

Volume 12, issue 2, June 2001 | Recherches

Functional characterization of the mouse lymphotoxin-beta receptor promoter

Abstract

The lymphotoxin beta-receptor (LTbR), a member of the tumor necrosis factor (TNF) receptor family, plays a crucial role in lymphoid organogenesis by signaling through its functional ligand LTa1b2. While the receptor is expressed on a wide range of cell types e.g. fibroblasts and monocytes, the ligand is expressed only on activated T, B and NK cells. Remarkably, no cell type has been identified so far that expresses both the receptor and the ligand. In order to characterize the mouse LTbR gene expression on a molecular level, we isolated about 1 kb of the

5' flanking region of the LTbR gene. Primer extension analysis revealed one transcriptional start site located at – 60 upstream of the ATG-containing first exon. Northern blot analysis showed that the LTbR is abundantly expressed in the mouse fibroblast cell line NIH 3T3, and to a lesser extent, in the mouse macrophage-like cell line RAW 264.7. To determine whether the 5' flanking region exerts functional promoter activity, we generated deletion mutants fused to the luciferase reporter gene. Transfection experiments using these reporter gene constructs showed that the isolated 5' flanking region is transcriptionally active in NIH 3T3 and RAW 264.7 cells, and determined a minimum length required for the transcriptional activity of the LTbR promoter in these cells. Further sequence analysis of the isolated 5' flanking region identified a number of putative DNA-binding sites for transcription factors. Interestingly, incubation of NIH 3T3 cells with dexamethasone resulted in an elevated mRNA level of the LTbR gene. This effect was abolished by using the specific glucocorticoid receptor inhibitor RU486, indicating an increased transcriptional activity of the LTbR promoter after glucocorticoid stimulation.

Full text

INTRODUCTION

The LTbetaR, a member of the tumor necrosis factor (TNF) family, is functionally activated upon binding of the membrane-bound, heterotrimer, lymphotoxin alpha$_1$beta$_2$ (LTalpha$_1$beta$_2$) molecule [1-3]. Interestingly, LTbetaR is expressed on a wide range of cell types with the exception of lymphocytes, whereas the expression of the ligand is restricted to activated lymphocytes [4]. Currently the biological functions of this ligand-receptor system is poorly characterized. It has been shown that signaling through LTbetaR induces cell death in some adenocarcinoma tumor lines in the presence of IFN-gamma [5, 6], although this receptor does not contain a classical death domain. In addition, signaling through LTbetaR has been reported to induce NFkappaB activation and chemokine production in a cell type-restricted manner [7-10]. Recent studies in LTbetaR-deficient mice indicated that this receptor is critically involved in lymphoid organogenesis and in the generation of adaptive humoral immune responses [11, 12]. In the adult animal, signaling through the LTbetaR is necessary for maintaining the lymphoid architecture [13]. The LTbetaR is expressed in major visceral organs and on stromal cells of lymphoid organs and thymus, as well as on cell lines of stromal origin. It is not found on T or B cells, or

peripheral blood-derived monocytes or dendritic cells. Some myeloid cell lines express the LTbetaR at low levels [14, 15]. In contrast, the specific ligand $LTalpha_1beta_2$ is expressed on activated lymphocytes, especially T cells, but also on B cells and NK cells [4, 16, 17]. Whereas some information about the regulated expression of the ligands LTalpha and LTbeta has been reported previously [18], only limited data exist about the transcriptional regulation of the LTbetaR [14]. To gain more insight into the mechanisms involved in the regulation of LTbetaR expression, the genomic organization of the 5'end of the mouse LTbetaR gene was identified and functionally characterized.

MATERIALS AND METHODS

*Isolation of the 5' flanking region of the mLT*beta*R gene*

About 1 kb of the 5' flanking region of the mouse LTbetaR gene was amplified by PCR, following the manufacturer's instructions (Perkin-Elmer), using the primers: 5'GGTACCTACACTGGCTGGGCTCTCAGG 3'and 5'GGATCCGGCGGCCTTTCGGCTCGGCTAACC 3' and a genomic clone [11], containing the entire open reading frame (ORF), and about 1.5 kb of the 5' flanking sequence of the mLTbetaR gene as a template. The subcloned sequence was characterized by restriction mapping and sequencing. Further sequence analysis was performed with the University of Wisconcin GCG Package, version 8.

Preparation of reporter gene constructs

The - 938 luciferase construct was prepared by PCR according to the manufacturer's instructions (Perkin Elmer). The following oligonucleotides were used as 3' and 5' primers, respectively, 5' CAGGGATCCGGCGGCCTTTCGGCTCGGCTAACC 3' and 5' GGTACCTACACTGGCTGGGCTCTCAGG 3'. The amplification product was digested with *Kpn*I and *BamH*I, subcloned in pGL2 Basic, and digested with *Kpn*I and *Bgl*II.

The promoter deletion mutant - 688 was constructed by digesting the - 938 luciferase construct with *Hind*III and subcloning in a *Hind*III-digested pGL2 basic vector. Correct orientation was verified by sequencing. The - 123 construct was created by digesting the - 938 construct with *Sma I,* immediately followed by

religation. The mutants - 269, - 239, and - 181 were prepared by PCR using the 3' primer mentioned above. The - 269 mutant was amplified using the primer 5' AGCGGTACCCTCTTCCCCCCCCCCGTCGCG 3'. The - 239 and the - 181 promoter frag- ments were amplified using 5' CGAGGTAGGCCGGCCTCTGCTCCCAGGGCT 3' and 5' AAAGGTACCCACTTCCTGAGCCCGGCGCTG 3', respectively. The resulting fragments were digested with *Kpn*I and *BamH*I, subcloned in pGL2 basic, and digested with *Kpn*I and *Bgl*II.

Cell lines and transient transfections of reporter gene constructs

NIH 3T3 mouse fibrosarcoma cells and macrophage-like cell line RAW 264.7 were subcultured every 3 days in DMEM, containing 1% L-glutamine, 0.05 mg/ml gentamycin, and 10% fetal bovine serum. For transfection of reporter plasmids, both cell lines were plated at a density of 5×10^5 cells per 35 mm tissue culture plate and transfected the following day with 2.5 mug of reporter plasmid using the transfection reagent DOTAP (Boehringer Mannheim, Germany) according to the manufacturer's instructions. After 16 hours cells were incubated for a further 24 hours with or without 10 muM dexamethasone for stimulation experiments. The glucocorticoid receptor antagonist RU486 was used at a concentration of 10muM and added to the cells one hour prior to addition of dexamethasone. Cells were harvested and lysed, and luciferase activity was assayed using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Each assay was normalized either to total protein content or beta-galactosidase activity. Protein concentration was measured by the Micro BCA* Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). beta-Galactosidase activity was determined by the beta-Galactosidase Enzyme Assay System (Promega). Each transfection was done in triplicate and repeated at least three times.

Primer extension analysis

Total RNA (20 mug each) isolated from NIH 3T3 was resuspended in 20 mul of hybridization buffer (10 mM Tris-HCl, pH 8.6, 300 mM NaCl, 1 mM EDTA) and incubated with 2×10^5 cpm of 5' end- labeled oligonucleotide (5' CAGGCGCATGGCGGCCTTTCGGCTCGGCTAACC 3'), at 55° C for 2 hours. Two hundred mul of 1 M ammonium acetate and 200 mul of isopropanol were added, and the samples were incubated at room temperature for 20 min. The precipitated nucleic acids were dissolved in 10 mul of 10 mM Tris-HCl, pH 8.6. Ten

mul of reverse transcriptase buffer (50 mM Tris-HCl, pH 8.6, 75 mM KCl, 10 mM $MgCl_2$, 0.25 mM spermidine), and 50 mug of actinomycin D (Boehringer Mannheim), 10 mM DTT, 10 U of RNasin (Promega), 0.5 mM dNTP each (dATP, dCTP, dGTP, dTTP), and 10 U of reverse transcriptase (Life Sciences, Inc., Hialeh, FL, USA) were added. The reaction was allowed to proceed for 2 hours at 45° C. The products of the primer extension reaction were analyzed on a 10% sequencing gel. The gel was fixed in 10% (v/v) acetic acid and 10% (v/v) methanol, dried, and autoradiographed.

RNA isolation and Northern blot experiments

For Northern blot analysis, total RNA was isolated from cultured cells using Trizol Reagent (Gibco-BRL, Germany) according to the manufacturer's recommendations, subjected to gel electrophoresis, blotted, and probed using a randomly P^{32}-labeled cDNA fragment coding for the extracellular domain of the mouse LTbetaR. Equal loading was verified by probing for GAPDH expression.

RESULTS

*Isolation and sequence analysis of the 5' flanking region of the mouse LT*beta*R gene*

In order to study the transcriptional regulation of the LTbetaR gene, specific primers were used to amplify and clone about 1 kb of the 5' flanking region of the mLTbetaR gene, relative to the translational start site. Sequence analysis of the 5' flanking region identified several putative binding sites for transcription factors that are known to be important in the regulation of other eukaryotic genes (Figure 1). There are no TATA-like sequences located in the proximal promoter region. However, several GC- and CACC-boxes that are able to bind the transcription factor Sp1 [19], are present in either a direct or inverted orientation (Figure 1). Eight out of twelve Sp1 binding sites are clustered within the proximal promoter region of the LTbetaR gene. A sequence located at position - 197 to - 202 contains only 1 bp mismatch compared to the consensus sequence for the transcription factor AP2. A putative binding site for the transcription factor AP1 was identified at position - 445 to - 451, relative to the translational start site. Both sequences are close to or overlap with a putative Sp1 binding site (Figure 1).

Moreover, two putative binding sites for the transcription factor NF-1 were identified (position - 146 to - 162 and position - 448 to - 435). Surprisingly, within the region - 895 to - 901 upstream of the translational start site, a sequence was identified that is homologous to a negative regulatory element described earlier in the NADPH cytochrome P-450 oxidoreductase gene [20].

Identification of the transcriptional start site

To define the initiation site of transcription, we performed primer extension analysis with RNA derived from NIH 3T3 cells. One oligonucleotide, complementary to nucleotides - 24 to + 9 in the cDNA was used as primer. The primer extention analysis (Figure 2) identified a single transcriptional start site at position - 60 upstream of the translational start site ATG within the 5' flanking region of the mLTbetaR gene. Interestingly, the nucleotide sequence 3' of the transcriptional start site at position - 54 to - 48, resembles an initiator element with a 1bp mutation compared to the corresponding consensus sequence ((C/T)(C/T)AN(T/A)(C/T)(C/T)) [21].

*Functional characterization of the mLT*beta*R promoter*

In order to characterize the mLTbetaR promoter function, several luciferase reporter constructs were generated (Figure 3a). The 3'end of each promoter fragment was at position - 1, 1 bp before the first ATG of the mLTbetaR open reading frame (ORF). After transient transfection of these reporter gene constructs into NIH 3T3 and RAW 264.7 cells, luciferase activity was determined and expressed as fold activity compared to the promoterless control plasmid (pGL2 Basic) (Figure 3b). The 1.0 kb (- 938 to - 1) mLTbetaR promoter construct showed about 35-fold higher luciferase activity in NIH 3T3 cells and about 15-fold higher activity in RAW 264.7 cells compared to the control plasmid, confirming functional promoter activity in both cell lines. Interestingly, LTbetaR promoter activity was increased more than twofold only in NIH 3T3 cells, when the region between - 688 bp and - 938 bp was deleted from the 5'end of the 1.0 kb reporter gene construct, suggesting a negative regulatory element within this region which represses the transcriptional activity of the LTbetaR gene in NIH3T3 cells. Surprisingly, this putative negative regulatory element in the distal promoter region in transfected NIH 3T3 cells seems not to repress LTbetaR promoter activity in RAW 264.7 cells since there is no significant difference in the luciferase activity when comparing the - 688 bp and - 938 bp reporter gene

construct in RAW 264.7 cells. Further 5' deletions (- 269 to - 1 and - 239 to - 1) resulted in a 50% loss of promoter activity in NIH 3T3 cells. The - 181 bp and - 123 bp deletion constructs (- 181 to - 1 and - 123 to - 1), both devoid of Sp1 and AP1 sites, resulted in a dramatic reduction of promoter activity to almost the level of the promoterless control plasmid, in both NIH 3T3 and RAW 264.7 cells (Figure 3b). Interestingly, while there seems to be a minimal length of about 269 bp of the LTbetaR promoter in RAW 264.7 cells for basal transcriptional activity, about the same reporter gene activity was obtained with the - 239 bp construct in NIH 3T3 cells. In contrast, transfection of the - 239 bp deletion mutant of the LTbetaR promoter in RAW 264.7 cells resulted in a strong reduction of luciferase activity to almost the level of the control plasmid. This result indicates that different 5' flanking regions of the mLTbetaR promoter are required for the transcriptional activation in different cell types. Computer based comparison of the DNA sequence between - 239 bp and - 269 bp did not identify any putative DNA binding site for known transcription factors.

*The transcriptional activity of the mLT*beta*R promoter is regulated by dexamethasone*

In order to further characterize the regulated expression of the LTbetaR gene, we stimulated different cell lines of fibroblastic and monocytic origin, with LPS, TNF, IL-1, IFN-gamma, and PMA. We were not able to observe any significant change in the mRNA expression under these experimental conditions (data not shown). However, incubation of NIH 3T3 cells with dexamethasone resulted in a significant upregulation of LTbetaR mRNA, as shown by Northern blot analysis (Figure 4a). The observation of an increased transcriptional activity of the LTbetaR gene in the presence of glucocorticoid was further supported by the use of reporter gene analysis. Luciferase activity was enhanced by at least 50% under the influence of dexamethasone in cells transiently transfected with the promoter fragments - 688 and - 269, respectively. Using the fragment - 938, no higher rate of induction could be achieved (data not shown), while using the - 239 fragment, moderate induction was detected that was completely absent in the - 181 to - 1 and - 123 to - 1 reporter gene constructs. Treatment of dexamethasone-stimulated and transfected NIH 3T3 cells with the specific glucocorticoid inhibitor RU486 [22], resulted in reporter gene activity that was almost reduced to the level of unstimulated cells while incubation of transfected but unstimulated NIH 3T3 cells with RU486 did not influence reporter gene activity (Figure 4b).

The positive regulatory effect of dexamethasone stimulation on the activity of the LTbetaR promoter constructs might suggest a functional relevance of glucocorticoid in regulation of LTbetaR expression.

DISCUSSION

The promoter region of the LTbetaR gene was analyzed by sequence analysis and by transfection of luciferase reporter gene constructs. Sequence analysis of a 1.0 kb promoter fragment revealed the presence of consensus elements for transcription factors, *e.g.*, AP1, AP2, SP1, and NF-1-like elements, as well as one transcriptional start site at position - 60, relative to the translational start site ATG. Furthermore, the proximal promoter region comprises a high GC content and seems to fall into the category of the so-called housekeeping-type promoters. The structure of the LTbetaR 5' flanking region suggests that different regulatory mechanisms are involved in the transcriptional regulation of the mLTbetaR gene. The functional characterization of the mouse LTbetaR promoter revealed that different 5' flanking regions of the mLTbetaR promoter are required for the transcriptional activation in different cell types. Further investigations will be necessary to identify transcription factors that contribute to the cell-specific regulation of the mLTbetaR promoter activity. Moreover, our studies indicated an upregulation of the mLTbetaR mRNA by treatment of transfected NIH 3T3 cells with dexamethasone as shown by Northern blot analysis. This observation directly originates from the transcriptional activation of the LTbetaR gene as shown by reporter gene studies. Interestingly, in many experimental systems the glucocorticoid receptor modulates gene expression by two major modes of action: 1) by ligand-dependent binding to its glucocorticoid receptor response element and 2) by cross-talk with other transcription factors *e.g.* Ap1 and NFkappaB/Rel through protein-protein interactions [23]. Further investigations will be necessary to identify the transcription factors that contribute to the cell-specific regulation of the mLTbetaR promoter activity, and to gain more information about the mechanisms involved in the LTbetaR gene expression.

CONCLUSION

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Figures

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      -938 (Kpn I)                                    neg. RE
-938  GTACCTACAC TGGCTGGGCT CTCAGGTCCC TCGGTTTAAG AGAGTTATAG GCTTATGTGC

-878  ACACACGCTG GAACTAGGCT ACCCAGCCCC AGCCCAGAAG CCCCCACTCA CCAGGACCTG

                SP1
-818  GGTTTACACA CGCCCACCCT TCCGTGAGAG AGGTCCCAGA GGAGAGGACA GATTCAGGGC

                                          SP1
-758  CAGCTGAACT CTCTCCAGTG TCTTGTGGTG TGCGCCCTGG TGTGTGGCGT GGGTGGGCTT

                -688  (Hind III)
-698  GTTACTGTGG AAGCTTCTTT TTAAAAAGTC ACAGAGTGGA GCAGGCCCTC AGTCTCTGCC

-638  AAGTGGGATG CCTGGCCAGA CGCTGGCTGC ATCTGCTAAC CACCTCTGGG TATCCTGGCT

      CACCC
-578  GGGTGCACTG TCAATCCCTG GCGCCTCCTC TTTGCAAATC TGACACCCAG CTGTCCACAG

-518  CTCTCTGCCT CAACGTCCAC GGCAGGTCAA CCAAGTCAGC TCTGCCTCGG GCTCTCGGAG

      CACCC     AP1  NF1-like
-458  GTGGGCCTGA CTGATGGCTA GCCACTGTCT CTGCTGCCCC CCTTTCGGCC AGCAAGCGAT

                                                  CACCC
-398  CCTAATCCGC AATCCCCTCT GAGAGCCAGG CTTCCGAAGA AAGGTGGAGG CCGGGTTCCG

                                      CACCC       SP1             SP1
-338  GGCCTGCAGC TCTCACGTGC TTTCCCGGCC ACCCCCTCCC GCCCTGCGTC GAGGCGGCCA

            -269                                  -239
-278  AGCCTGTTCC TCTTCCCCCC CCCCGTCGCG ATTGCGACAG GCCGGCCTCT GCTCCCAGGG

                  SP1    AP2                   -181
-218  CTCCCTGCTT CCGCCCCCGG CCGGCTCGCT CCACTCCCAC TTCCTGAGCC CGGCGCTGGA

      NF-1-like                         SP1    -123 (SmaI)
-158  GCCCTGGAGG CCAGGCCCGG CCGCTCCCGG CCCCCGGGGG CACGTCGGCC CAGCCGCCAG

                                     ↱(-60)            CACCC
 -98  GCTTGGGAAG TCGTGGCCAA CGCTGCTCAG GACGTCCGGG CTTCCCACCT TCCTCCTAGG

        CACCC                                  +1
 -38  ACTCCACCCG GTCTGGTTAG CCGAGCCGAA AGGCCGCCATG
```

Figure 1

Sequence analysis of the 5' flanking region of the mLTbetaR gene.

The identified transcriptional start site is indicated by an arrow. Putative binding sites for transcription factors are underlined. The A at the translational start site is designated + 1.

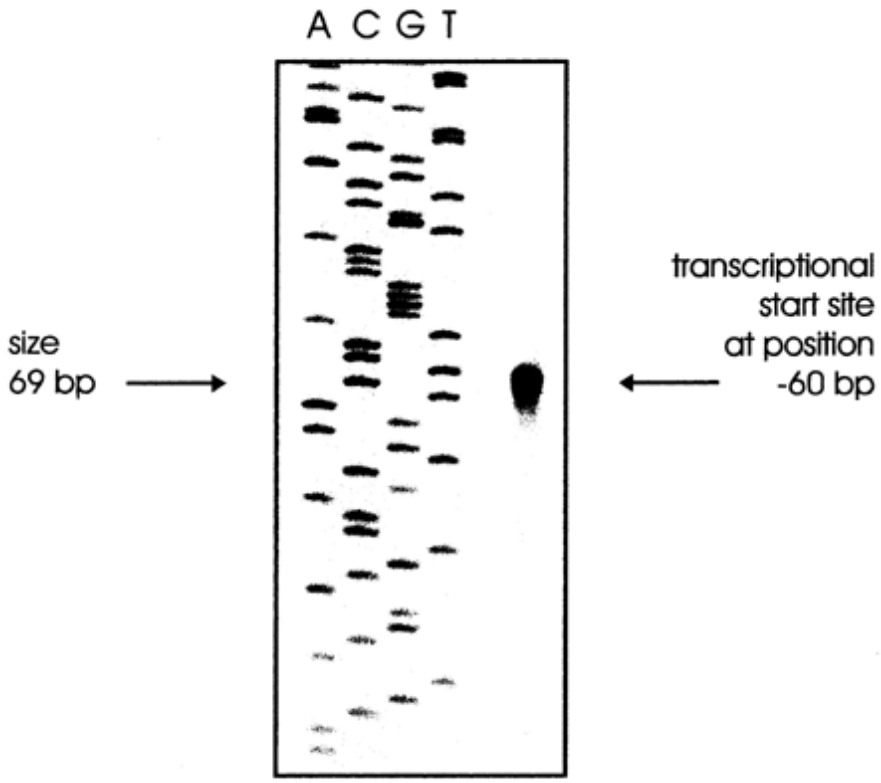
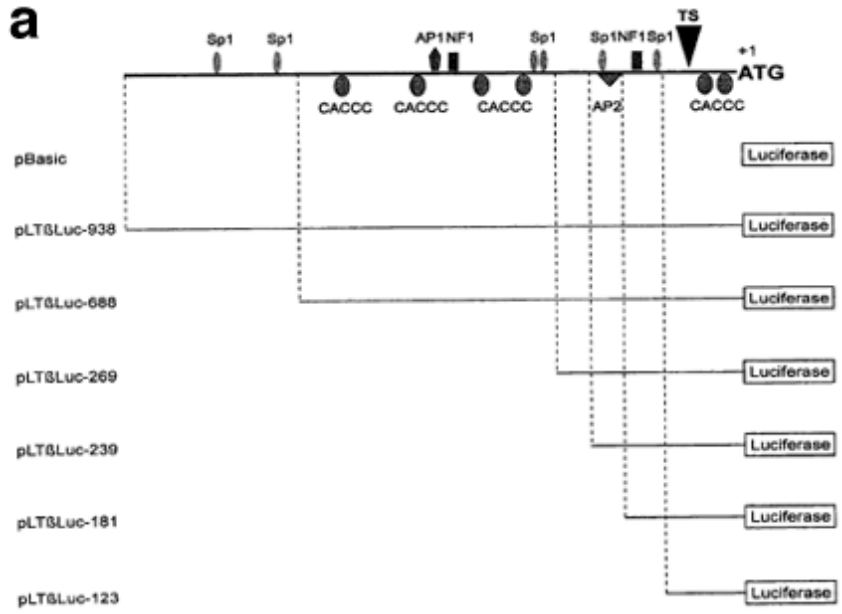


Figure 2

Analysis of the mLTbetaR transcriptional start site.

RNA derived from NIH 3T3 cells was hybridized with a specific primer (see Materials and Methods) and reverse transcription products were analyzed on a 10% sequencing gel along with a sequencing reaction serving as a size marker. The size of the reaction product is indicated on the left, while the position of the corresponding transcriptional start site is shown on the right.



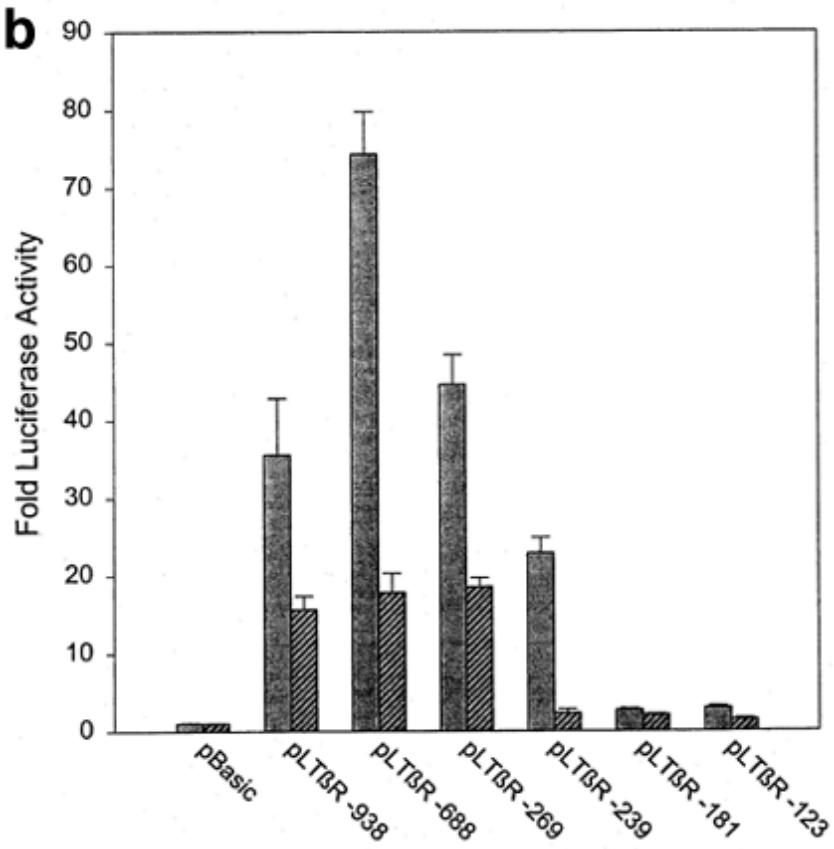
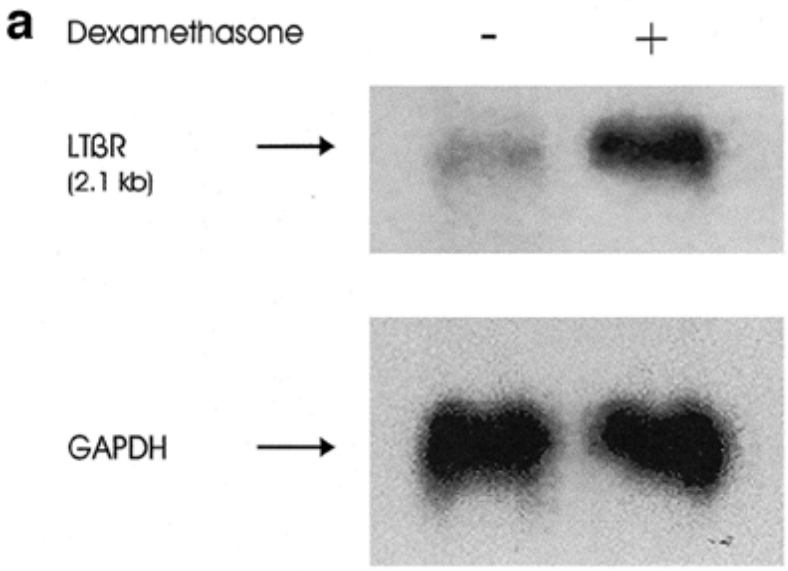


Figure 3

Functional analysis of the LTbetaR promoter.

a: LTbetaR promoter constructs used for functional analysis were cloned in front of the luciferase gene of pGL2 Basic. The position of the identified transcriptional

start site and putative DNA-binding sites for transcription factors are represented schematically. **b:** Promoter constructs were transiently transfected into NIH 3T3 cells (open bars) and RAW 264.7 cells (hatched bars), respectively. Fold luciferase enzymatic activities, relative to a promoterless vector (pGL2 Basic), are represented as means ± SD of three independent transfection experiments, each done in triplicate.



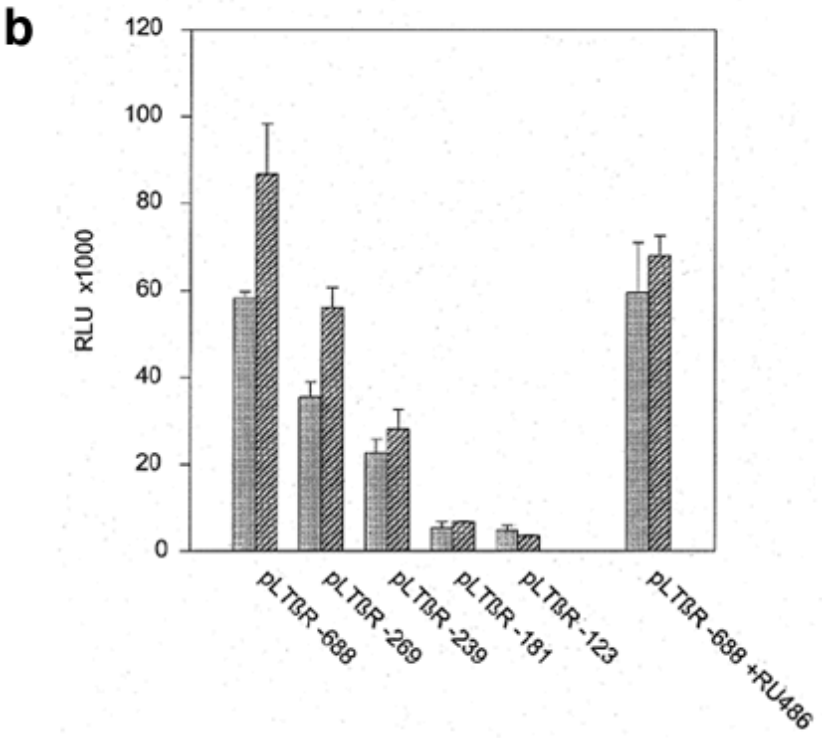


Figure 4

Regulation of the transcriptional activity of the mLTbetaR promoter by dexamethasone.

a: Northern blot analysis of mLTbetaR mRNA in NIH 3T3 cells incubated with or without dexamethasone (10 muM) for 21 hours. Hybridization to mGAPDH was used as an internal control. **b:** Reporter gene activity of LTbetaR promoter constructs in NIH 3T3 cells, untreated (open bars) or treated with dexamethasone (10 muM) for 21 hours (hatched bars). Fold luciferase enzymatic activities relative to a promoterless vector (pGL2 Basic) are represented as means ± SD of three independent transfection experiments, each done in triplicate.

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V. Barak, T. Halperin, I. Kalickman
Sambucol, inflammatory cytokines, IL-1b, TNF-a, IL-6, IL-8.

Interferon (IFN)-g and Herpes simplex virus/tumor necrosis factor-a synergistically induce nitric oxide synthase 2 in macrophages through cooperative action of nuclear factor-kB and IFN regulatory factor-1

S.R. Paludan, L. Malmgaard, S. Ellermann-Eriksen, L. Bosca, S.C. Mogensen
macrophages, NOS2, Herpes simplex virus, NF-kB, IRF-1.

Negative regulation of onconstatin M signaling by suppressor of cytokine signaling (SOCS-3)

F. Magrangeas, O. Boisteau, S. Denis, Y. Jacques, S. Minvielle
cytokine signaling, inhibition, Jak-STAT pathway.

Increased expression of interleukin-8 and aminopeptidase N by cell-cell contact: interleukin-8 is resistant to degradation by aminopeptidase N/CD13

A. Kehlen, I. Egbert, K. Thiele, K. Fischer, D. Riemann, J. Langner
cell co-culturing, leucine aminopeptidase, plasma membrane preparations, CD13, proteolysis of IL-8, interleukin-8.

Functional characterization of the mouse lymphotoxin-beta receptor promoter

P. Muller, D.N. Männel, T. Hehlgans
LTbR-promoter, transcriptional activation, dexamethasone.

Glucose potentiates interleukin-1b (IL-1b)-induced p38 mitogen-activated protein kinase activity in rat pancreatic islets of Langerhans

A.M.E. Sprinkel, N.A. Andersen, T. Mandrup-Poulsen
interleukin-1b, p38, ERK1/2, JNK, nitric oxide, diabetes mellitus.

Sustained parasite burden in the spleen of Leishmania infantum-infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge

D. Rousseau, S. Demartino, F. Anjuère, B. Ferrua, K. Fragaki, Y. Le Fichoux, J. Kubar
murine leishmaniasis, acquired immunity, inflammation, chemokines, spleen cell subpopulations.

Effects of cytokines and prolactin on the replication of Toxoplasma gondii in murine microglia

N. Benedetto, A. Folgore, C. Romano-Carratelli, F. Galdiero
T. gondii, cytokine, PRL, MGC.

Strip test for bedside detection of interleukin-6 in cervical secretions is predictive for impending preterm delivery

H. Trébéden, F. Goffinet, G. Kayem, F. Maillard, E. Lemoine, D. Cabrol, B. Weill, F. Batteux
cytokine, IL-6, prematurity, cervicovaginal secretions, strip test.

Serum levels of interleukin-18 in patients with uncomplicated Plasmodium falciparum malaria

D. Torre, M. Giola, F. Speranza, A. Matteelli, C. Basilico, G. Biondi
interleukin-18, malaria, Plasmodium falciparum, gamma interferon.

The ecdysone inducible gene expression system: unexpected effects of muristerone A and ponasterone A on cytokine signaling in mammalian cells

S. Constantino, R. Santos, S. Gisselbrecht, F. Gouilleux
ecdysone, PI 3-kinase, AKT, survival.

Presence of estrogen receptors in human myeloid monocytic cells (THP-1 cell line)

M. Cutolo, B. Villaggio, A. Bisso, A. Sulli, D. Coviello, J.M. Dayer
THP-1 cells, macrophages, estrogen receptors, estrogens, monocytes.

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