2.) Inhibition der GM-CSF Freisetzung durch Glukokortikoide

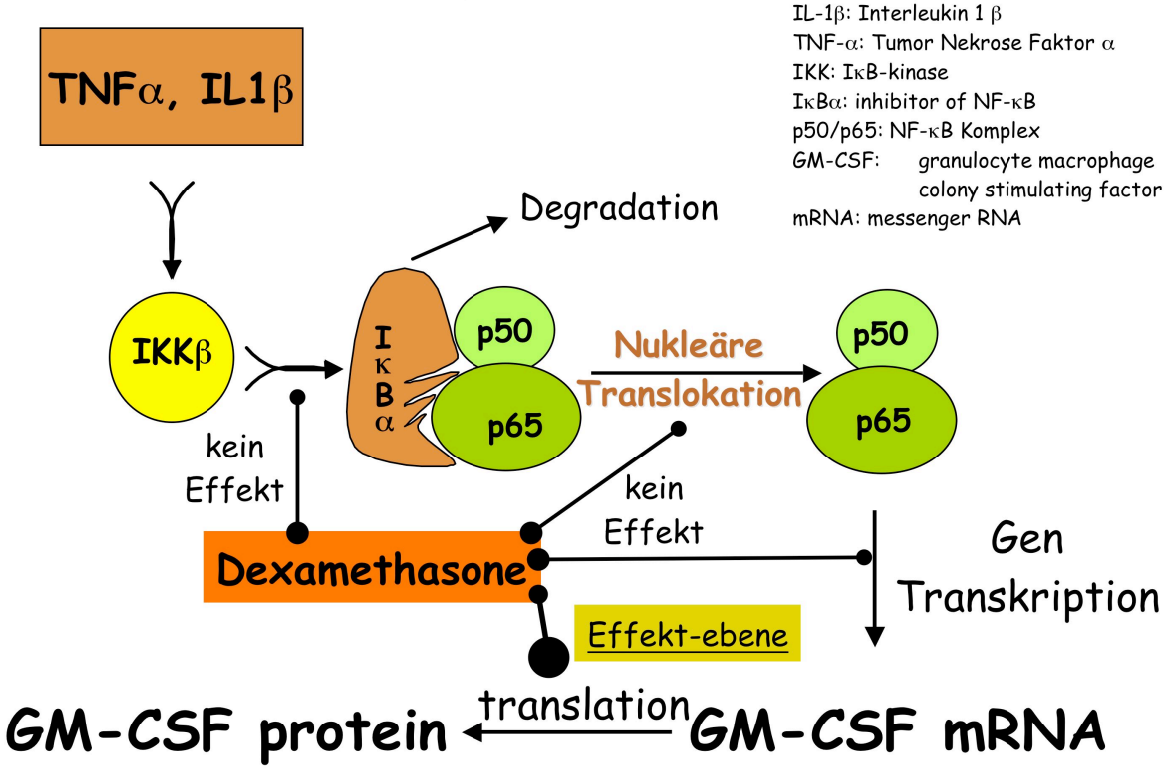
Neben dem Bronchialepithel spielen T-Lymphozyten eine entscheidende Rolle in der Unterhaltung der chronischen Entzündungsreaktion. Die für das Asthma bronchiale charakteristische Vermehrung eosinophiler Granulozyten in der Bronchialschleimhaut wird unter anderem durch die Freisetzung des Zytokins GM-CSF reguliert. Untersuchungen zur Regulation von GM-CSF wurden bisher im Wesentlichen an Zelllinien durchgeführt. Unser Ziel war es, das gültige Modell der inhibierenden Wirkung von Glukokortikoiden an einem primären T-Zellmodell zu überprüfen. Die Untersuchung der Genregulation von GM-CSF erfolgte in primären humanen T-Zellen sowie an isolierten peripheren, mononukleären Zellen (PBMC), die mit Hilfe eines Ficoll-Gradienten aus humanem Vollblut gewonnen wurden. Nach PMA/PHA (Lektin) - Stimulation wird GM-CSF Protein freigesetzt, diese Synthese lässt sich vollständig durch Dexamethason hemmen. Der durch semiquantitative RT-PCR ermittelte mRNA Zeitverlauf zeigt ein Maximum 12h nach Stimulation; nach Zugabe von Dexamethason ist die GM-CSF mRNA zu allen Zeitpunkten unterdrückt. Dieser Effekt ist dosisabhängig (Bergmann et al., 2004c).

Zur Analyse der transkriptionellen Effekte wurde ein System zur transienten Transfektion von primären humanen T-Zellen entwickelt. Die Isolation erfolgte mit Hilfe einer magnetischen, negativen Selektion aus PBMC durch eine Kombination von Antikörpern gegen T-Zell fremde Oberflächenantigene. Nach transienter Transfektion der verschiedenen GM-CSF Promotorkonstrukte konnte eine maximale Stimulation schon mit dem proximalen Promotorkonstrukt nachgewiesen werden. Überraschenderweise wurde die Promotorinduktion nicht durch die Zugabe von Dexamethason beeinflusst, während in einer parallelen Messung im Zellüberstand die GM-CSF Synthese vollkommen unterdrückt wurde (Schema 2) (Bergmann et al., 2004c). Wenn die transkriptionelle Steuerung von GM-CSF tatsächlich durch die Transkriptionsfaktoren NF-κB und AP-1 gesteuert wird, widerlegen diese Ergebnisse die Ausgangshypothese: die Wirkung von Dexamethason ist offensichtlich nicht auf die Protein-Protein Interaktion mit diesen Transkriptionsfaktoren zurückzuführen (Schema 2). Um diese Schlußfolgerung zu untermauern führten wir Mutationen an den Bindungsstellen dieser beiden Transkriptionsfaktoren im GM-CSF Promotor durch. Es zeigte sich, dass diese Mutationen tatsächlich auch in primären T-Zellen die Induzierbarkeit der Konstrukte vollständig unterdrückte

(Bergmann et al., 2004c)). Dieser Effekt war zudem selbst in dem 3.3kb Promotorfragment nachweisbar, welches auch den Enhancer enthielt. Die Beobachtung konnte durch Untersuchungen mit NF-κB – und AP-1 (entspricht TRE-Bindungsstellen) abhängigen Promotorkonstrukten validiert werden: Auch hier war die Induktion nach PMA/PHA-Stimulation durch Dexamethason unbeeinflußt (Bergmann et al., 2004c).

Zusammenfassend ist daher das bisher gültige molekulare Modell der Glukokortikoid-vermittelten Unterdrückung von proinflammatorischen Zytokinen unzureichend. Andere molekulare Modelle, vor allem mit Angriffspunkten auf der posttranskriptionellen Ebene müssen jetzt überprüft werden (Schema 2).

Schema 2



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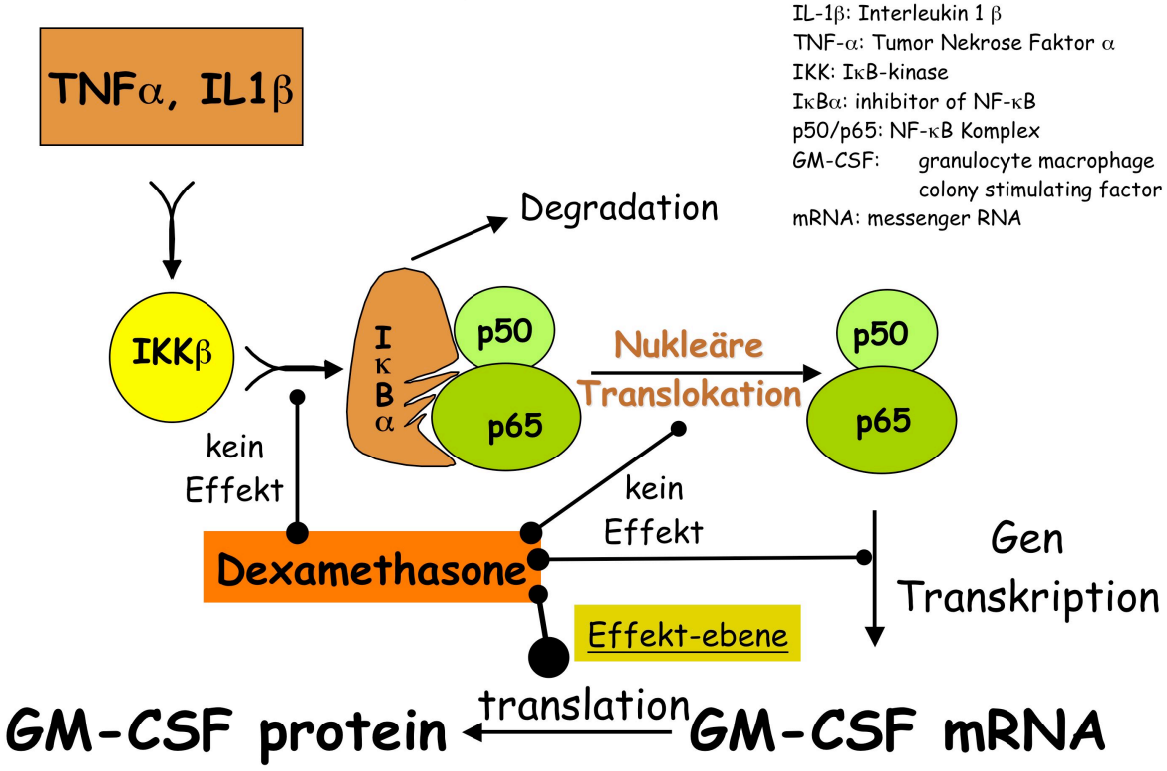
Schema 2



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Glucocorticoid Inhibition of Granulocyte Macrophage–Colony-Stimulating Factor from T cells Is Independent of Control by Nuclear Factor-κB and Conserved Lymphokine Element 0

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Release of granulocyte macrophage–colony-stimulating factor (GM-CSF) from T cells is important in the differentiation, maturation, and survival of inflammatory cells. Here the induction of GM-CSF expression from T cells was dependent on transcription and translation and was prevented by dexamethasone. In primary human CD3$^+$ T cells, up to 3.3 kb of human GM-CSF promoter was strongly activated by PMA + PHA. Mutations in either the −85/−76 nuclear factor (NF)-κB site or the activator protein-1 region in the −54/−31 conserved lymphokine element 0 (CLE0) site substantially reduced promoter activity. Both GM-CSF promoter and NF-κB–dependent constructs were unresponsive to dexamethasone whereas the release of GM-CSF was potently repressed. Analysis of GM-CSF mRNA and protein expression at various time points and the effect of adding dexamethasone after the stimulus revealed the existence of potent mechanisms of inhibition acting at a translational level. The expression of tristetraproline and HuR, proteins that bind the AU-rich element in the GM-CSF 3′-untranslated region was unaffected by dexamethasone and overall AU-rich element binding activity was unaltered. Taken together our data support an important role for the NF-κB and CLE0 sites in the transcriptional control of GM-CSF expression in primary human T cells and suggest that post-transcriptional/translational mechanisms are key mediators of glucocorticoid-dependent repression.

A two-signal model has been proposed for T cell activation whereby signaling from the TCR/CD3 complex plus a costimulus, such as ligand binding to CD28, is required for maximal activation of resting T lymphocytes (1). In inflammatory diseases such as asthma, activated T cells orchestrate the inflammatory response by upregulating cytokines such as granulocyte macrophage–colony-stimulating factor (GM-CSF), which then act on effector cells, including eosinophils, to potentiate the inflammation (2).

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Abbreviations: activator protein-1, AP-1; adenosine/uridine (AU)-rich element, ARE; conserved lymphokine element 0, CLE0; enzyme-linked immunosorbent assay, ELISA; glyceraldehyde phosphate dehydrogenase, GAPDH; granulocyte macrophage–colony-stimulating factor, GM-CSF; glucocorticoid receptor, GR; nuclear factor-κB, NF-κB; peripheral blood mononuclear cells, PBMC; phytohemaglutinin, PHA; phorbol 12-myristate 12-acetate, PMA; tristetraproline; untranslated region, UTR.

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The release of GM-CSF from activated T cells is regulated by both transcriptional and post-transcriptional mechanisms and stimuli such as phorbol 12-myristate 13-acetate (PMA), Ca^{2+} ionophore, phytohemaglutinin (PHA), or concanavalin A (ConA) are used to mimic these events experimentally (3, 4). In the Jurkat T cell line, the proximal GM-CSF promoter (−620 to +34) is inducible by PMA plus Ca^{2+} ionophore, and this response is dependent on either the conserved lymphokine element 0 (CLE0) (−54 to −31) or the nuclear factor-κB (NF-κB)–binding regions (−85 to −76) in the GM-CSF promoter (5). Various other factors, including NF-AT, AP-1, SP-1, ETS, and YY1, also bind this promoter region and are indicative of a complex regulatory function (4, 6). In T cells stimulated with PMA plus either calcium ionophore or ConA, the proximal NF-κB site is occupied by an inducible complex of the Rel-proteins p50 and p65 (7, 8). Additionally, an enhancer region has been characterized ~3 kb upstream, which contains functional NF-AT and AP-1 binding sites, that may also play a role in promoter activation and tissue specificity (9–11).

Glucocorticoids provide the mainstay treatment in chronic inflammatory diseases such as asthma via the suppression of proinflammatory genes, including GM-CSF, that are activated during inflammation (2, 12). However, the mechanisms of glucocorticoid-mediated repression are still incompletely understood and both transcriptional and post-transcriptional effects are implicated (12). Negative transcriptional effects (transrepression) include the binding of activated glucocorticoid receptor (GR) to DNA binding sites to block positive regulatory sites and direct protein–protein interactions with the transcription factors AP-1 and NF-κB (for review *see* Ref. 12). Post-transcriptional mechanisms of repression include alteration of mRNA half-life (13–15), inhibition of ribosomal protein synthesis (16), direct inhibition of translation (14, 17), intracellular degradation of proteins (14), and inhibition of protein release (14, 17). Although some studies point to a combination of mechanisms (13, 14), transcriptional interference with AP-1 and NF-κB has attracted particular attention, because binding sites for these transcription factors are found in the promoters of numerous proinflammatory genes (2, 12).

As previous studies on GM-CSF promoter activation were conducted in cell lines and nonhuman cells (4), we have evaluated the role of the proximal CLE0 and NF-κB binding sites in primary human T cells and examined this in the context of the dexamethasone-dependent repression of GM-CSF.

Materials and Methods

Isolation of Human Peripheral Blood Mononuclear Cells and T Cells

Human peripheral blood mononuclear cells (PBMC) were prepared from the peripheral blood of normal donors (following informed consent according to existing local ethics committee guidelines) using the Ficoll-Paque (Amersham Pharmacia Biotech, Amersham, UK) method and cultured at a density of 3×10^6 cells/ml as previously described (18). Primary human T cells were isolated by negative magnetic MACS isolation (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (19). Flow through from these columns was typically > 93% T cells, as determined by fluorescence-activated cell sorter analysis using fluorescein isothiocyantate-labeled anti-CD3. Cells were then resuspended at 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin (all Sigma, Poole, UK), plus 10 ng/ml human IL-2 (R&D Systems, Abingdon, UK). After 72 h, cells were typically > 97% CD3 positive as measured by fluorescence-activated cell sorter. Cells were stimulated by the addition of PMA (50 nM) and PHA (5 μg/ml; Sigma) or the activating antibodies (500 ng/ml) mouse anti-human CD3 (UCHT1) and CD28 (CD28.2) (BD Pharmingen, Oxford, UK) as previously described (18).

GM-CSF Determination

GM-CSF in supernatants was determined by enzyme-linked immunosorbent assay (ELISA; Pharmingen) as previously described (20). For intracellular GM-CSF determination, cells were harvested and washed in phosphate-buffered saline before lysis on ice in 1× reporter lysis buffer (RLB; Promega, Southampton, UK). After one freeze–thaw cycle, cellular debris was spun out and the supernatant used directly for ELISA.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

RNA was extracted and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) for GM-CSF and glyceraldehyde phosphate dehydrogenase (GAPDH) performed using primers and conditions as previously described (20). For each experiment the exponential phase of the PCR, where starting material is proportional to product formation, was determined as described (20). Cycle numbers for GM-CSF ranged between 28 and 34 and for GAPDH between 24 and 28. Reaction products were analyzed by agarose gel electrophoresis and gel images subject to densitometric analysis using GelWorks 1D Version 4.01 (NonLinear Dynamics Ltd) (UVP Ltd, Cambridge, UK). Southern blotting was used to confirm identity of products.

Transcriptional Reporters

Three human GM-CSF promoter fragments were generated: the full-length promoter (GM-FL) spanned the entire region from –3298 bp to +35 bp; GM-PE contained the proximal promoter fused directly to the enhancer region (−3,286/−2,572 and −624/+35); the proximal promoter (GM-P) from –624 bp to +35 bp was cloned into pGL3basic plasmid (Promega) and has been previously described (Genbank Acc. No. AJ22414) (20). Mutations in the proximal NF-κB (−85/−76 - TTTAGTTCCC) and CLE0 (−54/−31 - TTAA GAA) sites were introduced using the Quick-Change kit (Stratagene, Cambridge, UK) and were confirmed by sequencing. The NF-κB–dependent reporters, 6κB.tk and pGL3.6κB.BG, and the reporter containing mutated NF-κB sites, pGL3.6κB.BG.mut, have previously been described (20, 21). The AP-1 (TRE)-dependent reporter, pAD.TRE contains six TRE/AP-1–binding sites upstream of the rabbit β-globin minimal promoter in pADneo2BgLuci vector as previously described (22).

Electroporation of T Cells

Following electroporation of T cells, performed as previously described (19), cells containing each construct were washed and resuspended in 400 μl of serum-free RPMI medium. Cells were divided into 100-μl portions and placed into 96-well round bottom culture plates for treatments. After 12 h, the supernatants were harvested for ELISA and the cells suspended in 1× reporter lysis buffer (Promega, Southampton, UK). Luciferase activity was determined according to the manufacturers' specification (Promega) and was normalized to protein content as determined by Bradford assay (BioRad, Hemel Hempstead, UK).

Western Blot Analysis

Total cellular proteins were harvested in 1× RLB (as above) supplemented with the Complete protease inhibitor cocktail (Roche, Welwyn Garden City, UK). Cell lysates were subject to one freeze–thaw cycle before SDS-PAGE and transfer to Hybond-ECL membranes (Amersham Pharmacia Biotech, Amersham, UK). Western blot analysis for HuR was performed using a anti-human mouse monoclonal antibody (19F12; Molecular Probes, Leiden, The Netherlands) and for TTP using a C-terminal specific rabbit polyclonal SAK21A (gift from Andrew Clark, Imperial College London, UK) as previously described (23).

GM-CSF AU-Rich Element RNA Electrophoretic Mobility Shift Assay

Oligonucleotides (sense GTA ATA TTT ATA TAT TTA TAT TTT TAA AAT ATT TAT TTA TTT ATT TAT TTA AGA and antisense CTT AAA TAA ATA AAT AAA TAA ATA TTT TAA AAA TAT AAA TAT ATA AAT ATT ACA corresponding to the AU rich region of the human GM-CSF 3′UTR, bases 641–694 (Accession M10663), were annealed and inserted into the T-tailed EcoRV site of the pGEM5z T-vector (Promega). Double stranded sequencing confirmed identity. Plasmids were prepared, linearized, and high specific activity α-^{32}P sense riboprobes prepared exactly according to the method of Mahtani and coworkers (23). Likewise, the preparation of cytoplasmic extracts and RNA binding reactions was as described by Mahtani and colleagues (23). Samples were run on 4% (wt/vol) polyacrylamide gels in 0.5× TBE Buffer (1× TBS is 45 mM Tris-borate, 10 mM EDTA) before vacuum drying and autoradiography.

Statistical Analysis

Statistical analysis was performed by ANOVA with a Bonferroni post-test (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

Results

Release of GM-CSF from Human PBMC

Cells were treated with combinations of the phorbol ester, PMA, the lectin, PHA, as well as with activating antibodies, anti-CD3 and anti-CD28, to the T cell surface receptors

CD3 and CD28. In the case of PMA or anti-CD3, a robust increase in GM-CSF release was observed, whereas PHA or anti-CD28 alone failed to induce a substantial release of GM-CSF (Figure 1A). Costimulation with either PMA + PHA or anti-CD3 + anti-CD28 resulted in significantly greater release of GM-CSF than with a single treatment alone.

Incubation of cells in the presence of either the transcriptional inhibitor, actinomycin D, or the translational blocker, cycloheximide, before stimulation prevented the release of GM-CSF, demonstrating the requirement for new transcription and translation (Figure 1B). Likewise, stimulation of cells in the presence of the glucocorticoid, dexamethasone, completely blocked the release of GM-CSF (Figure 1C), which in the case of PMA + PHA occurred with an IC_{50} of 1.88×10^{-9} M (data not shown).

GM-CSF Promoter Activation in Primary Human T Cells

As release of GM-CSF in response to PMA + PHA was more robust than for anti-CD3 + anti-CD28 stimulation and at the RNA level the duration of this response was more sustained (Figure 1A and data not shown), PMA + PHA costimulation was selected for GM-CSF promoter analysis. Transfection of a full-length, −3286/+35 (GM-FL), proximal, −624/+35 (GM-P), and proximal promoter combined with the distal enhancer region (−3,286/−2,572 + −624/+35) (GM-PE) resulted in strong promoter activation by PMA or PMA + PHA in primary human T cells (Figure 2A). In each case PHA alone was a relatively poor activator and failed to produce any obvious synergy in combination with PMA. This contrasts with the release of GM-CSF from these same (transfected) cells, which showed clear synergy with combined PMA and PHA stimulation (Figure 2B). Furthermore, each of the three reporters, GM-FL, GM-PE and GM-P, gave rise to similar levels of inducibility, indicating that all the major regulatory sites are located within the +624/−35 promoter region.

To test the role of the −85/−76 NF-κB and the −54/−31 CLE0 sites, primary human T cells were transfected with wild-type GM-CSF reporters or reporters containing mutations in these sites (Figure 2C). In the context of the full-length, enhancer + proximal and the proximal promoters, mutation of both the NF-κB and CLE0 sites resulted in almost no inducibility in response to PMA + PHA stimulation.

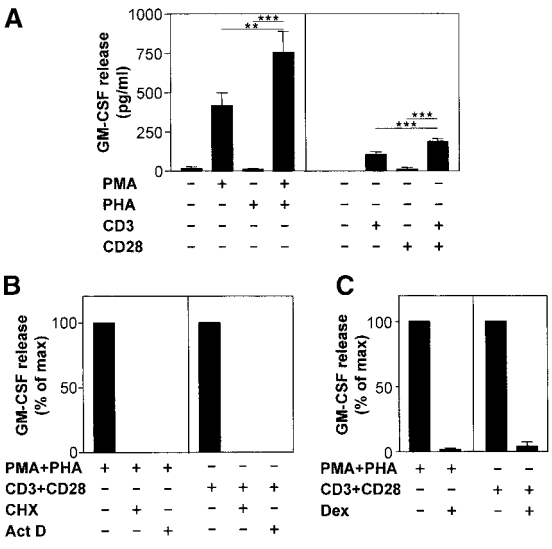

Figure 1. Effect of stimuli, inhibitors and dexamethasone on GM-CSF expression in human PBMC. (*A*) PBMC were treated, as indicated, with combinations of PMA (50 nM) and PHA (5 μg/ml) or anti-CD3 (UCHT1) (500 ng/ml) and anti-CD28 (CD28.2) (500 ng/ml). Supernatants were harvested after 24 h for GM-CSF determination. Data (n = 20 for PMA and PHA, n = 6 for anti-CD3 and anti-CD28) are shown as means ± SEM. Statistical analysis was performed using ANOVA for matched samples using a Bonferroni post-test. ***$P < 0.001$. (*B* and *C*) PBMC were treated with PMA + PHA or anti-CD3 + anti-CD28, as in *A*, in the presence or absence of cycloheximide (10 μg/ml), actinomycin D (10 μg/ml), or dexamethasone (1 μM), as indicated. Data (*B*, n = 6; *C*, n = 12 for PMA+PHA and n = 6 for anti-CD3 + anti-CD28) are plotted as a percentage of stimulated as means ± SEM.

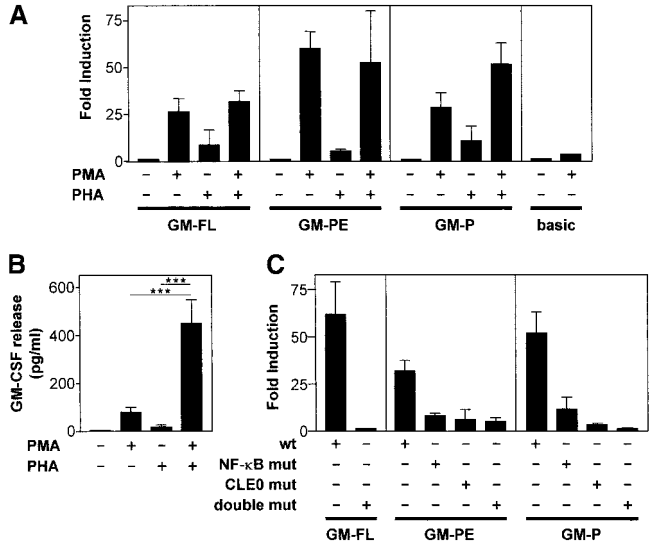

Figure 2. Effect of PMA and PHA on GM-CSF promoter activation and protein release in primary human T cells. (*A*) Primary human T cells were prepared and transfected with various reporter plasmids containing the indicated parts of the GM-CSF promoter (GM-FL = −3,298/+35, GM-PE = −3,298/−2,572 + −624/+35, GM-P = −624/+35, basic = parent luciferase vector) before incubation in the presence of PMA (50 nM) and PHA (5 μg/ml), as indicated. After 12 h cells were harvested for luciferase assay. Data (GM-FL, n = 3; GM-PE, n = 6; GM-P, n = 6; basic, n = 3) expressed as fold induction for each reporter compared with unstimulated cells are plotted as means ± SEM. (*B*) Supernatants from the experiments in *A* above were analyzed for GM-CSF release. Data (n = 6) are plotted as means ± SEM. Statistical analysis was performed by ANOVA with a Bonferroni post-test. ***$P < 0.001$. (*C*) Primary human T cells were prepared and transfected with wild-type or mutated (mut) GM-CSF promoter constructs, as indicated, and then either not stimulated or stimulated with PMA (50 nM) + PHA (5 μg/ml). Data (n = 3–7, except double muts where n = 2) were expressed as the fold induction of PMA + PHA compared with unstimulated for each reporter construct. Values are plotted as means ± SEM.

Likewise the single mutants, NF-κB mut or CLE0 mut, in the enhancer + proximal and the proximal promoter revealed a low level of inducibility by PMA + PHA.

Effect of Dexamethasone on GM-CSF Expression

PBMC were stimulated with PMA + PHA either in the absence or presence of dexamethasone. Release of GM-CSF into the supernatant was first detectable 6 h post-stimulation and the maximum levels were observed at 24 h (Figure 3A). At each time point, dexamethasone resulted in inhibition of GM-CSF indicating that steps at, or before release from the cells were being targeted. To more closely examine any effect on the *de novo* synthesis of GM-CSF, cells were treated as above and then harvested for measurement of intracellular GM-CSF protein. Consistent with the extracellular release data, a substantial rise in intracellular GM-CSF was observed by 6 h (Figure 3B) and by 2 h for GM-CSF mRNA (Figure 3C). As intracellular GM-CSF had peaked by 9 h and extracellular levels continued to rise, these data point to ongoing GM-CSF protein synthesis and continual release from the cell over the final 15 h of the experiment. Dexamethasone potently repressed the intracellular accumulation of GM-CSF and the level of GM-CSF mRNA.

Effect of Dexamethasone on GM-CSF Promoter Activation in Primary Human T Cells

Primary human T cells were transfected with various reporter plasmids and the response to PMA + PHA examined in the presence or absence of dexamethasone (Figure 4). The GM-CSF promoter constructs, GM-FL, GM-PE, and GM-P, were again highly inducible in response to PMA + PHA; however, simultaneous incubation with dexamethasone (1 μM) showed no effect on promoter activation (Figure 4A). Likewise, PMA + PHA treatment of two different NF-κB–dependent reporters resulted in a substantial increase in reporter activity that was unaffected by simultaneous treatment with dexamethasone (Figure 4B). Similarly, an AP-1–dependent reporter was also tested (Figure 4C). In this case the inducibility by PMA + PHA was considerably less (4-fold), but a 40% inhibition by dexamethasone was observed. To confirm the efficacy of these treatments, GM-CSF protein was assayed in the supernatants of the experiments from Figure 4A. This revealed a very potent induction of GM-CSF protein by PMA + PHA and a total inhibition of this release by dexamethasone (Figure 4D).

Effect of Dexamethasone on Steady-State GM-CSF mRNA and mRNA Half-Life

PBMC were isolated and either pretreated, or not treated, with PMA + PHA for 2 h (Figure 5). PMA + PHA produced a substantial induction of GM-CSF mRNA (Figure 5, *lane 2 in both panels*). At this time (designated t = 0), cells were then either not treated or treated with dexamethasone (Figure 5, *left panels*). Consistent with the above time course data (Figure 3C), RT-PCR analysis of PMA + PHA–treated cells revealed no substantial changes in steady-state mRNA expression over the following 6 h. Furthermore, the addition of dexamethasone at this time point also resulted in no change in steady-state GM-CSF mRNA levels. These data indicate that for both treatments the rate of mRNA synthesis balanced the rate of decay. To analyze effects on mRNA decay, we employed standard transcriptional arrest experiments using actinomycin D (Figure 5, *right panels*). This indicates a $t_{1/2}$ for PMA + PHA–induced GM-CSF mRNA of between 4 and 6 h. The addition of dexamethasone resulted in an apparent stabilization of GM-CSF mRNA such that the $t_{1/2}$ was no longer discernible. Although reasons for this effect are currently unclear, it is notable that similar effects have previously been reported (17).

Dexamethasone Blocks Further GM-CSF Synthesis in Prestimulated Cells

As in the previous experiment the addition of dexamethasone after the PMA + PHA stimulus, had little effect on steady state GM-CSF mRNA levels, the effect of dexamethasone on GM-CSF synthesis in pre-stimulated cells was examined. Since intracellular GM-CSF was maximal at 9 h post-stimulation, cells were treated for this time before the addition of dexamethasone. At this point (t = 0) brefeldin A (10 ng/ml), an inhibitor of vesicular transport, was added to block export of GM-CSF from the cytoplasm, and this effect was confirmed in pilot studies (data not shown). Thus, following the addition of brefeldin A, a time-dependent

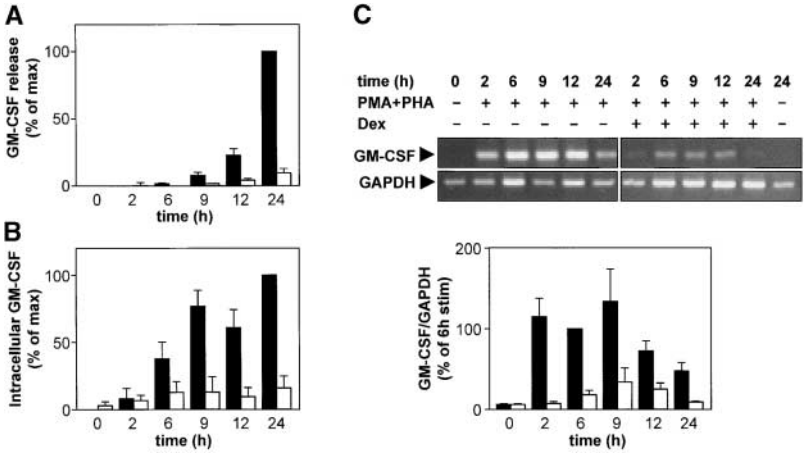

Figure 3. Effect of dexamethasone on GM-CSF expression. Human PBMC were prepared and treated with PMA (50 nM) + PHA (5 μg/ml) in the absence (*solid bars*) or presence (*open bars*) of dexamethasone (1 μM). (*A*) Supernatants or (*B*) cells were harvested for extracellular or intracellular GM-CSF determination, respectively. Data (*A*, $n = 9$; *B*, $n = 4$) were expressed as a percentage of stimulated at 24 h and are plotted as means ± SEM. (*C*) Cells were treated as above and harvested for RNA and RT-PCR analysis of GM-CSF and GAPDH. Representative ethidium stained agarose gels are shown. After densitometric analysis, data ($n = 4$) as a ratio of GM-CSF/GAPDH were expressed as a percentage of stimulated at 6 h and are plotted as means ± SEM.

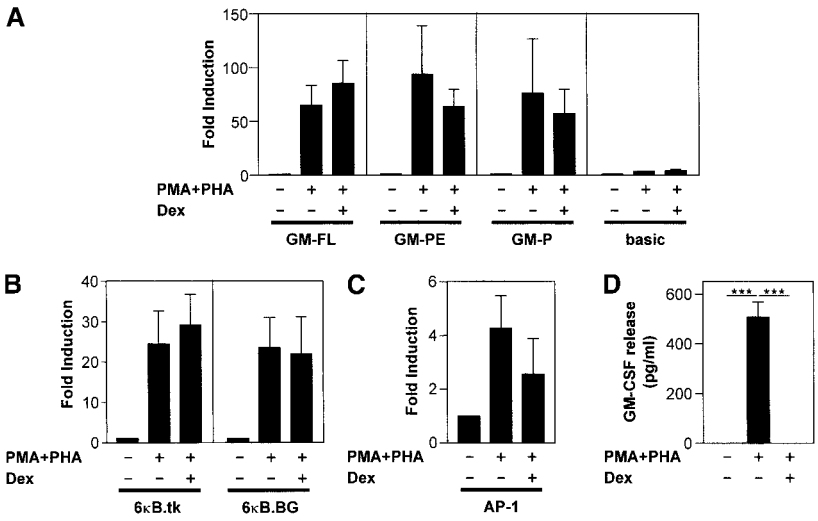

Figure 4. The effect of dexamethasone on promoter activation and GM-CSF release in primary human T cells. (*A*) Primary human T cells were prepared and transfected with various plasmids containing the indicated parts of the GM-CSF promoter (GM-FL = −3,298/+35, GM-PE = −3,298/−2,572 + −624/+35, GM-P = −624/+35, basic = parent luciferase vector) before incubation in the presence of PMA (50 nM) + PHA (5 μg/ml) and dexamethasone (1 μM), as indicated. After 12 h, cells were harvested for luciferase assay. Data (GM-FL, $n = 6$; GM-PE, $n = 6$; GM-P, $n = 8$; basic, $n = 3$) were expressed as fold induction for each reporter compared with unstimulated cells and are plotted as means ± SEM. (*B* and *C*) T cells were prepared and transfected with either NF-κB–dependent or AP-1–dependent reporter plasmids, as indicated, before treatment as in *A*. Data (6κB.tk, $n = 8$; 6κB.BG, $n = 6$; AP-1, $n = 6$) were expressed as fold induction for each reporter compared with unstimulated cells and are plotted as means ± SEM. (*D*) Supernatants from the experiments in *A* above were analyzed for GM-CSF release by ELISA. Data ($n = 6$) are plotted as means ± SEM. Statistical analysis was performed by ANOVA with a Bonferroni post-test. ***$P < 0.001$.

increase in intracellular GM-CSF was observed (Table 1). The addition of dexamethasone to samples at t = 0 (i.e., with the brefeldin A) reduced the increase in intracellular GM-CSF over the first 2 h (Table 1). After this time there was little further increase in intracellular GM-CSF protein, suggesting that any further synthesis had been prevented.

Inhibition of GM-CSF Synthesis Is Time-Dependent

In the preceding two experiments, the addition of dexamethasone after the PMA + PHA, had no effect on steady-state GM-CSF mRNA levels, but prevented the accumulation of GM-CSF protein over a similar time frame. Studies were therefore conducted to examine the time-dependence of dexamethasone, actinomycin D, and cycloheximide on the expression of GM-CSF protein. In these experiments, PBMC were treated with PMA + PHA (t = 0) and in all cases the supernatants were harvested after 24 h for GM-CSF determination. The addition of either dexamethasone or actinomycin D or cycloheximide contemporaneously with the stimulus (i.e., t = 0) resulted in the near complete prevention of GM-CSF release (Figure 6A). A similar level of repression was also observed when the drugs were added up to 2 h after the PMA + PHA stimulus, a time when GM-CSF mRNA is known to be highly elevated (Figure 3C). The first evidence for loss of repression occurred when the drugs were added as late as 4 h after the stimulus and a ~50% inhibition of GM-CSF release was even observed when the drugs were added 9 h after the stimulus. In the case of cycloheximide, the ability to repress GM-CSF expression at later time points is consistent with the delayed kinetics of translation from mRNA, GM-CSF protein having being first detected at 6 h post-stimulation.

To exclude the possibility that these effects occurred at the level of GM-CSF release from the cell, similar experiments

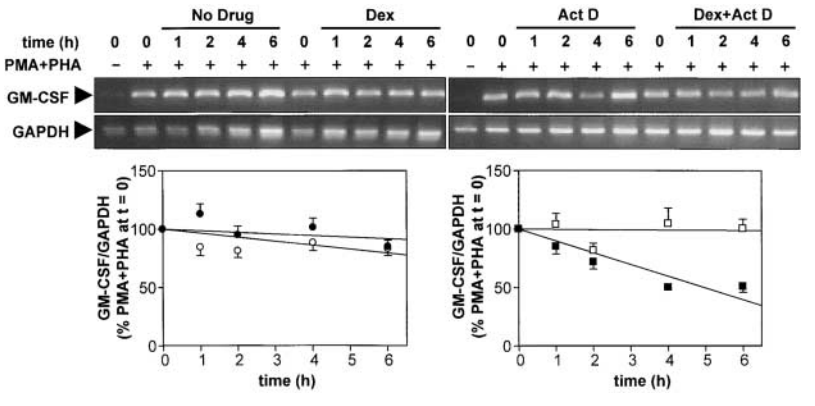

Figure 5. Effect of dexamethasone on pre-induced GM-CSF mRNA and mRNA half-life. Human PBMC were either prestimulated (t = −2) with PMA (50 nM) + PHA (5 μg/ml) or not stimulated. After 2 h (t = 0), cells were either not treated or treated with combinations of dexamethasone (1 μM) and actinomycin D (10 μg/ml), and were harvested for RNA and RT-PCR analysis at the times indicated. Representatives are shown. After densitometric analysis, data ($n = 7$ each performed in duplicate) as a ratio of GM-CSF/GAPDH are expressed as a percentage of PMA + PHA at t = 0 and are plotted as means ± SEM. *Filled circles*, PMA + PHA; *open circles*, PMA + PHA + Dex; *filled squares*, PMA + PHA + Act D; *open squares*, PMA + PHA + Dex + Act D.

TABLE 1
Effect of dexamethasone on intracellular accumulation of GM-CSF in pre-stimulated cells

	Time (h) after Addition of BFA/Dex			
Treatment	0	2	4	6
PMA + PHA	19.6 ± 2.4	53.9 ± 10.7	84.4 ± 12.8	100
PMA + PHA + Dex	17.3 ± 6.5	39.5 ± 9.9	48.1 ± 7.1	49.8 ± 6.3

Human PBMC were either pre-stimulated (t = −9) with PMA (50 nM) + PHA (5 μg/ml) or not stimulated. After 9 h (t = 0), brefeldin A (BFA, 10 ng/ml) was added and cells were either not treated or treated with combinations of dexamethasone (1 μM). Cells were then harvested immediately (t = 0) or at the times indicated for measurement of intracellular GM-CSF content by ELISA. GM-CSF concentrations in the cell lysate are given (n = 4) as a percentage of the maximum (at 6 h) as means ± SEM.

were conducted over a shorter time frame (8 h) in the presence of brefeldin A. As with GM-CSF release, dexamethasone, when added within 2 h of the stimulus, inhibited > 75% of the total intracellular GM-CSF, suggesting that the previous data were due to an actual effect on GM-CSF synthesis and not due to effects on GM-CSF release (Figure 6B). Likewise, actinomycin D was highly effective at inhibiting accumulation of GM-CSF when added 2 h after the stimulation, and was able to prevent over 50% of the total response even when added 4 h after the stimulus.

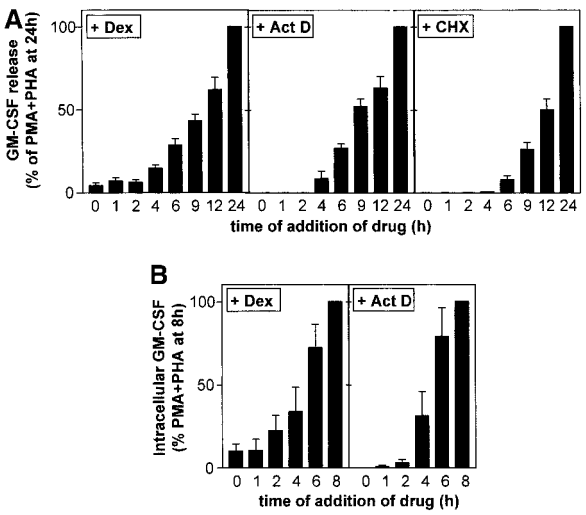

Figure 6. Effect of addition of dexamethasone, actinomycin D, or cycloheximide at times after the stimulus. (*A*) Human PBMC stimulated with PMA (50 nM) + PHA (5 μg/ml). Dexamethasone (1 μM), actinomycin D (10 μg/ml), or cycloheximide (10 μg/ml) were added either simultaneously with the PMA + PHA or at various times afterwards (as indicated). In all cases supernatants were harvested 24 h after the PMA + PHA stimulation and GM-CSF release was determined. Data were expressed as a percentage of the PMA + PHA treatment as means ± SEM. (*B*) Human PBMC were stimulated with PMA (50 nM) + PHA (5 μg/ml) in the presence of brefeldin A (10 ng/ml). Dexamethasone (1 μM) or actinomycin D (10 μg/ml) or were added either simultaneously with the PMA + PHA or at various times afterwards (as indicated). Cells were harvested 8 h after the PMA + PHA stimulation and intracellular GM-CSF was determined. Data were expressed as a percentage of the PMA + PHA treatment as means ± SEM.

Effect of Dexamethasone on AU-Rich Element-Binding Proteins

The data from the above experiments provides compelling evidence for repressive actions of dexamethasone occurring at post-transcriptional/translational levels. Furthermore, both these processes may be regulated via conserved AU-rich motifs or elements (AREs) in the 3′UTR of genes such as GM-CSF, and recent evidence has shown that T cells express the ARE-binding proteins HuR (also called HuA), a protein that is thought to mediate mRNA stabilization, and tristetraprolin (TTP), a protein that may destabilize ARE-containing mRNAs (24). As dexamethasone can elicit mRNA destabilization via AU-rich regions (15), it is possible that modulation of either HuR or TTP may explain the response to dexamethasone. Cells were therefore stimulated with PMA + PHA or anti-CD3 + anti-CD28, as above, in the presence of absence of dexamethasone. Western blot analysis for HuR revealed a modest increase in expression 6 h after PMA + PHA treatment, but not after anti-CD3 + anti-CD28 (Figure 7A). In each case dexamethasone had no effect on HuR expression. Similarly, the expression of TTP was substantially increased by PMA + PHA treatment, whereas anti-CD3 + anti-CD28 resulted in only a slight increase in expression (Figure 7A). In each case a similar, but reduced, pattern of expression was observed at 2 h post-stimulation.

To examine the possibility that dexamethasone was affecting the overall binding of these proteins to the GM-CSF ARE, cytoplasmic extracts were incubated with a ^{32}P-labeled probe corresponding to the AU-rich region in the 3′UTR of the human GM-CSF. In untreated cells, little or no ARE binding activity was detected (Figure 7B). Following anti-CD3 + anti-CD28 treatment, a small increase in binding activity was observed. This contrasts with the substantial increase in ARE binding seen 6 h after PMA + PHA treatment. A similar response was observed at 2 h post-stimulation (data not shown). Again dexamethasone showed no obvious effect on total RNA binding activity.

Discussion

In the present study, we have shown that the release of GM-CSF from human PBMC stimulated by a combination of PMA + PHA or by anti-CD3 + anti-CD28 is totally dependent on new transcription and translation. As GM-CSF mRNA and intracellular GM-CSF levels were initially low

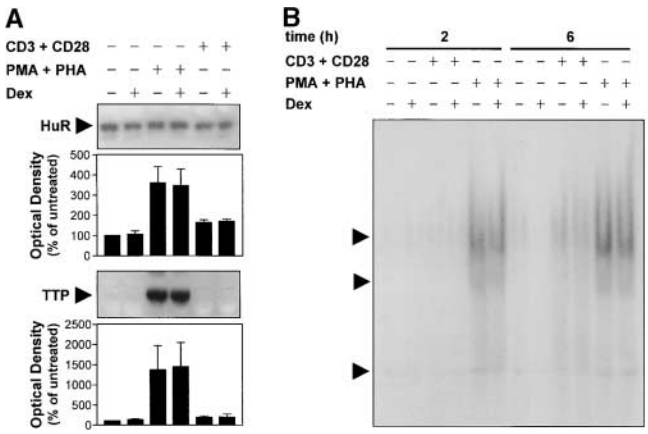

Figure 7. Effect of stimulation and dexamethasone on HuR and TTP expression and on proteins binding to the GM-CSF ARE. (*A*) Human PBMC were stimulated with PMA (50 nM) + PHA (5 μg/ml) or anti-CD3 (UCHT1) (500 ng/ml) and anti-CD28 (CD28.2) (500 ng/ml) in the presence or absence of dexamethasone (1 μM) as indicated for 6 h. Cells were then harvested for Western blot analysis of HuR (*upper panels*) and TTP (*lower panels*). In each case densitometric analysis was performed and data ($n = 4$), expressed as a percentage of untreated, were plotted as means ± SEM. (*B*) Cells were treated as in *A*, above, and cytoplasmic extracts prepared for RNA EMSA. After treatment with RNase T1 (50U/ml) and heparin (5 mg/ml) for 20 min on ice, binding reactions were analyzed on 4% nondenaturing polyacrylaminde gels. Specific RNA binding complexes identified by competition with excess cold probe is indicated. A representative gel from four similar independent experiments is shown. Free ribonulceotides have run to the bottom of the gel and are not shown.

but were highly induced following PMA + PHA treatment, these data indicate that the release of GM-CSF is primarily regulated by *de novo* mRNA and protein synthesis and argues against significant release of preformed protein. To examine the role of GM-CSF promoter activation, we have developed a technique of reporter gene assay using highly enriched human T cells. Consistent with an important role for transcriptional activation, we report that up to ∼ 3.3 kb of the human GM-CSF promoter was inducible in response to PMA but unresponsive to PHA stimulation in primary human T cells. Furthermore, and in contrast to the marked synergy between PMA and PHA on GM-CSF release from primary T cells, PHA showed little or no ability to potentiate the transcriptional response, suggesting that the effects of PHA were primarily mediated by post-transcriptional or translational mechanisms.

Transfection analysis of the GM-CSF promoter in various T cell lines, particularly Jurkat T cells, indicates that in addition to the proximal promoter region, an enhancer region, located ∼ 3 kb upstream of transcription start, is important for promoter activation (9). However, in primary human T cells these two constructs showed similar levels of inducibility, suggesting that, at least for PMA + PHA stimulation, the upstream enhancer is dispensable. Interestingly, some evidence of independent enhancer function was observed with the GM-PE (−3286/-2572 + −624/+35) construct, which has the upstream enhancer fused directly to the proximal promoter and tended to show greater levels of inducibility than the proximal promoter alone. Although these data are consistent with studies using a similar fusion in cell lines and mouse cells, the effect could also be attributed to the nonphysiologic arrangement of the promoter (11). The enhancer element contains a number of functional NF-AT– as well as AP-1–binding sites, and was identified using stimulation by PMA + ionomycin, which results in a strong and prolonged elevation in intracellular Ca^{2+} (9). In contrast, stimulation of T cells with PHA produces a more physiologic rise in intracellular Ca^{2+} and may therefore result in lower levels of enhancer activation (25). This is consistent with the upstream enhancer only responding to PMA and Ca^{2+} ionophore and thus, may explain the apparent differences between these two studies (4).

The NF-κB–like site at −85/−76 and a CLE0 element at −54/−31 are thought to be essential for activation of the proximal GM-CSF promoter (7, 10, 26). Importantly, GM-CSF promoter activation was substantially impaired by these same mutations in primary human T cells. These data confirm in a physiologically relevant system the role of NF-κB and CLE0 elements in the induction of GM-CSF. Therapeutic strategies that target these transcription factor complexes may therefore be expected to be of benefit in diseases, such as bronchial asthma, where T cell–derived GM-CSF plays a role.

Glucocorticoids are commonly used to treat chronic inflammation in allergic diseases, including asthma, and release of GM-CSF from activated T cells represents an important effector function of T cells that is potently downregulated by the glucocorticoids (2). In the present study this effect was confirmed in both PBMC and in primary human T cells. As transcriptional interference by glucocorticoids, acting via GR, on factors such as NF-κB and AP-1 is thought to account for a major part of the anti-inflammatory effects of glucocorticoids (2), we tested the effect of dexamethasone on promoter activation in primary human T cells. However, although a significant ∼ 40% decrease in AP-1–dependent transcription was observed, there was no clear effect of dexamethasone on NF-κB–dependent transcription or on the three GM-CSF promoter constructs. Because these constructs have been fully validated, in terms of both promoter upregulation and the necessity for both NF-κB and CLE0 sites, these data suggest that in respect of the dexamethasone-dependent repression of PMA + PHA–stimulated GM-CSF expression, mechanisms other than repression of promoter activity may be important. This conclusion is supported by Smith and coworkers, who showed that repression of the GM-CSF promoter by dexamethasone required the upstream enhancer element and did not occur in the context of the proximal promoter alone (27). These studies were conducted using conditions, PMA plus Ca^{2+} ionophore stimulus, where the upstream enhancer would be transcriptionally active and therefore susceptible to repressive effect of GR acting on AP-1–dependent transcription (4, 27). Consistent with this, we document the ability of dexamethasone to repress AP-1–dependent, but not NF-κB–dependent, transcription in primary human T cells. Furthermore, our studies reveal that the upstream

enhancer does not play a major role in the transcriptional activation of GM-CSF by PMA + PHA and consequently the data from Smith and colleagues would predict little effect of dexamethasone on this system (27). Although repression of NF-κB–dependent transcription is commonly reported, overexpression of GR and/or extensive glucocorticoid treatments are frequently used to demonstrate a repressive effect (28–31). In this respect, we should emphasize that in our study overexpression of GR or prolonged exposure to dexamethasone was not necessary for repression of GM-CSF release, indicating that the endogenous levels of GR are sufficient for full physiologic responses. An additional explanation for the lack of effect of dexamethasone observed in both the studies reported here and those of Smith and associates (27) is a failure of the transfected reporters to adopt the correct chromatin structure.

The first evidence that we provide for the existence of potent post-transcriptional mechanisms lies in the finding that despite the lack of glucocorticoid-dependent repression of the promoter, the addition of dexamethasone with the stimulus results in the almost complete absence of GM-CSF mRNA and protein. Given the apparently normal promoter activation, as discussed above, the lack of mRNA can only be explained by a failure to accumulate the mature cytoplasmic mRNA. This could be due to either reduced pre-RNA processing and export from the nucleus, thereby leading to nuclear degradation, or by a failure to stabilize the mRNA (i.e., degradation) in the cytoplasm. There is now mounting evidence that the nuclear processing and subsequent export of mRNA are highly complex regulated events (32), but as yet control by glucocorticoids has not been implicated. By contrast, glucocorticoid-dependent mRNA decay is an established phenomenon (12). However, as the addition of dexamethasone to cells that had been prestimulated resulted in little or no effect on steady-state GM-CSF mRNA (Figure 5), we can in this case specifically exclude mRNA destabilization as a target. Rather, it seems that dexamethasone may prevent the formation of a stable mRNA (i.e., a failure to stabilize mRNA), which once generated is resistant to the effects of glucocorticoids. Furthermore, a number of ARE-binding proteins are known that could mediate such an effect via AREs in the GM-CSF 3′UTR (33). In the present study, the expression of the ARE-binding protein, HuR, which causes mRNA stabilization, was mildly upregulated by PMA + PHA, whereas the expression of TTP, which binds AREs and causes destabilization, was dramatically upregulated. At the same time, binding of proteins to the GM-CSF ARE was markedly induced by PMA + PHA at 2 h (data not shown) and to an even greater extent at 6 h (Figure 7). As HuR and TTP may both bind and compete for the GM-CSF ARE (24), these data are consistent with a model in which cell stimulation initially results in activation of HuR binding. Subsequently as TTP expression is increased following stimulation (Figure 7), TTP is able to displace HuR from the ARE and will lead to mRNA destabilization. Thus the abundance of GM-CSF mRNA may be tightly regulated by a natural feedback control mechanism involving a switch from rapid initial stabilization, involving HuR, to later destabilization, by TTP (24). However, analysis of both HuR and TTP expression, as well as signal-induced proteins binding to the GM-CSF ARE, revealed no effect of dexamethasone, suggesting that gross changes in the expression of these proteins or overall changes in ARE binding do not account for repressive effects (Figure 7). Further analysis of the exact composition of ARE-binding proteins as well as their post-translational modifications will be required to reveal whether dexamethasone mediates ARE-dependent repression via more subtle mechanisms.

Another possible glucocorticoid target is protein translation, and indeed, modulation of translational initiation factor expression and ribosomal protein gene expression are major targets of glucocorticoid action (16). The studies presented here document a profound ability of glucocorticoids to repress GM-CSF protein expression even when added, as in clinical usage, subsequent to the stimulating agent (Table 1 and Figure 6). As steady-state levels of GM-CSF mRNA were unaltered by dexamethasone over a similar time frame (Figure 5), these data point conclusively to a major glucocorticoid action occurring at the level of translational control. To date, the ability of glucocorticoids to translationally repress various proinflammatory genes, including inducible nitric oxide synthase (14, 17) and interleukin-2 (34), have been demonstrated. Furthermore, the findings that glucocorticoids, and other steroid hormones, can negatively modulate the poly A tail length of repressible genes provides additional support for translation as a general target for steroid action (13, 35). Although the actual protein(s) involved in this translational repression is currently not known, is it possible than the actinomycin D sensitivity of GM-CSF synthesis from preformed mRNA (*see* Figures 6A and 6B) may provide a clue. In these experiments, the synthesis of GM-CSF protein was inhibited by actinomycin D added after the PMA + PHA stimulus (Figure 6). As GM-CSF mRNA is already present and persists for the duration of the experiment (Figure 5), these data implicate the need for ongoing transcription, presumably of another protein, for successful GM-CSF translation. It is possible that such proteins could themselves be targeted by dexamethasone to inhibit GM-CSF expression.

In conclusion, we present a body of data confirming the critical importance of the NF-κB (−85/−76) and CLE0 (−54/−31) sites in proximal region of the human GM-CSF promoter in primary human T cells. Furthermore, we demonstrate that promoter activation via these two sites is not *per se* a major target of glucocorticoid action in primary human T cells, and instead suggest that post-transcriptional and particularly translational mechanisms of glucocorticoid-dependent repression are overriding. Further studies into such mechanisms may reveal new strategies to combat T cell–mediated inflammatory diseases.

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