasmid containing the corresponding cDNA was digested with appropriate restriction enzymes. In all of the cases, the amplification products of the RT-PCR were controlled by sequence. All probes were labeled with [α-32P]dCTP (Valeant Pharmaceuticals, Costa Mesa, CA) with the oligolabeling kit method (Pharmacia Biotech, Uppsala, Sweden). The fold of the mRNA was measured using actinmixin D, as described (35).

Protein extraction and Western blot analysis

Western blot analysis was done, as previously described (36). A total of 100 μg of protein from cell lysates was loaded per lane and separated on a 7.5% SDS-PAGE. For inducible NO synthase immunoblotting, we used Tap1 Abs (Santa Cruz Biotechnology), LMP2 Abs (Biomol, Plymouth, PA), and an anti-β-actin Ab (Sigma-Aldrich) as a control. Peroxidase-conjugated anti-rabbit and anti-mouse IgG (Sigma-Aldrich) were used as secondary Abs. Incubations were performed for 1 h at room temperature. ECL detection was performed (Amersham Bioscics, Piscataway, NJ), and the membranes were exposed to x-ray films (Amersham Biosciences).

Quantitative RT-PCR analysis

RNA was treated with DNase (Ambion, Austin, TX) to eliminate DNA contamination. For cDNA synthesis, 1 μg of RNA and TaqMan reverse-transcription reagents (including Multiscribe reverse transcriptase and random hexamers) were used, as described by the manufacturer (Applied Biosystems, Foster City, CA). The primers used for real-time PCR for Tap1 were 5′-GAGGGGAGGAAGC and Lmp2 TACCGTGAGGACTTGTTAGCGC and GGCTGTCCTAGCATGACCACCCAG and AAGAAGACCGTCGGAGACG and Lmp2 TACCGGGACGTTGAGCAG and GCCCTGC GAATTACGATCCCC (Applied Biosystems). Real-time monitoring of PCR amplification of cDNAs was done using the TaqMan Universal master mix (Applied Biosystems) in the ABI Prism 7700 sequence detection system (Applied Biosystems). Relative quantification of gene expression was performed using β-actin, as described in the TaqMan users’ manual (GAACCA CACCTTTACAAATGACTGT and CTTGCGTGAAGGTGAGCAGCC GATAAAGCATCCT (Applied Biosystems). Threshold cycle was defined as the cycle number at which the fluorescence corresponding to the amplified PCR product is detected. The PCR efficiency of each gene was defined as the slope of the PCR amplification curve. The cycle threshold (CT) value for each cDNA sample was normalized to the β-actin expression level in each sample. The RT-PCR analysis was controlled by sequencing the products of the amplification. Also, we included a sample without RNA in each reaction.

Nuclear extracts and EMSA analysis

Nuclear extracts were prepared from bone marrow-derived macrophages, as previously described (37), with some modifications. Briefly, pellets of bone marrow macrophages were washed twice with cold PBS buffer, followed by resuspension in 5 vol of hypotonic buffer (10 mM HEPES, pH 2.0, 10 mM KCl, 0.5 mM MgCl2, 0.5 mM EDTA). Nuclei were isolated by sonication, and the nuclear protein was extracted and kept on ice until use. The protein concentration was determined using the bicinchoninic acid assay (Pierce). Nuclear extracts were lysis with proteinase K for 5 min at room temperature, followed by centrifugation to remove insoluble material. Then, 20 μg of protein was used for each EMSA. The probes used in the EMSA are listed in Table I. In some experiments, the specificity of the EMSA was examined by the addition of unlabeled competitor DNA. EMSA was performed using a previously described method (38). A 20-μg sample of nuclear extract was incubated with 10 ng of labeled probe at room temperature for 20 min in a reaction mixture containing 100 mM KCl, 10 mM MgCl2, 10 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1% Nonidet P-40, and 20 mM dithiothreitol. To block nonspecific binding, 2 μg of poly(dI-dC) was added to each reaction. The DNA-protein complexes were separated by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and were detected by autoradiography. The specific DNA-protein complexes were analyzed by PhosphorImager (Molecular Dynamics) and analyzed using the ImageQuant software (Molecular Dynamics).
The lysates were homogenized in a potter, and the homogenate was cen-
resuspended in 3 vol of hypotonic buffer and left to stand on ice for 10 min.
The lysates were homogenized in a potter, and the homogenate was cen-
resuspended in 3 vol of hypotonic buffer and left to stand on ice for 10 min.
and centrifugation at 1500 rpm for 5 min, at 4°C. The pellet was then
probed in the presence of 2

B

Lmp2 after 3 h (Fig. 1

A

B

FIGURE 1. Induction of Tap1 and Lmp2 expression by LPS, TNF-α, and IFN-γ in macrophages. A, Bone marrow-derived macrophages were
stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), or IFN-γ (300 U/ml)
for the times indicated. Tap1 and Lmp2 mRNA expression was analyzed
by Northern blot. The 18S gene expression was used as a control. Similar
results were obtained in three experiments. B, Quantitative RT-PCR anal-
ysis was determined for bone marrow-derived macrophages. Relative
quantification of gene expression was performed using the β-actin gene,
and data were expressed as the mean of five independent experiments.
Value of p < 0.01 between time 0 and after treatment of samples for 6 h
for LPS and 12 h for TNF-α in bone marrow macrophages and RAW cells.

observed until cells had been stimulated for ~6 h, and induction
was not detected at 12 h.

As a control, we incubated macrophages with IFN-γ, an induc-
tor of Tap1 and Lmp2 (13, 14, 17). In contrast to LPS, when cells
were stimulated with IFN-γ, Tap1 and Lmp2 were induced in the
first 3 h after treatment, and there was a steady state increase in the
level of mRNAs of both genes throughout the remainder of the time
course, until at least 24 h (Fig. 1). On the basis of our results, we
conclude that the time course of the induction of Tap1 and Lmp2 in
macrophages differs depending on whether they are induced by LPS
or IFN-γ.

The steady state of Tap1 and Lmp2 mRNA in macrophages was
followed as a function of time after stimulation with 10 ng/ml
TNF-α. Increased levels of mRNA were not detected until at least
24 h of stimulation (Fig. 1). Quantitative RT-PCR began to detect
mRNA after 12 h of TNF-α treatment (Fig. 1B). Thus, the expres-
sion pattern resulting from stimulation with TNF-α differs to that
obtained with LPS or IFN-γ, indicating that distinct mechanisms
regulate the signaling pathways that follow the interaction of the
ligands with the corresponding receptors. Similar results for LPS
and TNF-α induction were obtained when we used the RAW 264.7
macrophage cell line (Fig. 1).

To examine whether the increase in the levels of RNA induced by
LPS or TNF-α required the synthesis of new proteins, cells were
treated with LPS or TNF-α in the presence of cycloheximide
at 5 μg/ml. This dose of cycloheximide produced a >90% reduc-
tion in the incorporation of [3H]leucine into trichloroacetic acid-
precipitable material (35). No RNA for Tap1 or Lmp2 was de-
tected after addition of LPS or TNF-α plus cycloheximide to the
cells.
The rate of degradation of RNA for Tap1 and Lmp2 was measured in bone marrow macrophages after treatment with LPS for 6 h or TNF-α for 12 h. Actinomycin D was then added to a concentration (5 μg/ml) sufficient to block all further RNA synthesis determined by [3H]UTP incorporation (40), and the RNA was isolated from aliquots of cells at intervals of 30 min thereafter. Tap1 and Lmp2 mRNAs were very stable, and there were no differences in the t1/2 when the induction was mediated by LPS or TNF-α.

The expression of Tap1 and Lmp2 proteins was measured by Western blot after treatment of bone marrow macrophages with LPS or TNF-α. No significant differences were found after treatment for 24 or 48 h.

LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-α (41). To determine whether LPS stimulates a distinct signaling pathway to that of TNF-α or whether the effects of LPS are due to the autocrine-production of TNF-α, we used bone marrow-derived macrophages from TNF-α-R1/II double KO mice and the corresponding WT controls. As expected, TNF-α did not induce Tap1 or Lmp2 mRNA. In addition, our results indicate that LPS induction of these mRNA expressions did not depend on the presence of TNF-α-R1/II (Fig. 2). These data demonstrate that the induction of these two genes by LPS was not due to the autocrine synthesis of TNF-α. As a control, macrophages from WT and KO mice were stimulated with IFN-γ, which induces the expression of Tap1 and Lmp2 by a mechanism that is independent of TNF-α-R1/II.

Most genes induced by the IFN-γ, as well as LPS and TNF-α, signaling pathway are mediated by STAT1, which, after phosphorylation, dimerizes and translocates to the nucleus (18, 19). However, IFN-γ can produce the expression of some genes in the absence of STAT1 (42). Because IFN-γ induces Tap1 and Lmp2 expression through STAT1, we addressed whether this protein was involved in the signaling pathway that leads to the expression of these genes, with different kinetics, induced by LPS or TNF-α. Bone marrow macrophages from mice in which STAT1 expression has been inactivated (STAT1 KO) were used (30). These cells and the corresponding controls were stimulated with LPS or TNF-α for range of times, as indicated, and specific mRNA was detected. As expected, in macrophages from WT animals, Tap1 mRNA was detected 6 h after LPS treatment. Constitutive expression of Lmp2 was observed, but levels increased under the effect of LPS (Fig. 3). STAT1 KO mice showed no expression of Tap1 and only basal expression of Lmp2. Similar results were obtained when cells were stimulated with TNF-α for 24 h (Fig. 3). Our data demonstrate that LPS- and TNF-α-induced expression of Tap1 and Lmp2 requires STAT1, as previously reported in other systems (26, 27).

Having observed the capacity of LPS and TNF-α to induce the expression of Tap1 and Lmp2, and established the involvement of STAT1 in these two signal transduction pathways, we analyzed the functional activity of the promoter to delineate critical sequence elements for these inductions. The TFSEARCH program in the TRANSFAC databases was used to examine the promoter sequence for possible regulatory areas in which transcription factors bind (43). This analysis showed that the promoter region of Tap1 and Lmp2 contains various putative sites for the binding of transcription factors that may be involved in LPS or TNF-α signal transduction, such as an NF-κB box (−110 to −119 from the ATG of Tap1 gene) and an AP1 (−145 to −151) and an AP1.a (−410 to −415) box. In addition, we detected two GAS boxes (GAS1 between −163 and −172, and GAS2 between −240 and −247) and one IRF-1 box (−177 to −187). To examine the role of these putative areas, we determined the transcription steady state from the promoter activity in both directions.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** LPS-dependent Tap1 and Lmp2 induction is independent of the autocrine production of TNF-α. Bone marrow-derived macrophages from WT and from TNF-receptor KO were stimulated with IFN-γ (300 U/ml), TNF-α (10 ng/ml), or LPS (100 ng/ml) for the times indicated. Tap1 and Lmp2 mRNA expression was analyzed by Northern blot (A). The 18S gene expression was used as a control. Similar results were obtained in three experiments. Quantitative RT-PCR analysis was determined for bone marrow-derived macrophages (B). Relative quantification of gene expression was performed using the β-actin gene, and data were expressed as the mean of five independent experiments. Value of p < 0.01 between time 0 and after treatment of samples for 6 h for LPS and 12 h for TNF-α.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** LPS and TNF-α induction of Tap1 and Lmp2 is STAT1 dependent. Control (WT STAT1) and macrophages from STAT1-deficient mice (KO STAT1) were stimulated with TNF-α (10 ng/ml) or LPS (100 ng/ml) for the times indicated, and gene expression was analyzed by Northern blot. Results shown are representative of two independent experiments. Quantitative RT-PCR analysis was determined for bone marrow-derived macrophages. Relative quantification of gene expression was performed using the β-actin gene, and data were expressed as the mean of five independent experiments. No significant differences were found between time 0 and after treatment of samples for 6 h for LPS and 12 h for TNF-α.
The original construct in the direction of Tap1, which contained the WT promoter (pGL3-tap) when transfected into the macrophage cell line RAW 264.7, showed basal activity. After 6 h of stimulation with LPS, expression increased two to three times (Fig. 4A). When the GAS1 box was mutated, LPS inducibility was lost, showing that in macrophages, the GAS1 region is critical for the induction of Tap1 expression in response to LPS. Mutation of GAS2, IRF1, NF-κB, and the two AP-1 sites had no effect on the response to LPS stimulation.

The same constructs were made for the promoter cloned in the direction of Lmp2 gene into the luciferase plasmid (pGL3-lmp) (Fig. 4B). The assays were performed after 6 h of stimulation with LPS, and the promoter for Lmp2 gene was induced 2.5-fold over basal expression. When constructs containing the mutated GAS1 were used, the reporter plasmids did not respond to stimulation. Again, no effect was observed when the GAS2, IRF1, NF-κB, or AP-1 boxes were mutated. Taken together, these results demonstrate that the GAS1 element is the only sequence required for the response of the Tap1/Lmp2 promoter to LPS stimulation. These findings confirm our observations using macrophages from the STAT1 KO mice (Fig. 3).

Similar experiments were conducted with TNF-α. The sequences involved in the regulation of Tap1 and Lmp2 induction by TNF-α were the NF-κB and IRF1 boxes, because mutations in these sites completely abrogated the induction (Fig. 5). These data are essential to understand the distinct kinetics of Tap1 and Lmp2 induction when macrophages are stimulated with LPS or TNF-α. Although STAT1 was involved in both cases, our results show the presence of distinct signaling mechanisms.

To study the proteins that bound to the areas of interest in the Tap1/Lmp2 promoter, gel electrophoresis DNA-binding assays were performed. When nuclear extracts prepared from macrophages treated with LPS and a probe covering the GAS1 box were used, two retarded bands were observed with extracts prepared from macrophages incubated for 3 h with LPS (Fig. 6A). In the probe with the mutated GAS1 box, no retarded bands were detected. As a control, we used an oligonucleotide with a canonical GAS box (44). To further characterize the interaction between the GAS1 box and transcription factors, competition experiments were performed. For these experiments, we used nuclear extracts obtained from macrophages after 3 h of interaction with LPS. As expected, the upper retarded band produced by the extracts can be competed by a 100-fold excess of the same cold oligonucleotide, and the oligonucleotide containing the GAS canonical box (Fig. 6A). No competition was observed when we used an excess of oligonucleotide that contained the mutated GAS1 box or the IRF-1 box. Next, we identified the binding factor to the GAS1 box. For this, we used an Ab against STAT1. This Ab, but not nonspecific IgGs, blocked the binding of nuclear extracts to the GAS1 probe (Fig. 6B). No binding was obtained when we used the oligonucleotides covering the IRF-1 or the NF-κB areas. These results demonstrate that the induced protein that binds to the GAS1 sequence is specific and is recognized by anti-STAT1 Abs.

To characterize the other areas in the promoter involved in the TNF-α-dependent regulation, we used nuclear extracts of macrophages treated for 6 h with TNF-α and oligonucleotides that cover the NF-κB and IRF-1 boxes. Using an oligonucleotide covering the area with the NF-κB sequence, a retarded band was found in extracts after TNF-α treatment (Fig. 6C). The experiments with the oligonucleotides with the mutated NF-κB box and the competition experiments demonstrated the specificity of the binding (Fig. 6D). Finally, the extracts showed the induction of two complexes when the IRF-1 probe was used (Fig. 6E). However, in the IRF-1 mutant and competition experiments, these complexes induced bands that appeared to bind specifically (Fig. 6F). Moreover, an Ab against IRF-1, but not the Ig control competed for the binding.

**Discussion**

In this study, we characterize the transcriptional regulation of the murine Tap1 and Lmp2 genes by LPS and TNF-α. The products of these genes are critical to provide peptides from endogenously synthesized proteins to the endoplasmic reticulum for MHC class
LPS AND TNF-α REGULATE Tap1 AND Lmp2

fore, LPS and TNF-α could be critical in the regulation of the endogenously synthesized peptides presented by class II molecules.

LPS-induced Tap1 and Lmp2 expression show considerable differences, which are probably related to the type of macrophage used in each study. For example, similar induction by LPS or IFN-γ of these two genes has been described in monocyte-derived macrophages (51), while in the THP-1 cell line LPS alone is almost inactive (14, 17). It is for these reasons that we used preferentially primary cultures of bone marrow-derived macrophages, and we found that Lmp2, but not Tap1, was constitutively expressed.

In contrast to IFN-γ, which produces a sustained induction of Tap1 and Lmp2 (13, 14), LPS and TNF-α induce a transient expression. In addition, the kinetics differs. For LPS, the induction is at 6 h, and for TNF-α at 12–24 h. When we used the macrophage cell lines RAW 264.7, the pattern expression was similar to that of primary cells. The fact that both LPS and TNF-α require STAT1 implies that TNF-α may be the mediator produced autocrinally by LPS, as we previously showed (41). However, by using TNF-α-receptor KO mice, we demonstrate that there are two distinct pathways of activation for LPS and TNF-α.

Several areas in the bidirectional promoter of Tap1 and Lmp2 are susceptible to regulation by STAT1 directly or proteins whose synthesis depends on STAT1, such as IRF-1. Using bone marrow macrophages, we found that IFN-γ-induced Tap1 and Lmp2 expression requires STAT1, which controls a GAS and an IRF-1 box (52). LPS is, together with IFN-γ, one of the most powerful agents in the activation of macrophages. Although the mechanisms involved in the response to LPS are still not clearly defined, we do know some DNA sequences that act in the regulation. Of these sequences, the AP-1 box and NF-κB boxes are two of the most important (53), and are present in the Tap1 and Lmp2 bidirectional promoter. However, in our functional studies, we found that mutation of a precise GAS box was sufficient to eliminate the LPS-dependent inducibility for Tap1 and Lmp2, and no functional activity was found for the AP-1 box and NF-κB boxes. This is supported by our observation that LPS did not induce the expression of Tap1 or Lmp2 in macrophages from STAT1 KO mice. In response to LPS, first Jak1 and Jak2 are activated, followed by STAT1, which forms homodimers that translocate to the nucleus and bind a GAS sequence in the Tap1/Lmp2 promoter (18, 19). However, the time course of the mRNA induction by LPS was too long to require only the binding of STAT1 to the GAS box. Thus, some elements must be presynthesized in the cell at the moment of LPS stimulus and either translocate to the nucleus or suffer posttranslational modifications, while others are synthesized. Our results, in part, confirm the previous data of Cramer et al. (14), who reported that, in the THP-1 monocytic cell line, LPS requires not only the GAS box, but also that of IRF-1. These differences may be related to the distinct cell lines used. For example, for IFN-γ-induced Tap1 and Lmp2 expression, only the GAS box is necessary in HeLa cells (54) and macrophagic cells (14). By contrast, in a renal carcinoma cell line, IFN-γ acts only through the IRF-1 sequence (55). The role of IRF-1 in the regulation of Tap1 and Lmp2 has been related to the paucity of CD8+ T cells observed in the IRF-1-/- mice (56). In some cases, such as in endothelial cells, the IFN-γ response is blocked only when the GAS and the IRF-1 boxes are mutated (13). Finally, in some melanoma cell lines, binding to either IRF-1 or GAS is sufficient to induce Tap1 expression, but for Lmp2 the binding of both transcription factors is required (23).

I binding. Although LPS and TNF-α induce the expression of MHC class I molecules (45, 46), they are particularly important for the regulation of MHC class II molecules in macrophages (47). There are several examples of the presentation of intracellular proteins with MHC class II molecules (48, 49). Moreover, the TAP complex influences allore cognition of these molecules (50). There-

FIGURE 6. Binding of nuclear extracts from LPS- or TNF-α-treated macrophages to the Tap1/Lmp2 promoter. A, Binding of nuclear extracts from LPS-treated macrophages to the GAS1 box. Nuclear extracts were prepared from bone marrow-derived macrophages incubated with LPS (100 ng/ml) for the times indicated. The probe was an oligonucleotide of 20 bases covering the GAS1 box from −157 to −177. The oligonucleotide GAS1mut covers the GAS1 box 5′-CTGGCGTTTAGAGGAAGAAG-3′ mutated by 5′-CTGGCGTAGATCCCGAAG-3′. Retarded complexes were detected by autoradiography. B, The binding of nuclear extracts from LPS-treated macrophages to the GAS1 box is specific. Nuclear extracts were prepared from bone marrow-derived macrophages incubated with LPS (100 ng/ml) for 3 h. The probe was the oligonucleotide of 20 bases covering the GAS1 box. Competition experiments were made adding 100× excess of the cold oligonucleotides indicated to the nuclear extracts before adding the radiolabeled GAS1 oligonucleotide. Anti-STAT1 Ab or control IgG (2 μg) was added to nuclear extracts before the binding assay. C, Binding of nuclear extracts from TNF-α-treated macrophages to the NF-κB box. Nuclear extracts were prepared from bone marrow-derived macrophages incubated with TNF-α (10 ng/ml) for the times indicated (C) or for 6 h (D). The oligonucleotide NF-κB covers the NF-κB box 5′-GCCGCCTAGTCCTCCGGGAAC-3′ mutated by 5′-GCCGCCTAGTCCTCCGGGAAC-3′. D, Competition experiments were made adding 100× excess of the cold oligonucleotides indicated to the nuclear extracts before adding the radiolabeled NF-κB oligonucleotide. E, Binding of nuclear extracts from TNF-α-treated macrophages to the IRF-1 box. Nuclear extracts were prepared from bone marrow-derived macrophages incubated with TNF-α (10 ng/ml) for the times indicated (E) or for 6 h (F). The oligonucleotide IRF-1mut covered the IRF-1 box 5′-CGAAGAACCAG AAAGCCGAC-3′ mutated by 5′-CGAAGACCCGTCCTCCGGGAC-3′. F, Competition experiments were made adding 100× excess of the cold oligonucleotides indicated to the nuclear extracts before adding the radiolabeled IRF-1 oligonucleotide. Anti-IRF-1 Ab or control IgG (2 μg) was added to nuclear extracts before the binding assay. These experiments were performed three times, and the results of one representative experiment are shown.
Because TNF-α regulates a series of genes, among which are those involved in the regulation of immune response such as cytokines, growth factors, receptors, and others (25), we tested its effect on the regulation of Tap1 and Lmp2, because these are also involved in the activation of the immune response. The induction of some of the genes that respond to TNF-α is mediated by transcription factors such as AP-1 and, more specifically, NF-κB (57). Although STAT1 is necessary for TNF-α-mediated induction, the areas that control the expression of Tap1 and Lmp2 differ from the GAS box required for that mediated by LPS. For TNF-α, the NF-κB and IRF-1 boxes are necessary. The time required for IRF-1 synthesis may explain the delay in the expression of Tap1 and Lmp2 expression induced by TNF-α in relation to LPS. In fact, the promoter of the transcription factor IRF-1 contains a GAS sequence that mediates its expression (58). Our results confirm, in part, the requirement of the NF-κB box, previously reported (7). Although the maximal binding to the GAS box occurred after 3 h of treatment with LPS, the maximal levels of RNA for Tap1 and Lmp2 were detected after 6 h. Similarly, NF-κB binding was maximal at 6 h, but mRNA levels were maximum at 24 h. This is probably due to a slow rate in transcription together with a reduced degradation (long life of RNA) that allows for the accumulation of RNA in the cell.

One of the questions remaining is to establish the role of STAT1 in LPS or TNF-α regulation of Tap1 and Lmp2 expression. In some reports, LPS or TNF-α can directly activate a STAT-like factor (59, 60), although other studies failed to detect direct activation of STAT1s in LPS-stimulated macrophages (61). This latter possibility is more plausible because LPS induces TNF-α in a STAT1-independent manner (26). Because LPS (62) and TNF-α (63) induce type I IFNs, this may be the mechanism by which STAT1 exerts its action (26). LPS uses two distinct TLRs: TLR2, which induces STAT1-dependent genes, and TLR4, which induces IFN-β, an IFN that is MyD88 and dsRNA-dependent protein kinase-independent, but Toll-IL-1R domain-containing adapter protein-dependent (64, 65).

The differential constitutive expression of Lmp2 and Tap1 is not explained by our results on gene regulation. In fact, we found that the same elements controlled the expression of these genes by either LPS or TNF-α. There are examples of other genes divergently transcribed from a single promoter. The H2A and H2B genes in the Xenopus xh3 histone gene cluster are divergently transcribed because of the activity of overlapping promoter sequences that share multiple regulatory elements (66). Furthermore, the human and murine COL4A1 and COL4A2 genes are expressed discordantly, although they share the same promoter. Lack of binding activity at the CCAAT and CTC regions markedly reduces transcription of the COL4A2 gene without affecting that of the COL4A1 gene (67). Recently, it has been shown that constitutive Lmp2 expression is due to the binding of a complex of unphosphorylated STAT1 and IRF-1 to the partially overlapping ICS2 and GAS sites in the human Lmp2 promoter to activate transcription (20). In the murine promoter, this ICS2 sequence does not overlap with the GAS site, and another mechanism may be involved for the constitutive expression of Lmp2. In our experimental model, we did not take into account several factors that may be important for the regulation of gene expression in vivo. For example, chromatin structures create barriers for each step in eukaryotic transcription, and the remodeling of histones builds DNA templates that are accessible to the general transcription apparatus (68). The way in which histones interact with the common promoter of Tap1 and Lmp2 may condition a distinct basal expression for each gene.

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References


