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Induction of STAT and NFκB Activation by the Antitumor Agents 5,6-Dimethylxanthenone-4-acetic Acid and Flavone Acetic Acid in a Murine Macrophage Cell Line

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ABSTRACT. The antitumor agents flavone-8-acetic acid (FAA) and its dose-potent analogue 5,6-dimethylxanthenone-4-acetic acid (DMXAA), currently in clinical trials, have a novel mechanism of action that is mediated through their ability to induce a spectrum of cytokines. Since NFκB and STAT transcription factors participate in the regulation of a number of genes involved in immune and cytokine responses, we investigated whether these transcription factors were activated in the ANA-1 murine macrophage cell line by DMXAA and FAA compared with lipopolysaccharide (LPS), a bacterial component that induces an overlapping spectrum of cytokines. Activation of STAT1 and STAT3 was observed distinctly 4 hr after DMXAA and FAA stimulation. DMXAA and FAA induced NFκB translocation with slower kinetics of activation compared with LPS. STAT activation by DMXAA and FAA was inhibited by cycloheximide, indicating a requirement for *de novo* protein synthesis. The ANA-1 cells produced high titres of interferons (IFNs) in the culture supernatant after stimulation with DMXAA and FAA, and the addition of antibodies to IFNα/β inhibited STAT activation, indicating that IFNs mediated STAT activation. NFκB activation, on the other hand, was not inhibitable with cycloheximide or with antibodies to IFNα/β. NFκB activation appeared to be a direct action of the anticancer agents, whereas activation of the STAT proteins was due, in part, to the high titres of IFNs induced. These results demonstrate the significance of the IFN response in initiating the cascade of secondary events that may contribute to the overall antitumor efficacy of DMXAA and FAA in murine models. BIOCHEM PHARMACOL **58**;7:1173–1181, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. antitumor; xanthenones; NFκB; STAT; transcription factors

FAA¶ (Fig. 1), synthesised by Atassi and co-workers [1], and its more dose-potent analogue DMXAA (Fig. 1), synthesised by Rewcastle *et al.* [2], both demonstrate high activity against vascularised transplantable murine tumors [3, 4]. They are prototypes of a novel approach to cancer therapy utilising low molecular weight synthetic molecules to stimulate host reactivity and tumor vascular damage. A striking feature of their antitumor action is the rapid induction of tumor vascular collapse leading to tumor ischemia and necrosis [5, 6]. The induction of haemorrhagic necrosis, while necessary, is insufficient for long-term regressions, and several studies have implicated an essential role for T-cells for cures to be achieved [7–9]. Both FAA and DMXAA have been shown to possess broad immune-stimulating abilities: elevation of natural killer cell activity [10, 11], stimulation of tumoricidal macrophage activity [12], and enhancement of the generation of lymphokine-activated killer cells [13].

The vascular and immune-modulatory effects of FAA and DMXAA have been attributed to their ability to induce a panel of cytokines. The early vascular effects appear to be largely TNF-mediated, since antibodies to TNF will abolish FAA-induced [14] or DMXAA-induced [15] vascular collapse, and the histological appearance of treated tumors resembles that of those treated with TNF [4]. The immune modulation has been attributed to the induction of IFNs. IFNα/β mediates FAA-induced elevation of natural killer cell activity [11], whereas IFNγ boosts T-cell immunity [16]. Other cytokines that are induced by these

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¶ *Abbreviations*: FAA, flavone-8-acetic acid; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; DMEM, Dulbecco's Modified Eagle's Medium; EMSA, electrophoretic mobility shift assay; IFNs, interferons; IL, interleukin; LPS, lipopolysaccharide; SSC, standard saline citrate; STAT, signal transducer and activator of transcription; and TNF, tumor necrosis factor-α.

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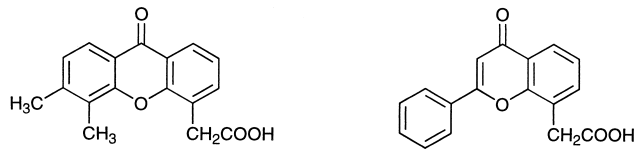

FIG. 1. Chemical structures of DMXAA and FAA.

agents include IL-6, MIP-1α, and IP-10 [17, 18], and as these chemokines can affect endothelial cell permeability and leukocyte migration and inhibit angiogenesis [19, 20], they also may be contributing to the overall antitumor action of these agents.

FAA has been subjected to extensive trials, but did not show clinical activity [21]. Consistent with its lack of response against human malignancies, FAA did not induce mRNA for IFNα and γ, or for TNF in human peripheral blood leukocytes, although excellent induction of these genes was obtained using FAA on murine splenocytes [22]. DMXAA is currently in Phase I trials and was selected as a clinical candidate on the basis that it induced TNF mRNA in the human HL-60 myelocytic cell line as well as in the murine J774 macrophage cell line, whereas FAA was active only in the murine line [23, 24]. Subsequently, DMXAA, but not FAA, was shown to induce TNF synthesis in cultures of human peripheral blood leukocytes [25]. These results demonstrated that a similar pathway for activation existed in human and murine cells, but that FAA activated the murine pathway only.

The action of DMXAA and FAA has been compared with that of LPS, and whereas all induced haemorrhagic necrosis, LPS did not elicit cures or growth delays against the Colon 38 tumor [26]. In cultures of primary macrophages, DMXAA induced a subset of the early LPS-inducible genes and was a better inducer of IP-10 and the IFNs than LPS, but induced lesser amounts of TNF than LPS and did not induce IL-1 [18]. In contrast to the action of LPS, gene induction with DMXAA in the macrophage cultures did not involve tyrosine phosphorylation of the mitogen-activated protein kinases, indicating that LPS and DMXAA may act on different intracellular pathways [18].

The biochemical pathway and target for DMXAA are not known. The ANA-1 macrophage cell line has been shown to express mRNA and protein of a number of cytokines, including IFNβ, IL-6, and TNF, in response to FAA, and has been used previously as a model for investigating the effects of FAA [17]. In this study we have compared activation of NFκB and STAT transcription factors by DMXAA and FAA with that by LPS using the ANA-1 model, with the purpose of elucidating differences in gene activation that may provide insights into the superior antitumor action of the synthetic molecules over LPS. We show here that distinct differences occur in the kinetics of NFκB activation and translocation by LPS compared with DMXAA and FAA. Greater activation of STAT1 and STAT3 proteins was observed with DMXAA and FAA as a result of IFNβ production than with LPS in the ANA-1 cells.

MATERIALS AND METHODS

Materials and Reagents

The sodium salt of DMXAA, obtained from the Auckland Cancer Society Research Laboratory, was dissolved directly in medium. The sodium salt of FAA was obtained from the Developmental Therapeutics Program, dissolved in a minimal volume of 5% sodium bicarbonate, and then diluted to the desired concentration in medium. LPS from *Escherichia coli* serotype 055:B5 was purchased from the Sigma Chemical Co., and was dissolved in water to 1 mg/mL for further dilutions in culture medium. Cell culture solutions, DMEM, Dulbecco's PBS, stock L-glutamine, 1 M HEPES, and penicillin and streptomycin solutions were obtained from Bio-Whittaker. Cycloheximide, poly(dI:dC), Nonidet P-40, and all protease inhibitors were purchased from Sigma. Glycerol and other liquid solvents were purchased from the Fisher Chemical Co.

Cell Line

The ANA-1 murine macrophage cell line, a gift from Dr. Luigi Varescio (NCI), was maintained in DMEM, supplemented with 10% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin, at 37° in a humidified atmosphere of 5% CO_2. For experiments, the cells were fed with fresh medium the day before treatment to ensure exponential growth. Then cells were cultured in 6-well plates at 10^6 cells/mL and allowed to settle overnight before LPS (100 ng/mL final concentration), DMXAA, or FAA (250 μg/mL final concentration) was added. The drug concentrations used were those determined previously to be optimal for maximal activity [17, 23]. In experiments where it was used, cycloheximide was added at 10 μg/mL 30 min before the addition of the stimulants. In experiments involving antibodies to IFNα/β, the antibodies (rabbit anti-mouse IFNα/β, Cat. No. 21032, Lee Biomolecular Research Laboratories) were added at the same time as the stimulants at a 1/50 final dilution (10,000 units/culture).

Preparation of Cytoplasmic and Nuclear Extracts

Cells were harvested at indicated times after stimulation, washed twice in ice-cold PBS, and lysed in low salt buffer (50 mM KCl, 25 mM HEPES) with protease inhibitors: leupeptin (10 μg/mL), aprotinin (20 μg/mL), dithiothreitol (100 μM), phenylmethylsulphonyl fluoride (1 mM), and Nonidet NP-40 (0.5%). The lysate was centrifuged at 400 *g* for 5 min at 4°. The nuclear pellet was washed with low salt buffer without NP-40 and then suspended in high salt buffer (500 mM KCl, 25 mM HEPES, 10% glycerol) with protease inhibitors, frozen and thawed to extract the

proteins, and finally centrifuged at 20,000 *g* for 10 min at 4°. The supernatant was dialysed against 500 mL of dialysis buffer (50 mM KCl, 25 mM HEPES, 10% glycerol) with protease inhibitors. Protein concentrations were measured using the BCA protein assay kit (Pierce).

EMSA

The following oligonucleotides were used

S1E: 5′-AGCTTGTCGACATTTCCCGTAAATCGTCGAG [27];

FcγR1: 5′-AGCTTGTATTTCCCAGAAAAGG [28];

NFκB: 5′-AGCTTACAAGGGACTTTC [29]

Oligonucleotides and their complementary strands were synthesised using an Applied Biosystems 394 DNA/RNA synthesiser, deprotected at 50° overnight, lyophilised, resuspended in water, and reprecipitated with 0.1 vol. of 3 M sodium acetate:0.7 vol. of isopropanol, washed with 70% ethanol, and resuspended in water. Complementary strands (50 μg) were annealed by heating to 92° for 2 min and then cooled to room temperature. Double-stranded oligonucleotides were radiolabelled using the Klenow fragment of DNA polymerase I (Pharmacia Oligolabeling Kit, 27–9250-01) and [α-^{32}P]dCTP (50 μCi, 3000 Ci/mmol, Amersham Corp.), in a fill-in reaction for 5′-protruding ends. EMSA was performed as previously described [30]. Briefly, protein (5 μg) was incubated in a total volume of 20 μL containing 12 mM HEPES, 2.5 mM $MgCl_2$, 60 mM KCl, 200 μM EDTA, 500 μM dithiothreitol, 25 μM *p*-nitrophenyl *p*-guanidinobenzoate, 12% glycerol, and 1 μg poly(dI:dC), for 10 min on ice. The labelled probe was added (1 μL of double strands, 15,000 cpm/reaction mixture), and incubated for 15 min at room temperature. The reaction products were resolved by electrophoresis (2 hr, 150 V) on a native polyacrylamide gel (59:1 cross-linking ratio) in 0.25 × TBE buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA from Digene Diagnostics). The gel was dried in a Bio-Rad model 583 gel dryer and examined by autoradiography. For supershift analyses, 1 μL of antibodies was added before the addition of the oligonucleotide. Antibodies to the NFκB family of proteins were a gift from Dr. Nancy Rice (NCI). Antibodies to STAT1, STAT3, STAT5a, and STAT5b were provided by Dr. Andrew C. Larner (Center for Biologics Evaluation and Research, FDA), and antibodies to STAT4 were purchased from Santa Cruz Biotechnologies, Inc.

Northern Blot Analysis of mRNA

Total cellular mRNA was isolated from ANA-1 cells using Trizol (Gibco-BRL), according to the manufacturer's instructions. The RNA samples (10 μg) were fractionated by electrophoresis on a formaldehyde-denaturing 0.8% agarose gel and transferred overnight to a nylon membrane (MSI Inc.) After UV-crosslinking (120 mJ, UV-Stratalinker, Stratagene), the membrane was baked (20 min, 72°) under vacuum, and each membrane was pre-hybridised for 30 min in 5 mL of Fastpair preformulated hybridisation reagent (Digene Diagnostics), at 42°. A cDNA probe for the cytokine mRNA of interest was labelled with [α-^{32}P]dCTP (Amersham) using a random priming kit (Prime-It, Stratagene). Excess radioactivity was removed by elution through a NACS 52 prepac column (Gibco-BRL). ^{32}P-Labelled probe (10^6 cpm/mL of hybridisation reagent) was added to the membrane, which then was hybridised for 36 hr at 42°. The blots were washed twice in 2 × SSC plus 0.1% SDS for 10 min at 42°, followed by a final wash for 10 min at 65° in 0.2 × SSC, 0.1% SDS. Blots were exposed to Kodak X-OMAT X-ray film for 1–3 days at 70°. After hybridisation with one probe, membranes were stripped by boiling in 0.01% SDS, 0.02% SSC for 20 min, and re-hybridised with another probe. Loading of the lanes was determined from the signal obtained from hybridisation of the blot with the probe for human β-actin.

Cytokine Assays

Analysis of culture supernatants for cytokine activity was performed by the Clinical Immunology Services, Program Resources, Inc./Dyn Corp., NCI-FCRDC. IFNα/β activity was determined using the vesicular stomatitis viral inhibition assay, where one unit of activity is the amount of IFN that inhibits viral lysis by 50% in the bioassay. IL-6 was determined using an ELISA kit from R & D Products.

RESULTS

Activation of STAT Proteins Following DMXAA and FAA Stimulation

We investigated whether the family of STAT transcription factors were activated in response to DMXAA, FAA, and LPS. Exponentially growing ANA-1 cells were stimulated with DMXAA (250 μg/mL), FAA (250 μg/mL), or LPS (100 ng/mL), and nuclear proteins were extracted after 0.5, 2, and 4 hr. Inducible complexes were detected 4 hr after stimulation with DMXAA and FAA using both the FcγR1 (Fig. 2) and SIE (Fig. 3) oligonucleotides containing STAT-responsive elements, with only faint bands detectable in the LPS-stimulated samples (Figs. 2 and 3, lanes 1–3). Supershift analyses were carried out on the 4-hr DMXAA nuclear extracts to identify the STAT proteins that were activated. Since the SIE probe preferentially binds STAT1, STAT3, and STAT4, we used antibodies to STAT1, STAT3, and STAT4 with the SIE probe. Antibodies to STAT1, STAT3, STAT5a, and STAT5b were used with the FcγR1 probe since those STAT proteins were bound preferentially with that probe [30, 31]. STAT1 antibodies supershifted the bottom (band 3) and the middle (band 2) bands, and antibodies to STAT3 blocked formation of the top (band 1) and middle (band 2) bands with the SIE (Fig. 4, lanes 2 and 3) and the FcγR1 probe (Fig. 4, lanes 7 and 8). Thus, band 1 contained STAT3 homodimers, band 2 contained heterodimers of STAT1 and STAT3, and band 3 contained STAT1 homodimers. An-

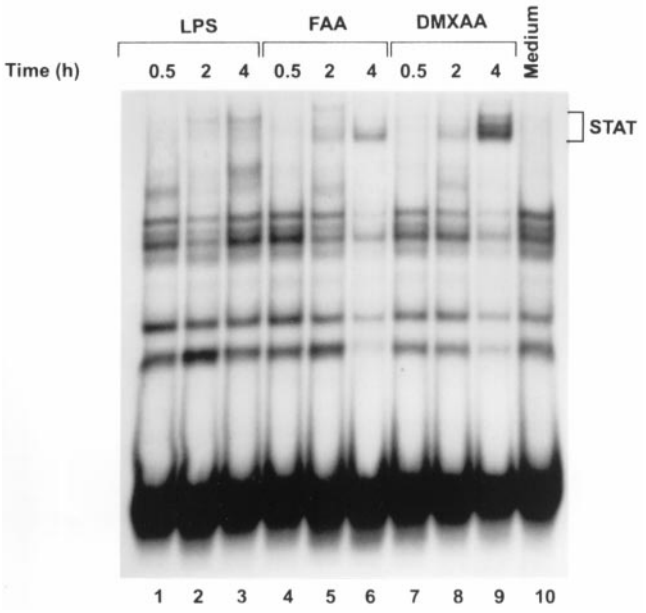

FIG. 2. STAT activation induced in ANA-1 cells after 0.5-, 2-, and 4-hr stimulation with LPS (100 ng/mL), FAA (250 μg/mL), or DMXAA (250 μg/mL), compared with unstimulated medium controls, as detected using the FcγR1 probe for EMSA.

tibodies to STAT6 had no effect on binding to the SIE probe, and no complexes were detected when the same nuclear extracts were assayed using two different probes containing the STAT6 RE, one from the IL-4 gene [32] and the other from the IgE promoter [33], confirming the lack of STAT6 activation. The specific complexes in lane 4 of Fig. 4, which had antibodies to STAT4 added, were fainter than those in the control lane 1, and antibodies to STAT5a and STAT5b (Fig. 4, lanes 9 and 10) induced a slower

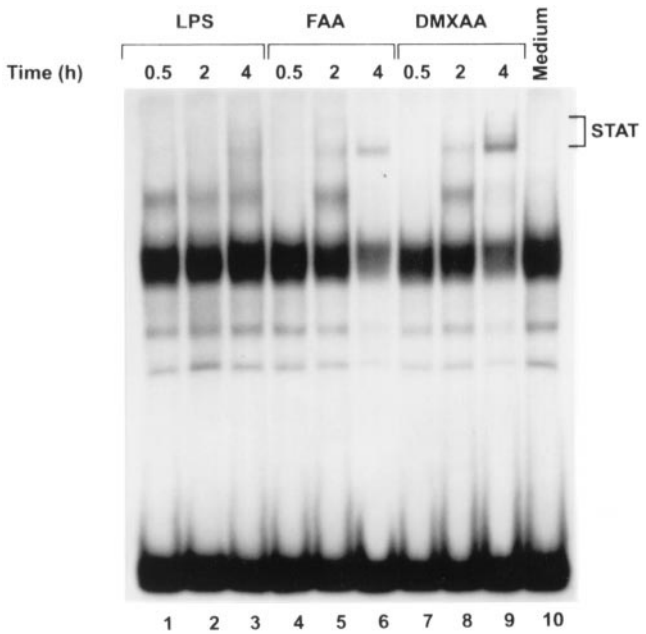

FIG. 3. STAT activation studied as for Fig. 2, using the SIE probe.

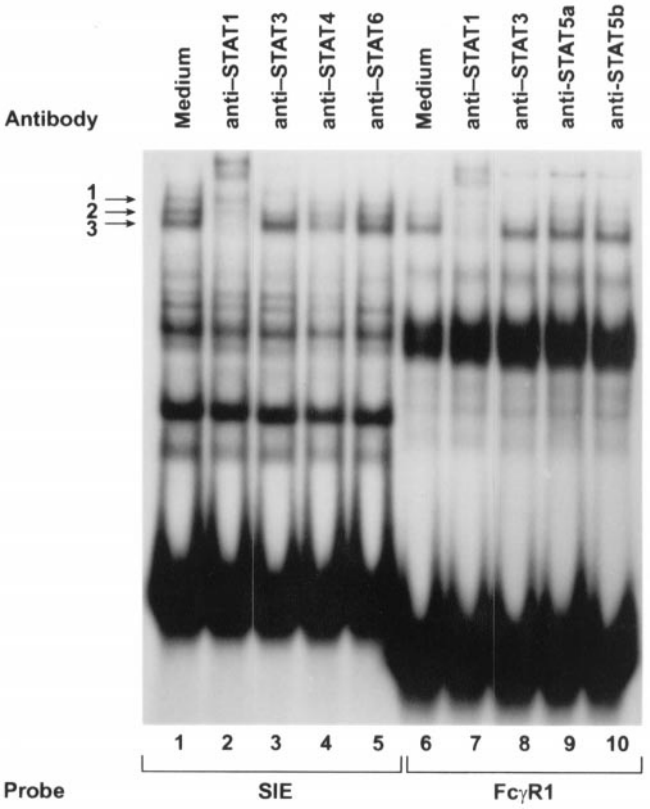

FIG. 4. Analysis of STAT complexes induced with DMXAA. Supershift analyses were performed on nuclear extracts from ANA-1 cells stimulated for 4 hr with DMXAA (250 μg/mL) with antibodies against STAT-1, STAT-3, STAT-4, and STAT-6 with the SIE probe (lanes 1–5), and antibodies to STAT1, STAT3, STAT5a, and STAT5b with the FcγR1 probe.

mobility complex without significant changes in the intensity of the unshifted bands. The results show that DMXAA primarily activated STAT1 and STAT3. STAT4, STAT5a, and STAT5b also may be activated, but to a lesser extent.

NFκB Activation by LPS, DMXAA, and FAA

The nuclear extracts were also examined for NFκB binding using a probe containing the NFκB consensus element from the human immunoglobulin κ light chain enhancer. NFκB activation by FAA and DMXAA was markedly slower than that observed with LPS (Fig. 5). High levels of NFκB binding were obtained within half an hour with LPS and were maintained for 4 hr. With DMXAA and FAA, maximal NFκB binding was detected at 2 hr, and by 4 hr the levels had declined significantly. Three independent experiments all showed similar kinetics of induction, and with a faster decline observed with DMXAA than with FAA.

To determine whether LPS, FAA, and DMXAA activated different NFκB family members, supershift analyses were carried out using nuclear extracts from cells 2 hr after stimulation with each agent. Identical results were obtained

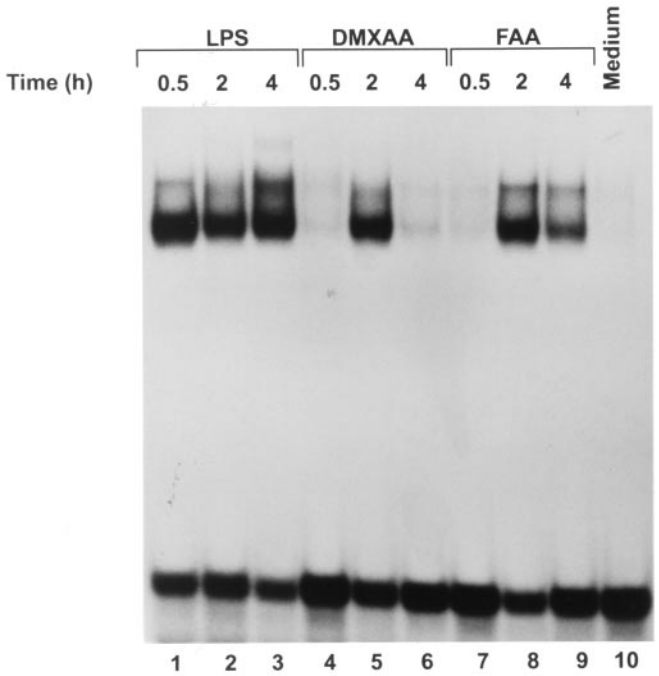

FIG. 5. Kinetics of NFκB activation induced with LPS, DMXAA, or FAA. Nuclear extracts from ANA-1 cells unstimulated or stimulated with LPS (100 ng/mL), DMXAA (250 μg/mL) or FAA (250 μg/mL) for the indicated times were analysed, using EMSA, for NFκB binding.

with all three agents. Partial supershifting of the upper band was obtained using antibodies to the p50 subunit, while antibodies to the p65 protein completely shifted the lower band (Fig. 6). Antibodies to c-*rel*, *rel* B, and the p52 family members and normal rabbit serum had no effect. Thus, in the ANA-1 cells, dimers consisting of p65 and p50 appeared to be the main proteins translocated by LPS, FAA, and DMXAA.

Effect of Cycloheximide on STAT and NFκB Activation

NFκB and STAT activation following treatment with DMXAA and FAA occurred after a considerable delay, raising the possibility that their activation was dependent on synthesis of new protein. To address this issue, cycloheximide (10 μg/mL) was added to the cultures of ANA-1 cells 30 min before the addition of the stimulants to determine whether the STAT and NFκB complexes could be induced in the absence of *de novo* protein synthesis. Cycloheximide inhibited STAT induction, but potentiated NFκB activation in response to DMXAA and FAA (Fig. 7). This was confirmed in a separate experiment shown in Fig. 8, which also showed that cycloheximide alone induced NFκB activation, but at a much lower level than that induced with DMXAA or FAA, and insufficiently to account for the increased levels observed in the nuclear extracts where both cycloheximide and DMXAA or FAA had been added (Fig. 8).

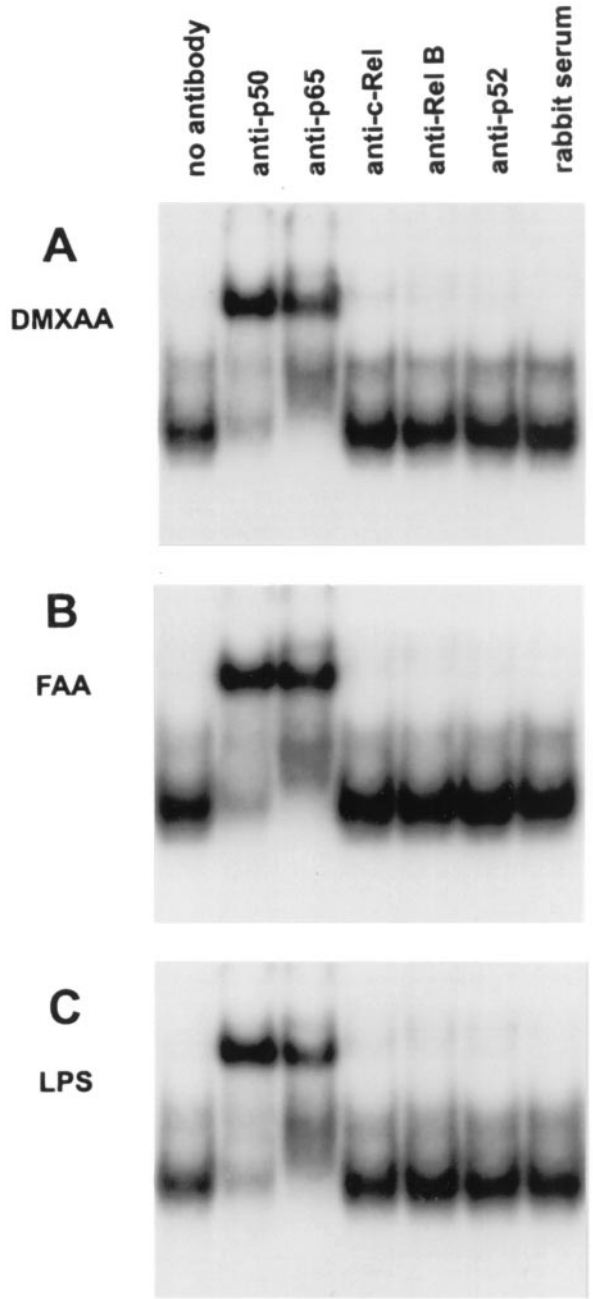

FIG. 6. Supershift analysis of NFκB proteins translocated in response to DMXAA, FAA, or LPS. Nuclear extracts from ANA-1 cells stimulated for 2 hr with (A) DMXAA (250 μg/mL), (B) FAA (250 μg/mL), or (C) LPS (100 ng/mL) were incubated with antibodies specific for each member of the NFκB family of proteins, p50, p65, c-*rel*, *rel* B, and p52, and resolved using EMSA for NFκB binding.

Role of IFNβ Production on FAA- and DMXAA-Induced STAT Activation

The STAT proteins activated in response to DMXAA in ANA-1 cells are the same as those induced with IL-6 or IFNα/β [34]. Previous work showed that FAA up-regulated mRNA for IL-6 and IFNβ but not IFNα in the ANA-1 cells, and induced large amounts of active IFN within 3 hr of stimulation [17]. Activation of the STAT transcription

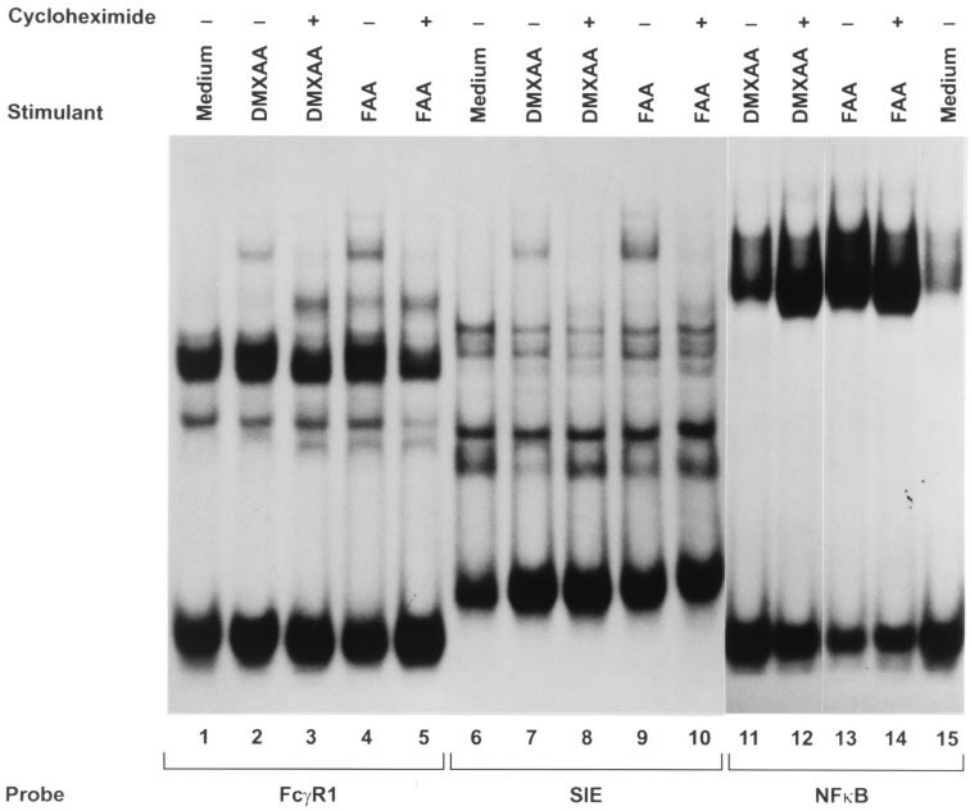

FIG. 7. Effect of cycloheximide on STAT and NFκB activation by DMXAA and FAA. ANA-1 cells were treated with DMXAA or FAA (250 μg/mL) with or without cycloheximide (100 ng/mL); 4-hr nuclear extracts were analysed for STAT binding using the FcγR1 (lanes 1–5) and the SIE probe (lanes 6–10), and 2-hr extracts were analysed for NFκB binding (lanes 11–15).

factors could be a secondary effect of the drugs, resulting from the endogenously induced cytokines. We examined induction of IL-6 and IFNβ by the three agents using the same conditions as those used for STAT and NFκB activation. No induction of mRNA for IFNβ was observed with LPS compared with DMXAA and FAA (Fig. 9). Similar amounts of IL-6 mRNA were obtained with LPS and FAA, with much lower amounts induced with DMXAA (Fig. 9).

Cytokine activity in the culture supernatants 24 hr after stimulation correlated with the pattern of mRNA induction. DMXAA and FAA induced high titres of IFN (>50,000 units), with minimal induction by LPS (<10 units). DMXAA, however, induced lower amounts of IL-6 (3,779 pg/mL) than FAA (22,510 pg/mL) or LPS (14,740 pg/mL).

Since STAT activation correlated with IFNβ induction by the two agents, we examined whether antibodies to IFNα/β blocked STAT activation. As seen in Fig. 10 with the FcγR1 probe, antibodies to IFNα/β added to the ANA-1 cells at the same time as the stimulant reduced STAT activation with respect to that observed with normal rabbit serum. With LPS as the stimulant, the formation of the lower bands, which were mainly STAT1 complexes (Fig. 4), was blocked preferentially. With DMXAA, formation of the lower bands containing STAT3 complexes was blocked preferentially (Fig. 10). This was seen also when the extracts were assayed using the SIE probe, but no reduction of NFκB activation was seen with the antibodies (data not shown).

DISCUSSION

These present studies show activation of NFκB and STAT1 and STAT3 transcription factors in response to FAA and DMXAA in ANA-1 cells (Figs. 2–5). NFκB activation with DMXAA and FAA was apparent at 30 min, but required 2 hr to be maximal and declined at 4 hr (Fig. 5). The kinetics of NFκB activation by DMXAA were markedly slower than those for LPS, which within 30 min induced significant translocation that was maintained at high levels for at least 4 hr (Fig. 5). The reason for the difference in kinetics of NFκB activation between LPS and the synthetic agents is not clear, but correlates with a slower production of TNF in mice treated with DMXAA compared with LPS [35]. LPS activates cells via surface receptors, and transfection of 70Z/3 B cells with the human CD-14 receptor increases responsiveness to LPS such that NFκB activation occurs at 1000-fold lower concentrations of LPS [36]. In contrast to LPS, cytokine induction by DMXAA in CD-14 knockout mice is not impaired [37], and DMXAA is thought to act intracellularly. A possible explanation for the faster action of LPS compared with DMXAA and FAA is that the CD-14 receptors endow cells with a particularly efficient and rapid signalling system.

NFκB has been shown to take part in the regulation of IL-6, TNF, and IFNβ gene expression, all of which are induced by FAA in ANA-1 cells [17]. The lack of IFN production by LPS in the ANA-1 cells in these studies was surprising. Using a more potent preparation of LPS, the ANA-1 cells were shown to respond comparably to thio-

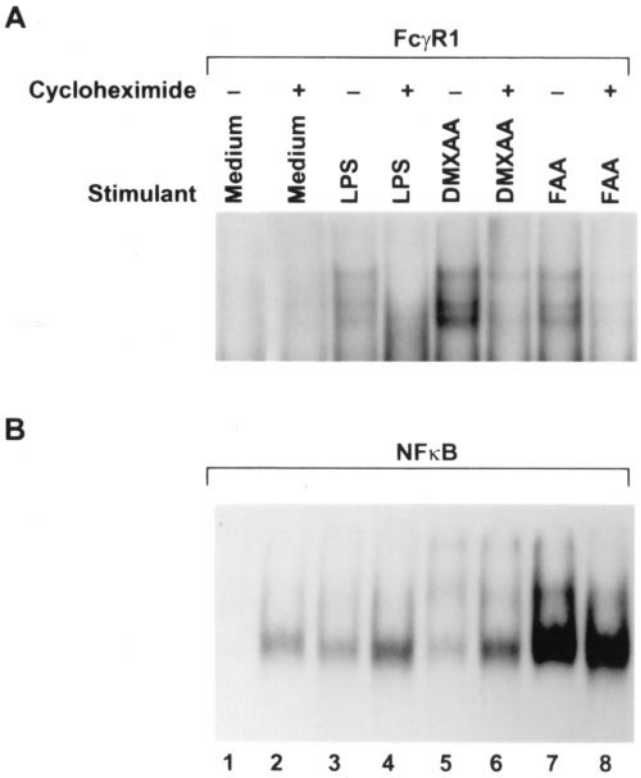

FIG. 8. Effect of cycloheximide alone or in combination with LPS, FAA, or DMXAA on STAT and NFκB activation. Nuclear extracts from ANA-1 cells cultured for 4 hr with cycloheximide (100 ng/mL, lane 2), LPS (100 ng/mL, lane 3), DMXAA (250 μg/mL, lane 5), or FAA (250 μg/mL, lane 7) alone, and cycloheximide in combination with LPS (lane 4), with DMXAA (lane 6), and with FAA (lane 8) were analysed using EMSA for (A) STAT complexes using the FcγR1 probe, and for (B) NFκB.

glycollate-elicited C3H/OuJ macrophages, but both cell types produced 8- to 10-fold higher titres of type I IFNs in response to DMXAA than to LPS. DMXAA and LPS induced an overlapping but different spectrum of cytokines in primary macrophages, and induced different levels of activity in those cytokines that they activated in common [18]. Differential expression of the various NFκB dimers in different cells led to altered patterns of expression of cytokines [38]. In this study, we found that the p50 and p65 subunits were the primary members that were translocated in response to LPS, DMXAA, and FAA in the ANA-1 cells (Fig. 6). Thus, another mode of regulation in addition to NFκB must mediate the differential response to each of the stimulants, perhaps in a similar manner to viral induction of IFNβ, which has been shown to be mediated by two virus-inducible activators, one of which is NFκB. In this case, both activators must bind to different sites on the DNA and act together for induction to occur [39].

The potentiation of NFκB activation in the presence of cycloheximide (Fig. 8) and induction by cycloheximide alone is consistent with the observations of Sen and Baltimore [29]. The early studies on the effects of cycloheximide on NFκB activation were interpreted by these authors to indicate that NFκB induction involved the conversion of a precursor into an active form, and cycloheximide prevented the synthesis of a labile inhibitor. It has now been established that dimeric NFκB complexes are stored in the cytoplasm as an inactive precursor complexed with its labile inhibitor, IκB [40, 41]. For our purposes, however, the experiments with cycloheximide established that NFκB induction by DMXAA did not involve *de novo* protein synthesis and appeared to be a direct response to the drug, in contrast to STAT induction.

Activation of STAT proteins was detected 4 hr after stimulation with DMXAA and FAA (Figs. 2 and 3) and was completely inhibitable with cycloheximide (Fig. 7). Since STAT proteins also exist in a latent form in the cytoplasm and are activated within 15 min upon phosphorylation by Janus kinases in response to cytokines or growth factors [42, 43], the delayed response to DMXAA and FAA and its inhibition by cycloheximide is consistent with STAT activation being a secondary response to primary cytokines induced with the drugs. STAT activation was more evident in response to DMXAA and FAA than to LPS. Under the same conditions to those used for STAT activation, ANA-1 cells produced much higher titres of IFN in response to DMXAA and FAA than to LPS, and anti-IFNα/β antibodies partially blocked STAT activation (Fig. 10). The results indicated that the activation of STAT proteins was mediated, in part at least, by IFNβ. It is not clear if the residual activity is due to activation by the production of other cytokines or to incomplete neutralisa-

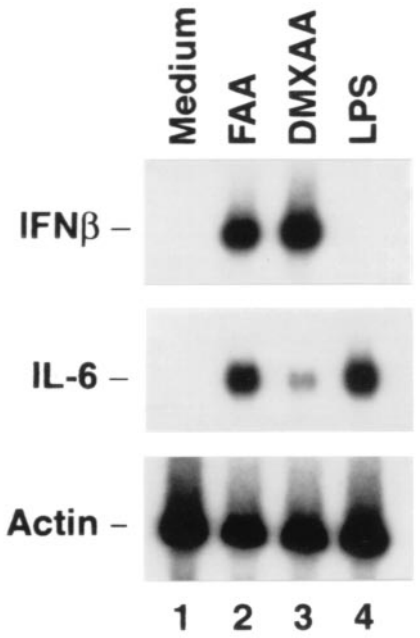

FIG. 9. Induction of mRNA for IL-6 and IFNβ in ANA-1 cells in response to DMXAA, FAA, and LPS. Cellular mRNA extracted from ANA-1 cells stimulated with LPS (100 ng/mL) or FAA or DMXAA (250 μg/mL) for 4 hr was analysed by northern blotting for IL-6 and IFNβ mRNA. Binding to the actin probe was used to show the mRNA loading in the individual lanes.

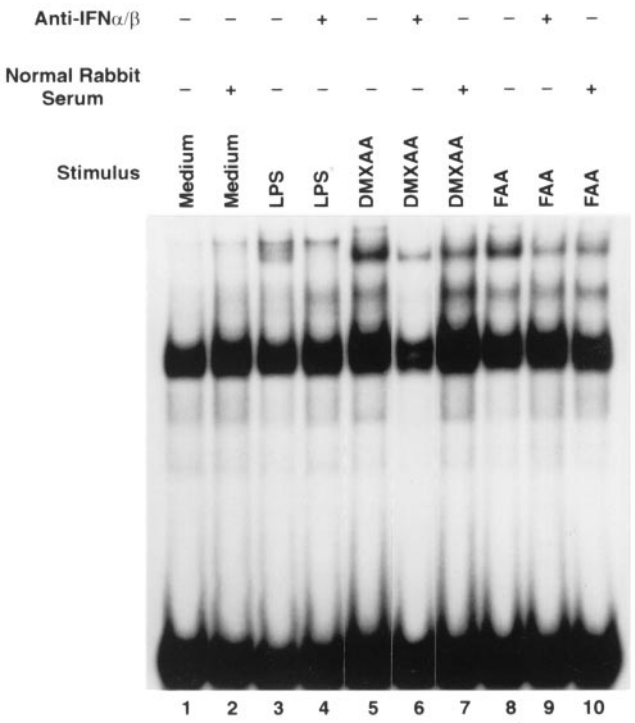

FIG. 10. Effect of anti-IFNα/β antibodies on STAT activation by DMXAA, FAA, and LPS. ANA-1 cells were stimulated with LPS (100 ng/mL) or DMXAA or FAA (250 μg/mL) for 4 hr with or without normal rabbit serum or antibodies to IFNα/β present in the culture, and the nuclear extracts were examined for STAT binding to the FcγR1 probe for EMSA.

tion of IFN with the concentration of antibodies used. These results are compatible with those of Perera and co-workers [18], who showed that anti-IFNα/β antibodies caused a significant but not complete inhibition of IP-10, D3, IRF-1, IRF-2, and ICSBP gene expression induced with DMXAA in primary macrophage cultures. These studies suggest an important role for IFNs in inducing secondary effects of DMXAA and FAA. An example of such an effect, the elevation of natural killer cells, has been suggested to contribute to the overall antitumor efficacy of FAA [9]. However, in studies on the molecular pathway of induction, it is necessary to dissociate the direct effects of the compounds from the secondary effects. The activation of NFκB, in contrast to activation of STAT by DMXAA and FAA, did not involve *de novo* protein synthesis and was not inhibitable with antibodies to IFNα/β, and thus appears to result from a primary effect of the compounds. We conclude that DMXAA and FAA activate murine cytokine genes through an NFκB-mediated regulatory pathway.

References

1. Atassi G, Briet P, Berthelon J-J and Collonges F, Synthesis and antitumor activity of some 8-substituted 4-oxo-4H-1-benzopyrans. *Eur J Med Chem* **20**: 393–402, 1985.
2. Rewcastle GW, Atwell GJ, Baguley BC, Calveley SB and Denny WA, Potential antitumor agents. 58. Synthesis and structure–activity relationships of substituted xanthenone-4-acetic acids active against the colon 38 tumor *in vivo*. *J Med Chem* **32**: 793–799, 1989.
3. Plowman J, Narayanan VL, Dykes D, Szarvasi E, Briet P, Yoder OC and Paull KD, Flavone acetic acid: A novel agent with preclinical antitumor activity against colon adenocarcinoma 38 in mice. *Cancer Treat Rep* **70**: 631–638, 1986.
4. Baguley BC, Calveley SB, Crowe KK, Fray LM, O'Rourke SA and Smith GP, Comparison of the effects of flavone acetic acid, fostriecin, homoharringtonine and tumour necrosis factor α on Colon 38 tumours in mice. *Eur J Cancer Clin Oncol* **25**: 263–269, 1989.
5. Bibby MC, Double JA, Loadman PM and Duke CV, Reduction of tumor blood flow by flavone acetic acid: A possible component of therapy. *J Natl Cancer Inst* **81:** 216–220, 1989.
6. Zwi LJ, Baguley BC, Gavin JB and Wilson WR, Blood flow failure as a major determinant in the antitumor action of flavone acetic acid (NSC 347512). *J Natl Cancer Inst* **81**: 1005–1013, 1989.
7. Bibby MC, Phillips RM, Double JA and Pratesi G, Antitumor activity of flavone acetic acid (NSC-347512) in mice—Influence of immune status. *Br J Cancer* **63**: 57–62, 1991.
8. Pratesi G, Rodolfo M, Rovetta G and Parmiani G, Role of T cells and tumor necrosis factor in antitumor activity and toxicity of flavone acetic acid. *Eur J Cancer* **26**: 1079–1083, 1990.
9. Hornung RL, Back TC, Zaharto DS, Urba WJ, Longo DL and Wiltrout RH, Augmentation of natural killer (NK) activity, induction of interferon and development of tumor immunity during the successful treatment of established murine renal cancer using flavone acetic acid (FAA) and interleukin 2. *J Immunol* **141**: 3671–3679, 1988.
10. Ching L-M and Baguley BC, Induction of natural killer cell activity by the antitumor compound flavone acetic acid (NSC 347512). *Eur J Cancer Clin Oncol* **23**: 1047–1050, 1987.
11. Hornung RL, Young HA, Urba WJ and Wiltrout RH, Immunomodulation of natural killer cell activity by flavone acetic acid: Occurrence via induction of interferon α/β. *J Natl Cancer Inst* **80**: 1226–1231, 1988.
12. Ching L-M and Baguley BC, Enhancement of *in vitro* toxicity of mouse peritoneal exudate cells by flavone acetic acid (NSC 347512). *Eur J Cancer Clin Oncol* **24**: 1521–1525, 1988.
13. Ching L-M and Baguley BC, Effect of flavone acetic acid (NSC 347512) on splenic cytotoxic effector cells and their role in tumor necrosis. *Eur J Cancer Clin Oncol* **25**: 821–828, 1989.
14. Mahadevan V, Malik STA, Meager A, Fiers W, Lewis GP and Hart IR, Role of tumor necrosis factor in flavone acetic acid-induced tumor vasculature shutdown. *Cancer Res* **50**: 5537–5542, 1990.
15. Browne WL, Wilson WR, Baguley BC and Ching L-M, Suppression of serum tumour necrosis factor-α by thalidomide does not lead to reversal of tumour vascular collapse and anti-tumour activity of 5,6-dimethyl-4-acetic acid. *Anticancer Res* **18**: 4409–4414, 1998.
16. Sayers TJ, Wiltrout TA, McCormick K, Husted C and Wiltrout RH, Antitumor effects of α-interferon and γ-interferon on a murine renal cancer (Renca) *in vitro* and *in vivo*. *Cancer Res* **50**: 5414–5420, 1990.
17. Eader LA, Gusella L, Dorman L and Young HA, Induction of multiple cytokine gene expression and IRF-1 mRNA by flavone acetic acid in a murine macrophage cell line. *Cell Immunol* **157**: 211–222, 1994.
18. Perera PY, Barber SA, Ching LM and Vogel SN, Activation of LPS-inducible genes by the antitumor agent 5,6-dimethylxanthenone-4-acetic acid in primary murine macrophages. Dissection of signaling pathways leading to gene induction

and tyrosine phosphorylation. *J Immunol* **153**: 4684–4693, 1994.
19. Schrum S, Probst P, Fleischer B and Zipfel PF, Synthesis of the CC-chemokines MIP-1α, MIP-1β and RANTES is associated with a type 1 immune response. *J Immunol* **157**: 3598–3604, 1996.
20. Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S, Kleinman HK, Reaman GH and Tosato G, Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis *in vivo*. *J Exp Med* **182**: 155–162, 1995.
21. Kerr DJ and Kaye SB, Flavone acetic acid—Preclinical and clinical activity. *Eur J Cancer Clin Oncol* **25**: 1271–1272, 1989.
22. Futami H, Eader LA, Back TT, Gruys E, Young HA, Wiltrout RH and Baguley BC, Cytokine induction and therapeutic synergy with interleukin-2 against murine renal and colon cancers by xanthenone-4-acetic acid derivatives. *J Immunother* **12**: 247–255, 1992.
23. Ching LM, Joseph WR, Crosier KE and Baguley BC, Induction of tumor necrosis factor-α messenger RNA in human and murine cells by the flavone acetic acid analogue 5,6-dimethylxanthenone-4-acetic acid (NSC 640488). *Cancer Res* **54**: 870–872, 1994.
24. Patel S, Parkin SM and Bibby MC, The effect of 5,6-dimethylxanthenone-4-acetic acid on tumor necrosis factor production by human immune cells. *Anticancer Res* **17**: 141–150, 1997.
25. Philpott M, Joseph WR, Crosier KE, Baguley BC and Ching L-M, Production of tumour necrosis factor-α by cultured human peripheral blood leucocytes in response to the antitumour agent 5,6-dimethylxanthenone-4-acetic acid (NSC 640488). *Br J Cancer* **76**: 1586–1591, 1997.
26. Ching LM, Joseph WR, Zhuang L and Baguley BC, Interaction between endotoxin and the antitumor agent 5,6-dimethylxanthenone-4-acetic acid in the induction of tumor necrosis factor and haemorrhagic necrosis of colon 38 tumors. *Cancer Chemother Pharmacol* **35**: 153–160, 1994.
27. Sadowski HB, Shuai K, Darnell JE and Gilman MZ, A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science* **261**: 1739–1744, 1993.
28. Lin J-X, Bhat NK, John S, Queale WS and Leonard WJ, Characterization of the human interleukin-2 receptor p-chain gene promoter: Regulation of promoter activity by *ets* gene products. *Mol Cell Biol* **13**: 6201–6208, 1993.
29. Sen R and Baltimore B, Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. *Cell* **47**: 921–928, 1986.
30. Yu CR, Lin JX, Fink DW, Akira S, Bloom ET and Yamauchi A, Differential utilization of Janus kinase-signal transducer activator of transcription signaling pathways in the stimulation of human natural killer cells by IL-2, IL-12, and IFN-α. *J Immunol* **157**: 126–137, 1996.
31. Yu CR, Young HA and Ortaldo JR, Characterization of cytokine differential induction of STAT complexes in primary T and NK cells. *J Leukoc Biol* **64**: 245–258, 1998.
32. Kotamides H and Reich NC, Interleukin-4-induced STAT6 recognizes and activates a target site in the promoter of the interleukin-4-receptor gene. *J Biol Chem* **271**: 25555–25561, 1996.
33. Köhler I and Rieber EP, Allergy-associated Iε and Fcε receptor II (CD23b) genes activated via binding of an interleukin-4-induced transcription factor to a novel responsive element. *Eur J Immunol* **23**: 3066–3071, 1993.
34. Hill CS and Treisman R, Transcriptional regulation by extracellular signals: Mechanisms and specificity. *Cell* **80**: 199–211, 1995.
35. Philpott M, Baguley BC and Ching L-M, Induction of tumor necrosis factor-α by single and repeated doses of the antitumour agent 5,6-dimethylxanthenone-4-acetic acid. *Cancer Chemother Pharmacol* **36**: 143–148, 1995.
36. Lee JD, Kato K, Tobias PS, Kirkland TN and Ulevitch RJ, Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. *J Exp Med* **175**: 1697–1705, 1992.
37. Perera PY, Vogel SN, Detore GR, Haziot A and Goyert SM, CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol. *J Immunol* **158**: 4422–4429, 1997.
38. Lernbecher T, Muller U and Wirth T, Distinct NF-κB/Rel transcription factors are responsible for tissue-specific and inducible gene activation. *Nature* **365**: 767–770, 1993.
39. Fan C-M and Maniatis T, Two different virus-inducible elements are required for human β-interferon gene regulation. *EMBO J* **8**: 101–110, 1989.
40. Sun SC, Ganchi PA, Ballard DW and Greene WC, NF-κB controls expression of inhibitor IκBα: Evidence for an inducible autoregulatory pathway. *Science* **259**: 1912–1915, 1993.
41. Baeuerle PA and Baltimore D, Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. *Cell* **53**: 211–217, 1988.
42. Darnell JE, Reflections on STAT3, STAT5 and STAT6 as fat STATs. *Proc Natl Acad Sci USA* **93**: 6221–6224, 1996.
43. Ihle JN, STATs: Signal transducers and activators of transcription. *Cell* **84**: 331–334, 1996.