Role of Stat3 in Glucocorticoid-induced Expression of the human IL-10 Gene

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Abstract

In the present report we have determined the molecular mechanisms, which govern the expression of the human IL-10 gene when induced by the glucocorticoid Methyl-Prednisolone (MP). Treatment cells with MP at 10^{-6} M will readily induce IL-10 in CD19+ primary B cells and in a human B cell line. Analysis of the IL-10 promoter showed a robust 18-fold induction and demonstrated that a potential GRE motif was not required, while mutation of the -120 STAT-motif strongly reduced MP induced transactivation. A strong induction was also seen with a trimeric STAT-motif and over-expression of dominant-negative STAT3 could block MP induction of IL-10 mRNA. Finally, MP treatment induced binding of STAT3 to the promoter as shown by gelshift, supershift and by chromatin-immuno-precipitation. These data show that glucocorticoid-induced expression of the IL-10 gene is mediated by the transcription factor STAT3.

Keywords

Human, B-Cells, Cytokines, Inflammation, Transcription Factors

Introduction

IL-10 is a major immunosuppressive cytokine produced by T-cells, monocytes / macrophages, dendritic cells and B-cells (Chang et al., 2000; de Waal Malefyt et al., 1992; Fiorentino et al., 1989). IL-10 will suppress the immune response by decreasing cell surface expression of MHC class II and by down-regulating the expression of cytokines like Tumour Necrosis Factor (de Waal Malefyt et al., 1991).

The immunosuppressive activity of IL-10 becomes obvious when looking at IL-10 knock-out mice, which show spontaneous autoimmune disease and increased resistance to infection (Bettelli et al., 1998; Dai et al., 1997).

Changes in the expression of IL-10 upon activation of leukocytes are mainly transcriptional and several factors have been implicated in its control. For the murine IL-10 gene Sp1 was reported to have an important role in regulating LPS-stimulated promoter activity (Brightbill et al., 2000). The respective motif is promoter proximal and its mutation reduced both the inducible and the constitutive promoter activity in the murine RAW 264 macrophage cell line (Brightbill et al., 2000; Tone et al., 2000). Ma et al. (Ma et al., 2001) noted an Sp1 site at –636 bp of the human promoter, which appeared to be responsible for all the inducible activity in monoblastic cells. For the human gene transactivation by catecholamines appears to require the action of C/EBP (Brenner et al., 2003) with the most important motif 44 bp downstream of the TATA box. Furthermore, there is evidence for a role of c-Maf in IL-10 gene expression, but demonstration of c-

Maf binding to a –196 bp motif in the human IL-10 promoter requires over-expression of this transcription factor (Cao et al., 2002; Cao et al., 2005). Based on deletion and linker scanning analysis we have identified a STAT-motif at –120 that is crucial to gene expression in a human B cell line when stimulated with LPS (Benkhart et al., 2000). Also, this motif was shown to be essential together with an up-stream IRF site for IFNα-induced IL-10 expression (Ziegler-Heitbrock et al., 2003). The role of STAT3 in expression of IL-10 has been confirmed in other cell types and in knock-out mice (Cheng et al., 2003; Herbeuval et al., 2004; Maritano et al., 2004). Recently it was shown that SOCS3 deficiency in murine TCR-stimulated T cells led to increased STAT3 activity and IL-10 expression (Kinjyo et al., 2006) and that constitutive IL-10 expression in T cell lines can be blocked by depletion of STAT3 by RNA interference (Kasprzycka et al., 2006). Hence it appears that the STAT-motif in the human IL-10 gene may be a central cis-element in regulation of this gene.

Glucocorticoids (GCs) are steroid hormones, which can potently suppress immune response and inflammation. Their main mode of action is via the glucocorticoid receptor-alpha (GCR-alpha), which is present in the cytosol as a homodimer, where it is bound to heat-shock proteins. When the lipophilic glucocorticoids enter the cell they bind to the receptor, leading to dissociation of the heat-shock proteins and to the translocation of the GCR into the nucleus. Here the GC-GCR complex will induce gene expression by binding to cognate DNA sequences (AGAACAnnnTGTTCT) (Smoak and Cidlowski, 2004). On the other hand GC will act by binding to and interfering with the action of other transcription factors

like NF-kB (Rhen and Cidlowski, 2005), which will lead to suppression of genes like the pro-inflammatory Tumor Necrosis Factor.

Among the genes induced by glucocorticoids is the anti-inflammatory cytokine IL-10 and induction of this gene obviously will contribute to the anti-inflammatory action of GCs. GC therapy has been shown to induce IL-10 in serum (Dandona et al., 1999) and in mononuclear cells (Gayo et al., 1998). In addition, John et al demonstrated that in the BAL (bronchoalveolar lavage) of patients, treated with inhaled glucocorticoids, alveolar macrophages are the main producers of induced IL-10 (John et al., 1998). According to in-vitro studies GC can induce IL-10 in monocytes (Mozo et al., 2004) and macrophages (Frankenberger et al., 2005). Furthermore, GC treatment will induce IL-10 in T cells in therapeutic settings (Karagiannidis et al., 2004) and in in-vitro culture (Barrat et al., 2002; Richards et al., 2000).

The molecular mechanism involved in glucocorticoid mediated induction of IL-10 has, however, not been determined to date. In the present report we demonstrate GC-induction of IL-10 in primary human B cells and in a B cell line. Analysis in this system of the cis- and trans-elements that control the human IL-10 promoter in GC-treated cells demonstrates that GCs act by inducing binding of the transcription factor STAT3 to the –120 motif in the human IL-10 gene. While induction of gene expression by GCs typically involves binding of the glucocorticoid receptor to a cognate glucocorticoid response element, our study describes a novel mode of action of GCs via direct activation of STAT3.

Experimental

Cell Culture

The human RPMI 8226.1 B cell line (Ziegler-Heitbrock et al., 1994) was cultured in RPMI 1640 culture medium supplemented with L-glutamine 2 mM (#25030-024, Invitrogen, Karlsruhe, Germany), penicillin 200 U/mL streptomycin 200 µg/mL (#15140-114, Invitrogen), nonessential amino acids 1-2x (#11140-35, Invitrogen) and OPI-supplement (contains Oxalacetic acid, sodium Pyruvat, and Insulin) 10 mL for 1 litre (#O-5003, Sigma, Taufkirchen, Germany). This medium was passed through an Ultrafilter (#8394189, Ultra SteriSet; Gambro Hospal GmbH, Martinsried, Germany) to remove any LPS that might have inadvertently contaminated the medium. 10 % fetal calf serum (FCS), pre-tested for the absence of detectable LPS (477U; Seromed, Berlin, Germany) was added and cells were cultured in 75 cm^2 tissue culture flasks (#3275, Costar, Bodenheim, Germany) at a density of $5x10^6$/25 mL.

ELISA

For determination of IL-10 protein, RPMI 8226.1 cells were stimulated with and without 6α-Methyl-Prednisolone (MP) (#M-0639, Sigma) at 10^{-6} M for 24 h. Afterwards, cells were harvested and the supernatant was measured by the PeliKine Compact™ Human IL-10 ELISA Kit (#M1910, Sanquin, Amsterdam) according to the manufacturer´s instructions.

RT-PCR analysis of gene expression

For mRNA isolation, $2x10^4$ cells were lysed in 200 µL TRI Reagent (#T-9424, Sigma, Taufkirchen, Germany). Chloroform was added and mRNA was recovered from the upper phase, precipitated with isopropanol, washed with 75 % ethanol and dissolved in water. mRNA was then reverse-transcribed using oligo(dT) primers. Quantitative PCR was performed using the LigthCycler-FastStart DNA Master SYBR Green I Kit (#12239264004, Roche; Mannheim, Germany) in a LightCycler device (Roche) using the following cycling parameters: a single pre-incubation step at 95°C for 10 min, then 38-40 cycles with 95°C 0 s, 60°C for 10 s, 72°C for 25 s. All reactions were finished with a melting curve run to establish the specificity of the PCR.

IL-10 amplification was carried out in parallel with amplification of the housekeeping gene α-enolase. mRNA expression levels of IL-10 were normalized to expression levels of α-enolase. The following primers were used:

IL-10 primer (product length 204 bp)

5`primer: 5` GCC TAA CAT GCT TCG AGA TC 3`

3`primer: 5` TGA TGT CTG GGT CTT GGT TC 3`

α-enolase primer (product length 619 bp)

5`primer: 5` GTT AGC AAG AAA CTG AAC GTC ACA 3`

3`primer: 5` TGA AGG ACT TGT ACA GGT CAG 3`

β_2-microglobulin (β2MG) (product length 116 bp)

5`primer: 5′- GGC TAT CCA GCG TAC TCC AAA G -3′

3`primer: 5′- CAA CTT CAA TGT CGG ATG GAT G -3′

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from heparinised (10 U/mL) blood from healthy human volunteers by density gradient centrifugation (Lymphoprep, 1,077 g/mL, Oslo, Norway). All subjects gave written informed consent, and the Ethics Committee of the medical Faculty of Ludwig Maximilian University, Munich, approved the study. Cells were directly used for subsequent isolation of CD19+ B cells.

Isolation of primary CD19+ B Cells

For positive selection of CD19+ B cells, the MACS magnetic separation technique was used (all columns and reagents from Miltenyi Biotec, Bergisch-Gladbach, Germany). A total of 15 x 10^6 PBMC were resuspended in 80 µL of PBS containing 20 µL anti CD19-MicroBeads (130-050-301, Miltenyi Biotec). After incubation for 30 min at 4°C, cells were washed and resuspended in 1,5 mL PBS and then loaded onto a LS column (#120-000-475) in a midiMACS magnet. The column was washed five times with 2 mL PBS each. Cells were recovered from the column by flushing the column five times with 2 mL PBS using a plunger. CD19+ cells were washed and resuspended in RPMI 1640 Medium in a final concentration of 1x 10^6 cells/mL for stimulation. To determine purity of the CD19+ B cells, a sample was stained with FITC conjugated anti-CD19 antibody (#555412, Becton-Dickinson, Heidelberg, Germany) and measured by flow cytometry. CD19+ B cells with a purity of 95 % or higher were used.

Reporter plasmids, transfection and luciferase assay

In this project we employed a variety of luciferase reporter plasmids with wild type and mutant sequences of the human IL-10 promoter upstream of the luciferase reporter gene. The following constructs were used:

Construct name	**Promoter sequence**
pIL-10 (-1044).luci	-1044 bp upstream of the IL-10 transcription start
pIL-10 (-195). luci	-195 bp upstream of the IL-10 transcription start
pIL-10 (-195 IRF mut).luci	-195 bp, carrying a mutation at the IRF motif
pIL-10 (-195 STAT mut).luci	-195 bp, carrying a mutation at the STAT-motif
pIL-10 (-195 IRFmut+ STAT3 mut).luci	-195 bp, carrying a mutation at the IRF and STAT-motif
pIL-10 (3xLS4).luci	three STAT3 binding motifs
pIL-10 (-150).luci	-150 bp upstream of the IL 10 transcription start

In addition we used a linker scanning series based on pIL-10 (-150).luci, which has decameric parts of the –150 promoter sequence sequentially replaced by an inert sequence. These constructs are labelled pIL-10 LS1.luci through pIL-10 LS9.luci.

All these constructs are described in Benkhart et al. (Benkhart et al., 2000; Ziegler-Heitbrock et al., 2003). The RPMI 8226.1 cells were transfected according to the method described by Shakov et al. (Shakhov et al., 1990). In brief, 1 x 10^7 cells in 1 mL RPMI 1640 Medium, supplemented with 2 mM L-Glutamin (#25030-024, Invitrogen, Karlsruhe, Germany) and 66,6 µg/mL DEAE-

Dextran (#E1210, Promega, Mannheim) were cotransfected with one of the promoter luciferase reporter constructs (mentioned above) and Renilla luciferase (#E6291, phRG-TK Vector; Promega, Mannheim) as internal control and incubated for 90 minutes at 37°C. After addition of 10 % DMSO for 150 s, cells were washed and cultured for 3 days in 6 well plates.

Cells were stimulated for 6 h with and without 6α-Methyl-Prednisolone at 10^{-6} M. Cell lysates were prepared using the Dual-Luciferase® Reporter Assay System (#E1910, Promega, Mannheim) according to the manufacturer`s instructions. Luciferase activity was measured in a Luminometer (Berthold, Pforzheim) and expressed as RLU/5x10E5 cells relative to Renilla.

Adenoviral (AdV) vectors and viral infections

A recombinant replication-deficient AdV vector encoding the STAT3 $Tyr^{705} \rightarrow$ Phe mutation which is dominant negative (S3 DN) (Kunisada et al., 1998) was kindly provided by Y. Fasjio (Osaka University, Osaka, Japan). An identical construct, lacking the insert (AdV0), was provided by A. Byrnes and M. Wood (University of Oxford, Oxford, UK). The recombinant viruses were purified and concentrated as previously described (Foxwell et al., 1998). RPMI-8226.1 cells were infected with virus in 24-well ultra low attachment plates (#3473, Costar) at a multiplicity of infection (MOI) of 50 for 2 h in serum-free media. FCS was then added to cultures and cells were incubated at standard conditions overnight. Cells were washed and re-suspended in media containing 10 % serum. Cells were then

plated with 1 mL volumes /well on a 24 well ultra low attachment plate followed by stimulation.

Gel shift Analysis

Nuclear extracts were isolated according to the method described by Dignam et al. (Dignam et al., 1983) in the presence of a protease inhibitor mixture (10 µg/mL aprotinin (Sigma, #A6279), 1 mM PMSF (#P7626, Sigma), 40 µg/mL leupeptin-propionyl (#L3402, Sigma), 20 µg/mL leupeptin-acetate (#L2023, Sigma), 20 µg/mL antipain (#A6191, Sigma), 20 µg/mL pepstatin A (#P4265, Sigma), 400 µM ALLN (#A6185, Sigma) and 2 mM DTT (#19474, Merck). Three µg of nuclear protein was admixed with ^{32}P-labeled double-stranded LS4 oligonucleotide (sense 5`-AGC TAT CCT GTG CCG GGA AAC C-3`, anti-sense 5`-TCG AGG TTT CCC GGC ACA GGA T-3`) the presence of 0,5 µg poly(dI/dC) (#27-7880-02, Amersham, Freiburg), 2 mg/mL BSA, 10 % v/v nuclear extract buffer D^+ (20 mM HEPES pH 7,9; 25 % (v/v) glycerol, 100 mM KCL, 0,05 mM EDTA pH 8 and 0,2 % (v/v) Igepal CA-630 (#I3021 Sigma). After 20 min of incubation at room temperature samples were electrophoresed on non-denaturing polyacrylamid gels in 0,25x TBE buffer. For supershift analysis nuclear extracts were first incubated with antibodies at 0,2 µg/µL for 30 min on ice. Rabbit polyclonal antibodies against STAT3 (c-20), sc-482 and control antibody IgG, sc-2027 were from Santa Cruz Biotechnology (Santa Cruz, CA). Gels were run with a distance of 10 cm for standard gelshift and 15 cm for supershift analysis.

Chromatin Immunoprecipitation

For ChIP $2x10^7$ cells were stimulated with and without 6α-Methyl-Prednisolone (MP) at 10^{-6} M for 2 h. Cells were cross-linked in 1 % formaldehyde for 30 min. Cross-linking was stopped by the addition of glycine to a final concentration of 1.25 M. Cells were washed with ice cold PBS, Buffer B (0,25 % Triton X-100, 10 mM EDTA pH 8, 0,5 mM EGTA pH 8, 20 mM HEPES pH 7,6) and Buffer C (0,15 M NaCl, 1 mM EDTA pH 8, 0,5 mM EGTA pH 8, 50 mM HEPES pH 7,6). Cell extract was diluted in 1 mL Chromatin incubation buffer (0,75 % SDS, 5 % Triton X-100, 0,75 M NaCl, 5 mM EDTA pH 8, 2,5 mM EGTA pH 8, 100 mM HEPES pH 7,6) and 1 x complete protease inhibitor cocktail (PIC) (# 1697498, Roche, Mannheim) and sonicated according to the manufacturer`s protocol using the Diagenode Bioruptor (Diagenode, Liège, Belgium). One hundred µL chromatin was saved as input.

In preparation of immunoprecipitation protein A/G Plus-Agarose beads (sc-2003, Santa Cruz Biotechnology) were washed twice with incubation buffer containing bovine serum albumin at 1 µg/mL and subsequently incubated in the same buffer for 2 h at 4°C. For immunoprecipitation (IP) 120 µL chromatin were admixed with 6 µL 5 % BSA, 10 µL 1x PIC, 36 µL 5x incubation buffer, 30 µL bead-suspension, anti-STAT3 (c-20) (#sc-482, Santa Cruz Biotechnology) at 6µg/µL and H_2O (300 µL total volume) and incubated overnight on a rotating wheel at 4°C. Beads were washed at 4°C twice with washbuffer1 (0,1 % SDS, 0,1 % NaDOC, 1 % Triton X-100, 0,15 mM NaCl, 1 mM EDTA pH 8, 0,5 mM EGTA pH 8, 20 mM HEPES pH 7,6), once with washbuffer 2 (0,1 % SDS, 0,1 %

NaDOC, 1 % Triton X-100, 0,5 M NaCl, 1 mM EDTA pH 8, 0,5 mM EGTA pH 8, 20 mM HEPES pH 7,6), once with washbuffer 3 (0,25 M LiCl, 0,5 % NaDOC, 0,5 % NP-40, 1 mM EDTA pH 8, 0,5 mM EGTA pH 8, 20 mM HEPES pH 7,6) and twice with washbuffer 4 (10 mM EDTA pH 8, 5 mM EGTA pH 8, 200 mM HEPES pH 7,6). DNA-protein complexes were eluted with 400 µL elution buffer (1 % SDS, 0.1 M $NaHCO_3$) and decrosslinked by adding 0,2 M NaCl and shaking for 5 h at 65°C. DNA was extracted using phenol-chloroform and then chloroform and precipitated overnight with ethanol. The precipitated DNA was redissolved in 200 µL H_2O (sample) or 100 µL H_2O (Input) and real-time PCR analysis was performed to quantify the amount of input and immunoprecipitated DNA. The primer sets that were used are as follows.

human myoglobin (product length 76 bp)

5`primer: 5`AAG TTT GAC AAG TTC AAG CAC CTG 3`

3`primer: 5`TGG CAC CAT GCT TCT TTA AGT C 3`

human IL-10 promoter, including the LS4 sequence (product length 80 bp)

5`primer: 5`TAG AGA AGG AGG AGC TCT AAG CAG 3`

3`primer: 5`AGG CCT CTT CAT TCA TTA AAA AGC C 3`

Quantitative PCR was performed using iQ™ SYBR® Green Supermix (#10003253RevA, BioRad, munich) and a single-colour detection MyIQ iCycler (Bio-Rad, Hercules, CA, USA). Conditions were the following: a single pre-incubation step at 95°C for 3 min, then 40 cycles with 95°C 15 s, 60°C for 45 s,

95°C for 1 min, 65°C for 1 min. All reactions were finished with a melting curve. Signals were expressed as percentage of the input.

Western Blot analysis

Ten to twenty micrograms of protein were resolved on either a 4-12% Novex® tris-glycine gel or NuPage ® bis-tris gel (Invitrogen) and transferred to Hybond-N^{TM} membranes (Amersham) using a Novex® X-Cell II^{TM} Mini Cell. Membranes were blocked for 1 h at room temperature with blocking buffer (5% (w/v) fat-free milk and 0.1% (v/v) Tween 20 on TBS), followed by overnight incubation at 4°C with polyclonal STAT3 Ab (sc-482, Santa Cruz), polyclonal phospho-STAT3 (Tyr705) Ab (#9131, Cell Signaling Technology), polyclonal phospho-STAT3 (Ser727) Ab (#9134 Cell Signaling Techology) or anti-actin Ab (#A2066, Sigma) diluted 1:1000 in blocking buffer. Peroxidase-conjugated anti-rabbit IgG (A0545, Sigma) was used as a secondary antibody at a dilution of 1:1000. Bound antibody was detected using the ECL kit (Amersham) and was visualised on X-Ray film.

Statistics

Statistical analysis was performed using the Student`s T-test. A value $p<0.05$ was considered significant.

Results

Induction of IL-10 mRNA and protein by Methyl-Prednisolone

Human B cells are a ready source of IL-10. MP treatment of purified CD19+ B cells from human peripheral blood led to a 3-fold induction of IL-10 mRNA (n= 6, $p< 0.05$, see Figure 1A). In order to study the molecular mechanisms involved in the induction of this gene in B cells we turned to a model B cell line.

The RPMI 82226.1 B cell line has proven a useful model in previous studies on the expression of IL-10 when using stimuli like LPS and Interferon alpha (Benkhart et al., 2000; Ziegler-Heitbrock et al., 1994; Ziegler-Heitbrock et al., 2003).

Methyl-Prednisolone readily induced IL-10 protein in these cells as shown in Figure 1B. Overnight treatment with the glucocorticoid at 10^{-6} M led to a 23-fold increase of protein in the culture supernatant as detected by ELISA (Fig 1B). When analysing the mRNA expression we tested different concentrations of MP and found the highest induction (17-fold) when treating the cells for 4 h with 10^{-6} M of the glucocorticoid. The response was lower at $10E^{-7}$ M and still was evident as a 5-fold increase at $10E^{-8}$ M (Fig 1C). Using a dose of 10^{-6} M a time course study demonstrated maximum levels of IL-10 transcripts at 2 and 4 hours with the levels returning to background at 24 h (Fig 1D). These data show that MP can induce IL-10 in the model cell line in a dose and time dependent manner.

Induction of the IL-10 promoter by Methyl-Prednisolone

While the induction of IL-10 by MP has been known for some time, the molecular mechanisms involved thus far remained elusive. For the study of these mechanisms we transfected the RPMI 8226.1 cells with a luciferase reporter plasmid driven by the –1044 IL-10 promoter. Transfected cells were stimulated with MP for 6 h and luciferase activity was determined in cell lysates with reference to a control plasmid encoding Renilla luciferase. In these studies MP at 10^{-6} M led to a 18-fold induction of the promoter, while at 10^{-7} M it was 11-fold and at 10^{-8} M there was still a 2-fold induction (Fig 2). When comparing promoter activity and induction of IL-10 mRNA levels (Fig 1C), then a very similar degree of stimulation with a similar dose response is seen suggesting that the regulation of IL-10 mRNA expression by glucocorticoids is mainly transcriptional.

The schematic drawing of the promoter construct in the upper part of Figure 2 indicates the location of transcription factor motifs for Sp1, IRF and STAT that have been described in previous reports. In addition a potential GRE motif can be identified at –316 (Figure 2). In order to ascertain whether this motif is necessary for the induction of the IL-10 gene we turned to shorter constructs lacking this site.

When analysing a –195 IL-10 promoter luciferase reporter gene construct we noted an 8,8-fold induction of the wt construct by MP at 10^{-6} M (Figure 3), suggesting that the potential GRE motif is dispensable for induction by glucocorticoids.

Role of the –120 STAT-motif in induction of the IL-10 promoter by Methyl-Prednisolone

We then studied the role of the IRF and STAT sites within this short fragment by transfecting constructs with specific mutants of these sites. The IRF mutant (IRFm) construct did show an unaltered activity as compared to wt, but surprisingly the STAT mutant (STATm) showed strongly reduced activity and the same was found for a construct with mutation of both sites (Figure 3). These data indicate that the STAT motif may be crucially involved in the glucocorticoid induction of the human IL-10 gene. Since there still was some induction of the IL-10 gene even in the STAT mutant, we asked whether there might be another element within this –150 fragment, that may be involved in the induction. For this we employed a linker scanning series as described previously, which stepwise replaces promoter sequences with an inert decameric sequence(Benkhart et al., 2000). When cells were transfected with these constructs and treated with MP, a significant decrease in glucocorticoid inducibility was only observed for the mutation at –120 bp, i.e. of the STAT motif (LS4 in Figure 4). Hence, no additional elements involved in glucocorticoid mediated IL-10 promoter induction were identified within this –150 fragment of the promoter.

We next asked whether Methyl-Prednisolone would induce a promoter construct carrying a multimer of the STAT-motif. A construct with a trimer of the IL-10 promoter STAT-motif in front of the luciferase reporter gene did, in fact, show a robust induction by MP, which was in average 28,8-fold (Figure 5). This

demonstrates that glucocorticods can induce a strong transactivation through a STAT site.

Role of STAT3 in the induction of the IL-10 gene by Methyl-Prednisolone

Since the –120 STAT-motif of the IL-10 promoter has been demonstrated to mainly bind STAT3 we next asked whether interference with STAT3 may block MP induction of the IL-10 gene expression. For this we infected RPMI 8226.1 cells with an adenovirus expressing dominant negative STAT3. This led to a clear expression of exogenous STAT3 protein by the cells (Figure 6, upper panel). When such cells were treated with MP followed by determination of IL-10 mRNA by RT-PCR then there was a strong reduction of IL-10 transcript levels (Figure 6, lower panel). In the same experiments cells infected with empty adenovirus did show a greater than 30-fold induction of IL-10 mRNA comparable to cells stimulated with MP in the absence of virus. These data suggest that STAT3 may be crucially involved in glucocorticoid induced IL-10 expression.

We therefore performed gelshift analysis using a radiolabelled double-stranded oligonucleotides encompassing the IL-10 promoter STAT-motif. As shown in Figure 7A, LPS stimulation of the cells will induce a DNA-protein-complex with a maximum signal at 4 h, as demonstrated previously (Benkhart et al., 2000). MP treatment of the cells will induce no binding protein at 1h but there is a strong signal at 2 h, which decreases at 4 h. Supershift analysis of this binding protein was performed at the 2 h time point and demonstrated a complete shift of the band

by the anti-STAT3 antibody but not by control antibody (Figure 7B). These data support a crucial involvement of STAT3 in glucocorticoid-mediated induction of IL-10.

Methyl-Prednisolone induces binding of STAT3 to the IL-10 promoter in intact cells

In order to confirm that MP can, in fact, induce recruitment of the STAT3 transcription factor to the human IL-10 promoter in intact cells we performed ChIP analysis of cells treated with MP at 10^{-6} M for 2 h. After stimulation cells were fixed, DNA was fragmented, STAT3 was immuno-precipitated and the bound DNA was subjected to PCR using primers covering the 80 bp promoter proximal sequence including the STAT motif. Gel electrophoresis of PCR products shows that MP treatment led to a clear increase in STAT3 binding (Figure 8A, upper panel) while the input DNA was similar (Figure 8A, lower panel). Real time PCR analysis was then performed and the anti-STAT3 precipitated DNA was calculated as percentage of DNA. These studies showed a clear 3,5-fold induction of STAT3-binding to the promoter (Figure 8B). By contrast, precipitation from a myoglobin promoter fragment, which lacks any STAT-binding motif, shows only a weak signal without any induction by MP treatment (see Figure 8B). These findings confirm the central role of STAT3 in glucocorticoid induction of the human IL-10 gene.

Discussion

IL-10 is produced by B cells and this cytokine has been shown to act in an autocrine fashion in proliferation and differentiation of these cells (Masood et al., 1995; O'Garra et al., 1990; Rousset et al., 1992) but also in induction of apoptosis of leukemic B cells (Fluckiger et al., 1994).

IL-10 can be induced in B cells by engagement of the B cell receptor (Fillatreau et al., 2002), via CD40 ligation (Mauri et al., 2003), Interferon-alpha stimulation (Ziegler-Heitbrock et al., 2003) and stimulation with LPS (Dalwadi et al., 2003; Ziegler-Heitbrock et al., 1994), but induction by GCs in B cells has not been documented to date. We show herein, that primary peripheral blood B cells will up-regulate IL-10 when treated with Methyl-Prednisolone.

In order to study the molecular mechanism we have turned to the RPMI 8226.1 B cell line. Using this model we can demonstrate a time and dose dependent induction of IL-10 and we can show that the effect is largely transcriptional as demonstrated with a –1044 bp IL-10 promoter luciferase reporter construct (Figure 2). This sequence gives an 18-fold induction by MP and contains several transcription factor motifs including a Sp1 site, an IRF site, a STAT site and a potential GRE at –316. While the GRE motif matches the AGAACAnnnTGTTCT consensus a shorter IL-10 promoter construct lacking this motif did still show a robust 9-fold induction, i.e. 2-fold lower compared to the full length construct, suggesting that the –316 motif is not required for GC induced IL-10.

By contrast all the evidence points towards a role for the –120 STAT-motif TGCCGGGAA as the crucial site for GC-mediated regulation of the human IL-10 promoter. This includes the finding of a strong reduction in trans-activation when the site is mutated (Figure 3). Since with mutation of the STAT-site there was still some inducibility albeit at a very low level, we have asked whether there might be additional sites in the proximal promoter involved in GC induction of IL-10. A linker scanning series did however only detect a reduced trans-activation when the linker was inserted at the STAT-site and not in any other region of the promoter. Thus it appears that trans-activation by MP only occurs via the STAT-motif.
The role of STAT3 in this process is supported by 3 pieces of evidence: a) infection with an adenovirus carrying dominant negative STAT3 could block Methyl-Prednisolone-induced IL-10 mRNA induction, b) binding of STAT3 to the motif is demonstrable in gelshift and supershift analysis and c) Methyl-Prednisolone will induce STAT3 binding to the IL-10 promoter in intact cells as shown by ChIP (see Figures 6, 7 and 8).

When considering the possible mechanism leading to involvement of STAT3 in GC induction of IL-10 gene expression the question is, whether there is any role for the GCR. In fact, we could demonstrate a complete blockade of IL-10 mRNA production and promoter activity using the steroid receptor blocker RU-486 (data not shown). Based on this one might consider the possibility that the GCR directly interacts with the STAT3.

The interaction of glucocorticoids with STAT3 have been described earlier for the regulation of genes like fibrinogen and alpha 2 macroglobulin (Duan and Simpson-Haidaris, 2006; Lerner et al., 2003; Takeda et al., 1998). In all these cases GCs do have little activity on their own but they operate synergistically with IL-6 induced STAT3. This synergistic action is mediated by interaction of the GCR protein with the STAT3 protein (Lerner et al., 2003). This is clearly different from the present system where GC alone will induce substantial STAT3 and this will bind and transactivate the IL-10 promoter leading to gene expression without any apparent co-stimulus.

The question then is: how do GCs increase STAT3 binding to the IL-10 promoter in intact cells? One possibility is that binding of the GCR to STAT3 will stabilize its DNA binding and retention in the nucleus. This would imply that STAT3 shuttling between cytoplasm and nucleus is trapped by attachment to the GCR. To this end we failed to demonstrate GCR binding to the proximal IL-10 promoter by ChIP, but these preliminary results may be due to inappropriate conditions and reagents for detection of this transcription factor.

Another possibility is that GCR action leads to enhanced serine-phosphorylation of STAT3 with subsequent nuclear translocation of STAT3 homodimers. Such a non-genomic action of GCs via the GCR has been shown to act via PI-3 kinase and Akt (Hafezi-Moghadam et al., 2002). However, GC treatment of RPMI 8226.1 cells did not induce increased serine phosphorylation and PI-3 kinase inhibition did not prevent GC-induced IL-10 mRNA production (data not shown).

While all the data suggest that the -120 Stat binding motif in the human IL-10 gene is crucial to GC induction of IL-10, there may be some contribution by the –316 GRE motif and we cannot exclude that there are additional strong elements upstream of – 1044 bp.

Induction of IL-10 by GCs had been demonstrated previously and the current study demonstrates for the first time a pronounced induction in B lymphocytes which is dependent on the transcription factor STAT3. The GC induction in B cells may contribute to the immuno-suppressive function of what has been coined regulatory B cell (Mizoguchi and Bhan, 2006).

As discussed above GC can also induce IL-10 in monocytes (Mozo et al., 2004), in macrophages (Frankenberger et al., 2005) and in T cells (Barrat et al., 2002; Richards et al., 2000).

The mode of action of GC in inducing IL-10 in these cells is still unclear but the current data on the molecular mechanism in B cells may provide a paradigm for the way IL-10 is induced by GC in other leukocytes.

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References

Barrat F. J., Cua D. J., Boonstra A., Richards D. F., Crain C., Savelkoul H. F., de Waal-Malefyt R., Coffman R. L., Hawrylowicz C. M. and O'Garra A. (2002) In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 195, 603-16.

Benkhart E. M., Siedlar M., Wedel A., Werner T. and Ziegler-Heitbrock H. W. (2000) Role of Stat3 in lipopolysaccharide-induced IL-10 gene expression. *J Immunol* 165, 1612-7.

Bettelli E., Das M. P., Howard E. D., Weiner H. L., Sobel R. A. and Kuchroo V. K. (1998) IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol* 161, 3299-306.

Brenner S., Prosch S., Schenke-Layland K., Riese U., Gausmann U. and Platzer C. (2003) cAMP-induced Interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. *J Biol Chem* 278, 5597-604.

Brightbill H. D., Plevy S. E., Modlin R. L. and Smale S. T. (2000) A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. *J Immunol* 164, 1940-51.

Cao S., Liu J., Chesi M., Bergsagel P. L., Ho I. C., Donnelly R. P. and Ma X. (2002) Differential regulation of IL-12 and IL-10 gene expression in macrophages by the basic leucine zipper transcription factor c-Maf fibrosarcoma. *J Immunol* 169, 5715-25.

Cao S., Liu J., Song L. and Ma X. (2005) The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. *J Immunol* 174, 3484-92.

Chang C. C., Wright A. and Punnonen J. (2000) Monocyte-derived CD1a+ and CD1a- dendritic cell subsets differ in their cytokine production profiles, susceptibilities to transfection, and capacities to direct Th cell differentiation. *J Immunol* 165, 3584-91.

Cheng F., Wang H. W., Cuenca A., Huang M., Ghansah T., Brayer J., Kerr W. G., Takeda K., Akira S., Schoenberger S. P., Yu H., Jove R. and Sotomayor E. M. (2003) A critical role for Stat3 signaling in immune tolerance. *Immunity* 19, 425-36.

Dai W. J., Kohler G. and Brombacher F. (1997) Both innate and acquired immunity to Listeria monocytogenes infection are increased in IL-10-deficient mice. *J Immunol* 158, 2259-67.

Dalwadi H., Wei B., Schrage M., Spicher K., Su T. T., Birnbaumer L., Rawlings D. J. and Braun J. (2003) B cell developmental requirement for the G alpha i2 gene. *J Immunol* 170, 1707-15.

Dandona P., Aljada A., Garg R. and Mohanty P. (1999) Increase in plasma interleukin-10 following hydrocortisone injection. *J Clin Endocrinol Metab* 84, 1141-4.

de Waal Malefyt R., Haanen J., Spits H., Roncarolo M. G., te Velde A., Figdor C., Johnson K., Kastelein R., Yssel H. and de Vries J. E. (1991) Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via

downregulation of class II major histocompatibility complex expression. *J Exp Med* 174, 915-24.
de Waal Malefyt R., Yssel H., Roncarolo M. G., Spits H. and de Vries J. E. (1992) Interleukin-10. *Curr Opin Immunol* 4, 314-20.
Dignam J. D., Lebovitz R. M. and Roeder R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11, 1475-89.
Duan H. O. and Simpson-Haidaris P. J. (2006) Cell type-specific differential induction of the human gamma-fibrinogen promoter by interleukin-6. *J Biol Chem* 281, 12451-7.
Fillatreau S., Sweenie C. H., McGeachy M. J., Gray D. and Anderton S. M. (2002) B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3, 944-50.
Fiorentino D. F., Bond M. W. and Mosmann T. R. (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170, 2081-95.
Fluckiger A. C., Durand I. and Banchereau J. (1994) Interleukin 10 induces apoptotic cell death of B-chronic lymphocytic leukemia cells. *J Exp Med* 179, 91-9.
Foxwell B., Browne K., Bondeson J., Clarke C., de Martin R., Brennan F. and Feldmann M. (1998) Efficient adenoviral infection with IkappaB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappaB dependent. *Proc Natl Acad Sci U S A* 95, 8211-5.
Frankenberger M., Haussinger K. and Ziegler-Heitbrock L. (2005) Liposomal methylprednisolone differentially regulates the expression of TNF and IL-10 in human alveolar macrophages. *Int Immunopharmacol* 5, 289-99.
Gayo A., Mozo L., Suarez A., Tunon A., Lahoz C. and Gutierrez C. (1998) Glucocorticoids increase IL-10 expression in multiple sclerosis patients with acute relapse. *J Neuroimmunol* 85, 122-30.
Hafezi-Moghadam A., Simoncini T., Yang Z., Limbourg F. P., Plumier J. C., Rebsamen M. C., Hsieh C. M., Chui D. S., Thomas K. L., Prorock A. J., Laubach V. E., Moskowitz M. A., French B. A., Ley K. and Liao J. K. (2002) Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat Med* 8, 473-9.
Herbeuval J. P., Lelievre E., Lambert C., Dy M. and Genin C. (2004) Recruitment of STAT3 for production of IL-10 by colon carcinoma cells induced by macrophage-derived IL-6. *J Immunol* 172, 4630-6.
John M., Lim S., Seybold J., Jose P., Robichaud A., O'Connor B., Barnes P. J. and Chung K. F. (1998) Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am J Respir Crit Care Med* 157, 256-62.
Karagiannidis C., Akdis M., Holopainen P., Woolley N. J., Hense G., Ruckert B., Mantel P. Y., Menz G., Akdis C. A., Blaser K. and Schmidt-Weber C. B. (2004) Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* 114, 1425-33.
Kasprzycka M., Marzec M., Liu X., Zhang Q. and Wasik M. A. (2006) Nucleophosmin/anaplastic lymphoma kinase (NPM/ALK) oncoprotein induces the T regulatory cell phenotype by activating STAT3. *Proc Natl Acad Sci U S A* 103, 9964-9.

Kinjyo I., Inoue H., Hamano S., Fukuyama S., Yoshimura T., Koga K., Takaki H., Himeno K., Takaesu G., Kobayashi T. and Yoshimura A. (2006) Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor-beta 1. *J Exp Med* 203, 1021-31.

Kunisada K., Tone E., Fujio Y., Matsui H., Yamauchi-Takihara K. and Kishimoto T. (1998) Activation of gp130 transduces hypertrophic signals via STAT3 in cardiac myocytes. *Circulation* 98, 346-52.

Lerner L., Henriksen M. A., Zhang X. and Darnell J. E., Jr. (2003) STAT3-dependent enhanceosome assembly and disassembly: synergy with GR for full transcriptional increase of the alpha 2-macroglobulin gene. *Genes Dev* 17, 2564-77.

Ma W., Lim W., Gee K., Aucoin S., Nandan D., Kozlowski M., Diaz-Mitoma F. and Kumar A. (2001) The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. *J Biol Chem* 276, 13664-74.

Maritano D., Sugrue M. L., Tininini S., Dewilde S., Strobl B., Fu X., Murray-Tait V., Chiarle R. and Poli V. (2004) The STAT3 isoforms alpha and beta have unique and specific functions. *Nat Immunol* 5, 401-9.

Masood R., Zhang Y., Bond M. W., Scadden D. T., Moudgil T., Law R. E., Kaplan M. H., Jung B., Espina B. M., Lunardi-Iskandar Y. and et al. (1995) Interleukin-10 is an autocrine growth factor for acquired immunodeficiency syndrome-related B-cell lymphoma. *Blood* 85, 3423-30.

Mauri C., Gray D., Mushtaq N. and Londei M. (2003) Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197, 489-501.

Mizoguchi A. and Bhan A. K. (2006) A case for regulatory B cells. *J Immunol* 176, 705-10.

Mozo L., Suarez A. and Gutierrez C. (2004) Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes. *Clin Exp Allergy* 34, 406-12.

O'Garra A., Stapleton G., Dhar V., Pearce M., Schumacher J., Rugo H., Barbis D., Stall A., Cupp J., Moore K. and et al. (1990) Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Int Immunol* 2, 821-32.

Rhen T. and Cidlowski J. A. (2005) Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med* 353, 1711-23.

Richards D. F., Fernandez M., Caulfield J. and Hawrylowicz C. M. (2000) Glucocorticoids drive human CD8(+) T cell differentiation towards a phenotype with high IL-10 and reduced IL-4, IL-5 and IL-13 production. *Eur J Immunol* 30, 2344-54.

Rousset F., Garcia E., Defrance T., Peronne C., Vezzio N., Hsu D. H., Kastelein R., Moore K. W. and Banchereau J. (1992) Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* 89, 1890-3.

Shakhov A. N., Collart M. A., Vassalli P., Nedospasov S. A. and Jongeneel C. V. (1990) Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J Exp Med* 171, 35-47.

Smoak K. A. and Cidlowski J. A. (2004) Mechanisms of glucocorticoid receptor signaling during inflammation. *Mech Ageing Dev* 125, 697-706.

Takeda T., Kurachi H., Yamamoto T., Nishio Y., Nakatsuji Y., Morishige K., Miyake A. and Murata Y. (1998) Crosstalk between the interleukin-6 (IL-6)-JAK-STAT and the glucocorticoid-nuclear receptor pathway: synergistic activation of IL-6 response element by IL-6 and glucocorticoid. *J Endocrinol* 159, 323-30.

Tone M., Powell M. J., Tone Y., Thompson S. A. and Waldmann H. (2000) IL-10 gene expression is controlled by the transcription factors Sp1 and Sp3. *J Immunol* 165, 286-91.

Ziegler-Heitbrock H. W., Pechumer H., Petersmann I., Durieux J. J., Vita N., Labeta M. O. and Strobel M. (1994) CD14 is expressed and functional in human B cells. *Eur J Immunol* 24, 1937-40.

Ziegler-Heitbrock L., Lotzerich M., Schaefer A., Werner T., Frankenberger M. and Benkhart E. (2003) IFN-alpha induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3. *J Immunol* 171, 285-90.

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Figure Captions

Figure 1 Induction of IL-10 protein and mRNA by Methyl-Prednisolone

A: Induction of IL-10 mRNA in primary B cells. CD19+ B cells were isolated to > 90% purity from human peripheral blood using magnetic-bead technology. Cells were stimulated for 24 h without or with 6α-Methyl-Prednisolone (MP) at 10^{-6} M. Then mRNA was recovered and cDNA was amplified by real- time PCR with primers for IL-10 and Enolase. Data were normalised to Enolase and are expressed relative to unstimulated samples (=1) using LightCycler Relquant software.

B: Induction of IL-10 Protein. RPMI 8226.1 cells were stimulated with or without 6α-Methyl-Prednisolone (MP) at 10^{-6} M for 24 h. Supernatant IL-10 protein was measured. Results are the average of five independent experiments +/- SD.

C and D: RPMI 8226.1 cells were stimulated for various times with MP at various doses. mRNA was recovered and cDNA was amplified by real- time PCR with primers for IL-10 and Enolase. Data were normalised to Enolase and are expressed relative to 0 h unstimulated samples (=1) using LightCycler Relquant software.

C: Time course analysis. Shown is the average of four independent experiments +/- SD

D. Dose-Response Analysis. Shown is the average of three independent experiments +/- SD. * = $p < 0.05$.

Figure. 2 Effect of Methyl-Prednisolone on human IL-10 promoter activity

RPMI 8226.1 cells were cotransfected with -1044 IL-10 promoter luciferase reporter construct and Renilla luciferase. After 70 hours the cells were stimulated for 6 h with

and without MP at 10^{-6} M. Luciferase activity was measured and expressed as RLU/5x10E5 cells relative to Renilla. Results are the average of five independent experiments +/- SD. * = $p < 0.05$ The cartoon at the top denotes the documented STAT, IRF and SP1 sites and the potential GRE site identified using MatInspector software (Genomatix, Munich, Germany).

Figure. 3 Effect of Methyl-Prednisolone on IL-10 promoter mutants

Promoter activity of the -195 IL-10 promoter luciferase reporter construct or constructs carrying mutations at the IRF motif, the STAT3 motif or both, was determined after transfection together with Renilla luciferase. After 70 h cells were stimulated with and without MP at 10^{-6} M for 6 hours. Luciferase activity was measured and expressed as RLU /5x10E5 cells relative to Renilla. Data are the average of three independent experiments +/-SD. * = $p < 0.05$.

(■): untreated 0h; (▨): untreated 6h; (□): MP10^{-6} M 6h; (▩): MP^{-7}M 6 h.

The cartoon at the top shows schematically the IRF and the STAT site with X denoting their mutation as described (Benkhart et al., 2000).

Figure 4 Analysis of the effect of Methyl-Prednisolone on the -150 IL-10 promoter linker scanning series.

A 10-bp linker was consecutively introduced into the -150 construct, which was then cloned into pβTATA.luci. The scanning serie was transfected into RPMI 8226 cells. Afterwards, cells were stimulated with and without MP at 10^{-6} M for 6 h. Luciferase activity was measured expressed as RLU /5x10E5 cells relative to Renilla. Results are expressed as the fold trans-activation by MP and are the average of three independent experiments +/- SD.* = $p< 0.05$ vs pIL-10 (-150).luci. The left hand scheme depicts

the mutant sequences as black boxes, which consecutively replace the wildtype sequence.

Figure 5 Effect of Methyl-Prednisolone on a luciferase reporter construct containing 3 STAT binding motifs

Cells were transfected with the trimer construct LS 4 which contains three STAT sites taken from the human IL-10 promoter. 70 h after transfection, cells were stimulated with and without MP at 10^{-6} M for 6 hours, and luciferase activity was measured and expressed as RLU /5x10E5 cells relative to Renilla. Data are the average of three independent experiments +/-SD.

Figure 6 Adenoviral dominant-negative STAT3 inhibits IL-10 mRNA production

RPMI 8226.1 cells were cultured for 3 days before infection with or without AdV expressing dominant-negative STAT3, as indicated, at a MOI of 50 for 2 h in serum free media. After addition of serum, cells were cultured overnight before being re-suspended in fresh serum supplemented (10%) media. Cells were then treated with MP at 10^{-6} M for 4 h. Transcript levels for IL-10 and β2microglobulin mRNA levels were determined by real-time PCR. Data were normalised to β2MG and are expressed as fold induction of untreated samples. Data from 4 independent experiments are expressed as means +/- SD and are given in the lower panel. ***= $p< 0.05$ compared to cells infected with empty virus.

Upper Panel: Protein (5 µg) from each experiment was run on SDS-PAGE and immunoblotted with anti-STAT3 and a blot representative of 4 experiments is shown.

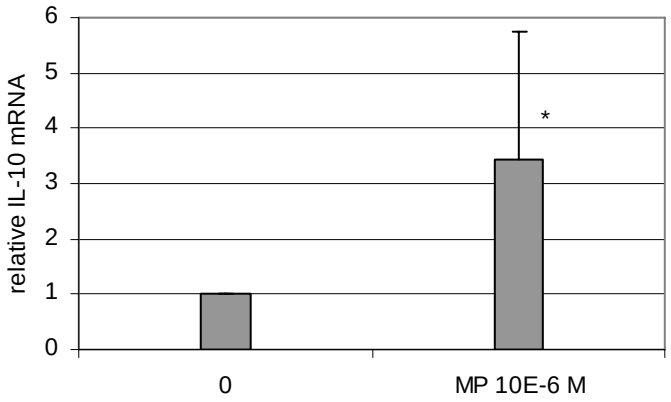
Figure. 7 Analysis of the LS 4 binding protein induced by Methyl-Prednisolone

A Gelshift**:** Nuclear extracts from cells stimulated for 1, 2 and 4 hours with MP at 10^{-6} M were admixed with radiolabeled LS4 oligonucleotide from the –120 motif of the human IL-10 promoter. Samples were separated on a nondenaturing polyacrylamide gel followed by exposure to an x-ray film. One representative of three experiments is shown. Arrow = inducible STAT-binding protein

B. Supershift**:** Nuclear extracts from cells stimulated for 2 hours MP at 10^{-6} M were admixed with radiolabeled LS4 oligonucleotide without or with anti-STAT3 antibody or control antibody (C). AB = antibody. Samples were separated on a nondenaturing polyacrylamide gel followed by exposure to an x-ray film. One of three experiments is shown.

Figure. 8 ChIP analysis of Methyl-Prednisolone induced STAT3 binding to the IL-10 promoter in intact cells10^{-6} M

Cells were stimulated with MP at 10^{-6} M for 2hours followed by formaldehyde fixation, sonication and precipitation with anti-STAT3. The 80 bp IL-10 promoter fragment encompassing the STAT-motif was amplified by RT-PCR. **A.** Agarose gel electrophoresis of the products for input (diluted 1:15) and for the STAT3 precipitate. **B.** Quantitation of the recovery for STAT3 as % of input. For comparison a 76 bp fragment of the myoglobin promoter was analysed. Average of n=3. * = $p < 0.05$



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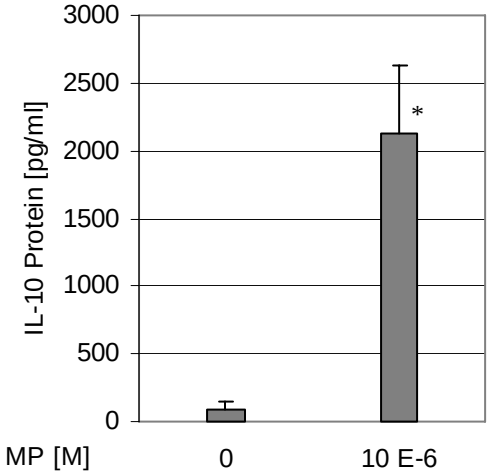

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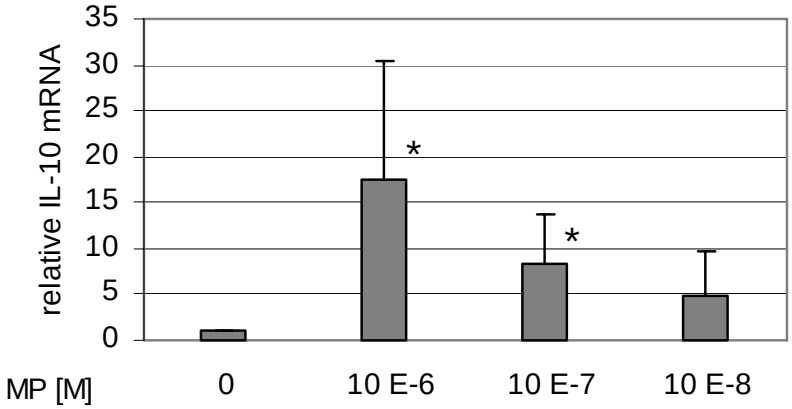

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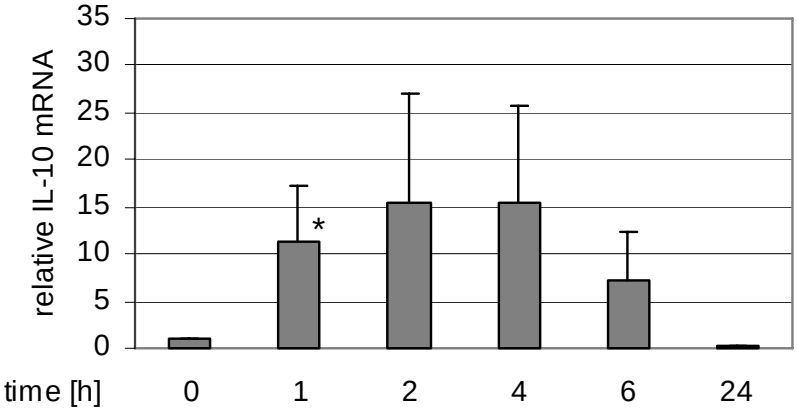


D

Figure 1

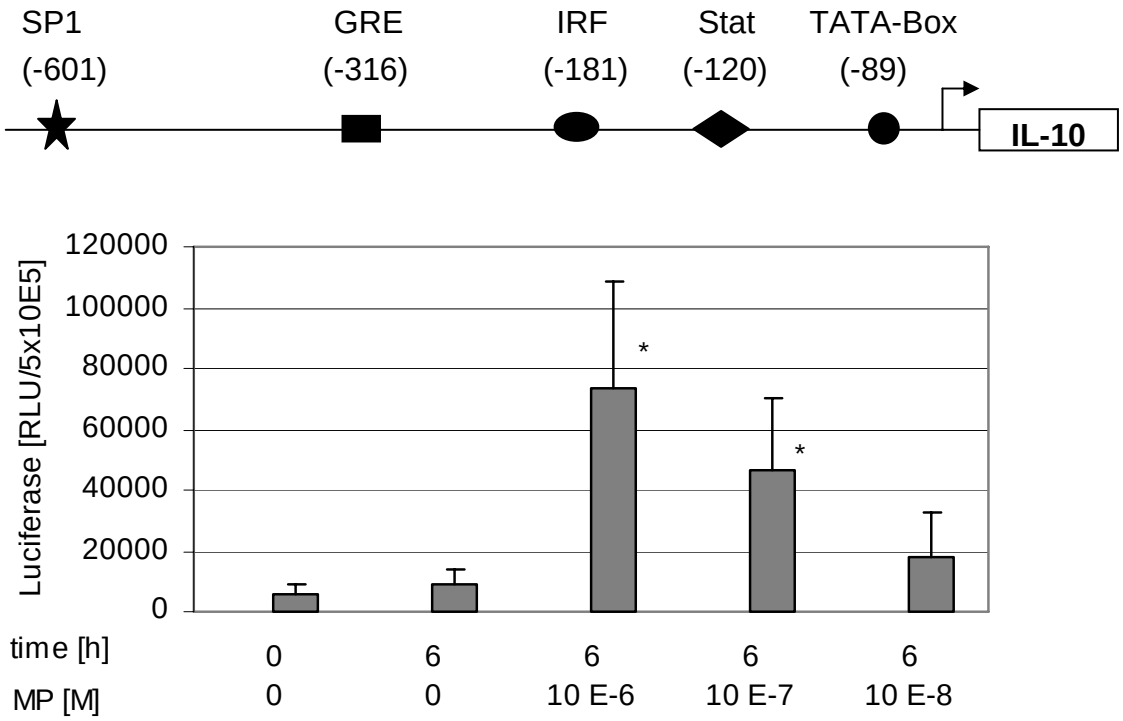


Figure 2

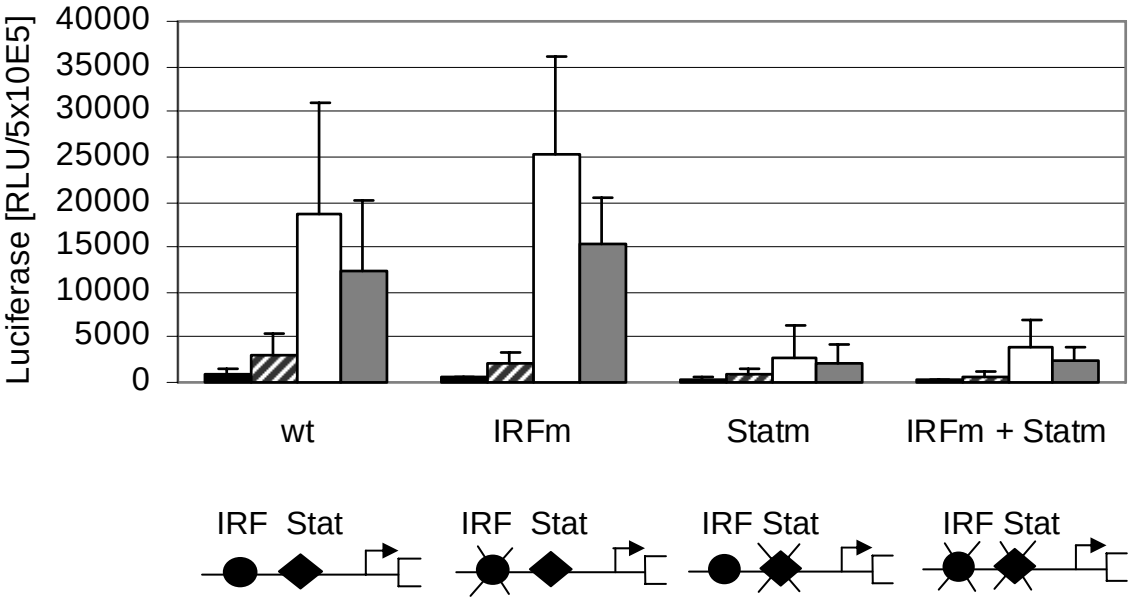
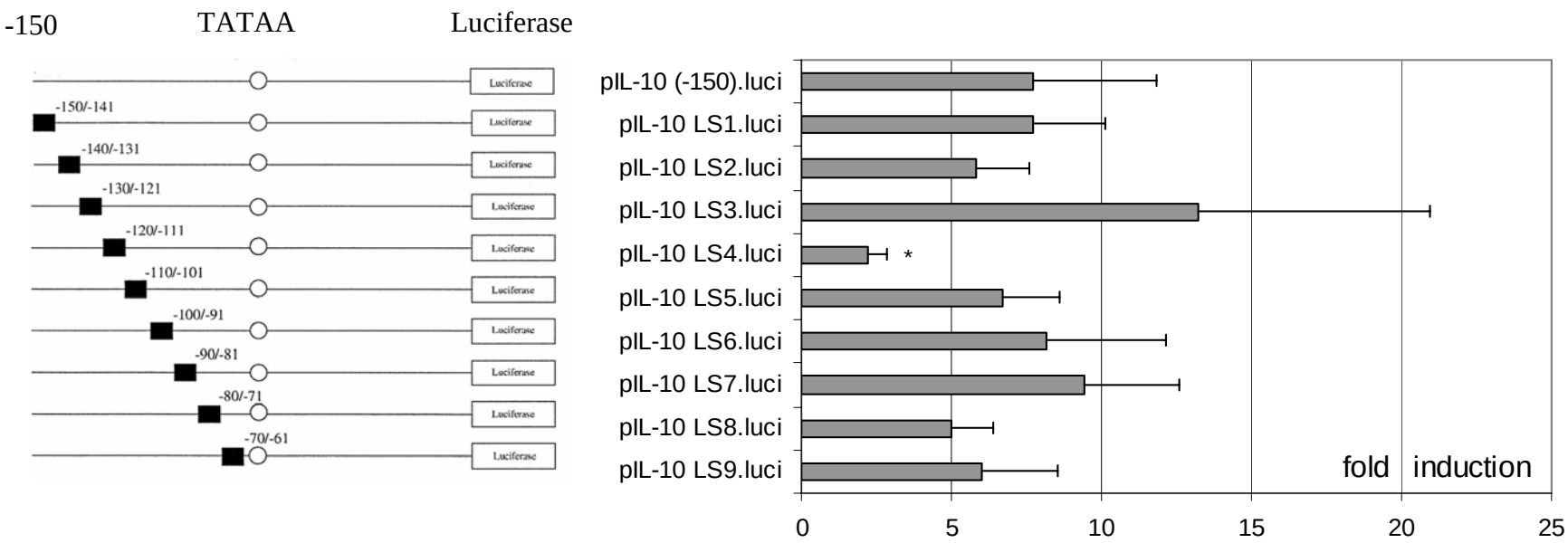


Figure 3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TATAA
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-140/-131
-130/-121
-120/-111
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-100/-91
-90/-81
-80/-71
-70/-61
pIL-10 (-150).luci
pIL-10 LS1.luci
pIL-10 LS2.luci
pIL-10 LS3.luci
pIL-10 LS4.luci
pIL-10 LS5.luci
pIL-10 LS6.luci
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Figure 4

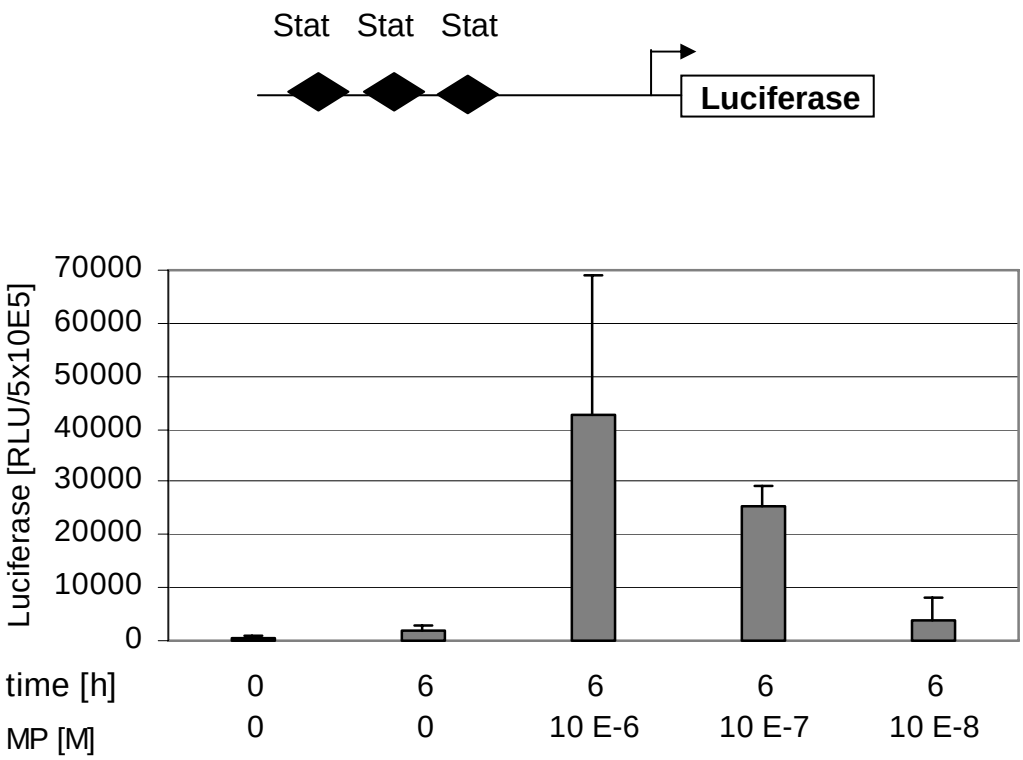
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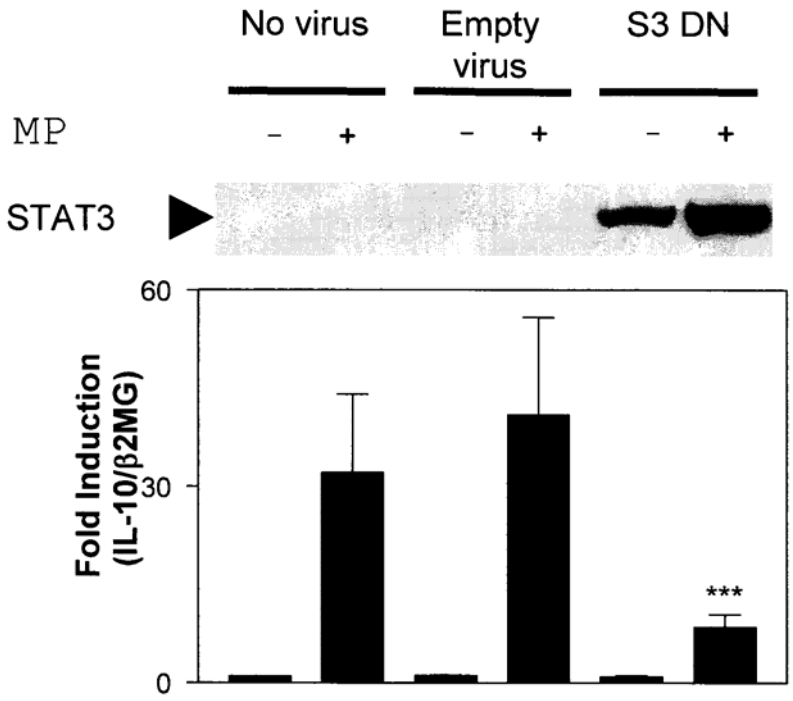
Figure 5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Fold Induction (IL-10/β2MG)
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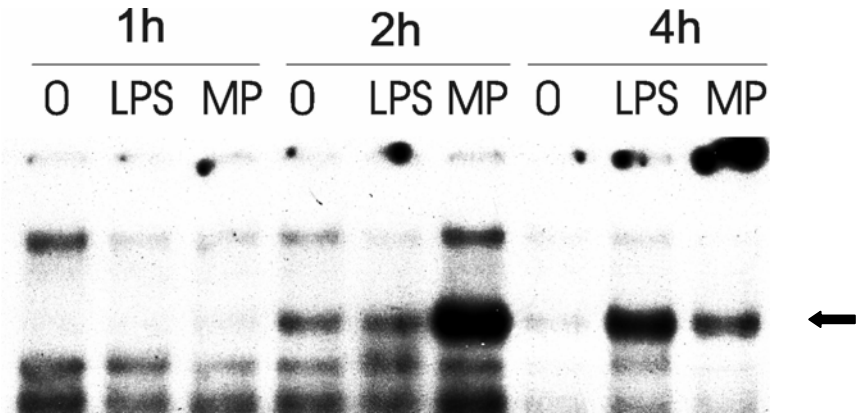
Figure 6

A



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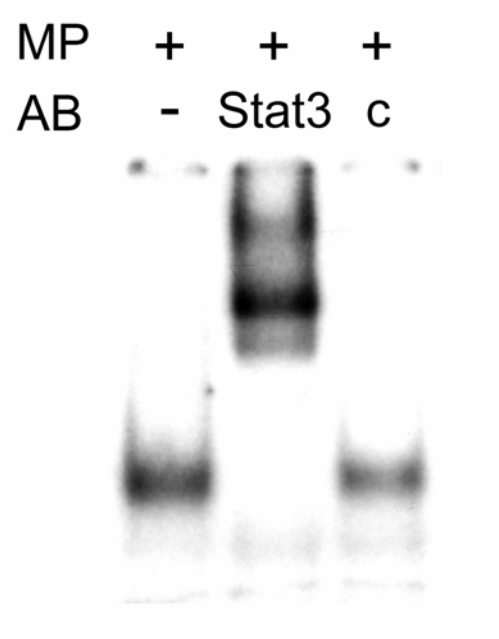

Figure 7

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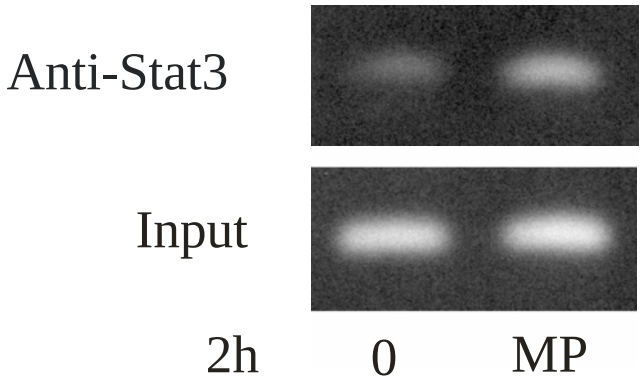
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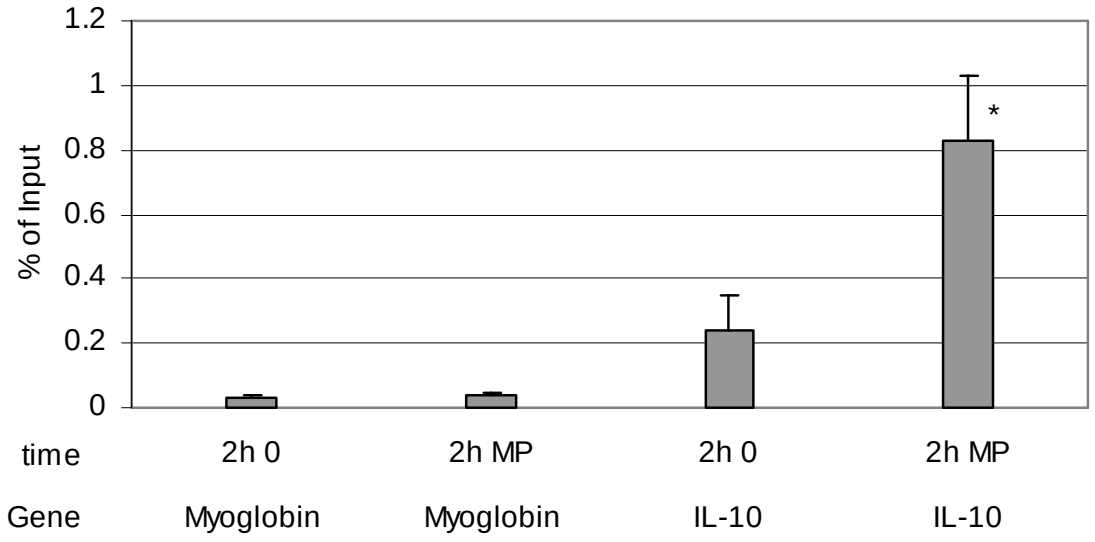
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Myoglobin
Myoglobin
IL-10
IL-10

Figure 8