CD40-induced activation of cytokine gene expression in dendritic cells (DC) is an important process in the initiation of primary immune responses. We have determined the intracellular signaling events that lead to CD40 ligation-induced activation of interleukin-6 (IL-6) gene transcription in a murine DC line, FSDC, that is phenotypically representative of bone marrow-derived DC. IL-6 reverse transcriptase-PCR and promoter assays established the responsiveness of FSDC to anti-CD40 ligation. Further promoter assays showed that the transcription factors NF-κB and AP-1 are downstream transcriptional mediators of CD40-induced IL-6 gene expression. Anti-CD40 treatment of FSDC stimulated increased expression of specific NF-κB (p50/p65) and AP-1 (c-Jun, JunB, JunD, and c-Fos) DNA-protein complexes. Overexpression of an IkBα super-repressor or a dominant negative JunD resulted in a strong inhibition of CD40-inducible IL-6 promoter activity supporting a role for both transcription factors. Upstream signal transduction events were studied by transfection of wild type and mutant human CD40 expression constructs into FSDC followed by stimulation with an anti-human CD40 antibody. These experiments revealed that anti-CD40 stimulation of NF-κB and IL-6 gene transcription requires specific amino acid residues in the cytoplasmic region of CD40 involved in the recruitment of TRAF2. Induction of IL-6 mRNA by anti-CD40 treatment was found to be a transient event (24 h) and was followed by a diminution of IL-6 transcript to levels below those found in unstimulated cells. This loss of IL-6 expression was associated with reduced p50/p65 NF-κB DNA binding and elevated binding of CBF1 to a site overlapping the NF-κB site. Overexpression of CBF1 resulted in a profound inhibition of basal and anti-CD40-induced IL-6 promoter activities indicating that prolonged induction of CBF1 may contribute to the transient nature of the IL-6 response. The physiological relevance of these molecular events to DC function is discussed.

Dendritic cells (DCs) are antigen presenting cells that are specialized to have the unique property of being able to prime naïve T cells. The powerful immunogenic properties of DCs have implicated these cells in the abrogation of peripheral T cell tolerance against viral, microbial, tumor, and transplant antigens (1). Following antigen capture the immature DC undergoes phenotypic and functional changes that promote its antigen presenting cells characteristics (1). Maturation events involving migration to the lymph nodes, alteration of intracellular major histocompatibility class II transport and surface expression, and decreased capacity to take up and process antigen are required for the DC to be able to activate T cells (1). Interaction of CD40 on DCs with CD40 ligand (CD40L) on naïve T cells is critical for DC maturation and the generation of antigen-specific T cell responses (1, 2). CD40L promotes DC survival, elevates expression of major histocompatibility and co-stimulatory molecules, and induces the expression of a variety of cytokines including tumor necrosis factor, IL-1, IL-6, IL-12, IL-15, and IL-18, all of which are involved in T cell activation and proliferation (1, 2).

IL-6 is a highly pleiotropic cytokine with properties that indicate it is not only a stimulus of the activation, proliferation, and survival of T cells (3–9), but is also able to modify DC function and survival (1, 10, 11). A direct effect of IL-6 on the proliferation of T cells has been documented by several groups (3–8), as has the ability of IL-6 to prevent the death of naïve T cells (9). Other studies have described the ability of IL-6 to induce IFNγ secretion in differentiating T cells; indeed lack of this response in IL-6 gene knockout mice renders animals unable to mount T cell responses against Mycobacterium tuberculosis and Toxoplasma gondii (12, 13). Production of IL-6 by CD40-stimulated DCs (1, 11) is therefore likely to be important in promoting the generation and survival of antigen-specific cytotoxic T cells. IL-6 can alter the manner in which antigen is processed by DCs enabling the activation of T cells against determinants that were previously cryptic (10). In addition, Grohmann and colleagues (11) reported that autocrine IL-6 mediates most of the anti-tolerogenic effects of CD40 ligation on CD8+ DC. Aberrant regulation of autocrine and paracrine stimulation of DCs by IL-6 may therefore be important in the generation of anti-self immunity and propagation of autoimmune disease.

The signal transduction events leading to the activation of cytokine gene transcription by stimulation of CD40 have mainly been studied in B cells. By contrast little is understood about these events in other cell types, including DCs (2). Studies regarding the transcriptional regulation of IL-6 in response to engagement of cell surface CD40 by either CD40L or anti-CD40 have so far revealed a potential role for NF-κB and the requirement for amino acids 202 to 225 in the cytoplasmic tail of CD40 (14). These amino acids in combination with other...
motifs in the cytoplasmic tail of CD40 are responsible for the recruitment of Janus kinase 3 (Jak3) and several members of the tumor necrosis factor receptor-associated factor (TRAF) protein family (2, 14–21). These factors function to link CD40 to the signaling pathways that activate NF-κB, c-Jun NH2-terminal kinase (JNK), and STAT3, which in turn stimulate transcription of various CD40-responsive genes. Studies in a variety of cell types have shown that induction of IL-6 gene transcription involves the coordinated regulation of factors that associate with the evolutionary conserved AP-1, NF-κB transcription. Since there is also a general scarcity of information regarding functional features of the IL-6 promoter required for this response (22–27). Similar detailed studies on the promoter and transcription factor requirements for stimulation of IL-6 gene transcription following CD40 engagement are currently lacking. Therefore undertaken a detailed investigation into events at both the IL-6 promoter and the cytoplasmic tail of CD40 that mediate induction of IL-6 mRNA synthesis in response to CD40 engagement.

Using the murine DC line FSDC (28), we show that anti-CD40 treatment of cells leads to a powerful, but transient induction of IL-6 mRNA expression. We delineate the structural features of the IL-6 promoter required for this response and in addition to demonstrating CD40 induction of AP-1 and NF-κB, and NF-IL-6 sites in the mouse and human IL-6 gene promoters (22–27). Similar detailed studies on the promoter and transcription factor requirements for stimulation of IL-6 gene transcription following CD40 engagement are currently lacking. Since there is also a general scarcity of information regarding the transcriptional regulation of DC function, we have therefore undertaken a detailed investigation into events at both the IL-6 promoter and the cytoplasmic tail of CD40 that mediate induction of IL-6 mRNA synthesis in response to CD40 engagement.

MATERIALS AND METHODS

Cell Culture—FSDC cell line (28), DC2.4 cell line (29), and bone marrow-derived dendritic cells (BMDC) were cultured in RPMI 1640 medium, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (Invitrogen). BMDC were obtained by extracting bone marrow from the leg bone of Balb/c mice (Harlan UK, Blackthorn, UK). The cells obtained were washed twice in sterile phosphate-buffered saline and plated out onto tissue culture dishes. Cells were grown in complete media supplemented with 5 ng/ml granulocyte macrophage colony-stimulating factor (30). Media was changed on alternating days for 10 days. Cells were used in studies on day 10 of primary culture.

CD40 Antibodies—Rat monoclonal antibody 323 was raised against mouse CD40 (31). Mouse anti-human CD40 monoclonal antibody LOB7.6 was obtained from Serotec, UK.

Plasmid DNA—All plasmid DNA was prepared using a commercial DNA extraction and isolation kit (Maxiprep, Qiagen). IL-6 promoter function was studied using the luciferase reporter vector pIL6-Luc651, containing nucleotides −651 to +1 of the human IL-6 gene. Construction of pIL6-Luc651 and derivatives carrying site-directed mutations in the AP-1 (−263 to −276), NF-IL-6 (−154 to −146), and NF-κB (−72 to −63) sequences have been described elsewhere (26). A luciferase reporter vector containing nucleotides −332 to +35 of the human ISB-α gene promoter was provided by Professor Ron Hay (St. Andrews, UK). The control Renilla luciferase vector pRL-TK was purchased from Promega (Southampton, UK). An expression vector (pHSV-JunD) for dominant negative JunD lacking amino acids 1–162 has been previously described (27). Expression vector pH5.829 for CBF1 was obtained from Dr. Diane Hayward (Baltimore, MD). Dominant negative TRAF2 was produced from an expression vector obtained from Professor David Brenner (Chapel Hill, NC) that generates a TRAF2 protein lacking nucleotides 87–501 which prevents interactions with downstream effectors (32). Dominant negative TRAF6 was overexpressed from a vector provided by Professor Luke O’Neil (33). An expression vector encoding an ISBα super-repressor protein (ISBαD) has been described elsewhere (34). Wild type human CD40 (hCD40) was amplified from Raji cell line cDNA using h40HindIIIF 5′-ccaagcttcacctcgccatggttcgtctgc-3′ and h40XbaIR 5′-tcgccggccgccaaactggatcagctctgagtaa-3′ antisense primer which incorporate HindIII and XbaI restriction enzyme sites to allow for cloning of hCD40 into pCDNA3 expression vector. Human CD40 expression constructs were generated by two-step recombinant PCR. The general method for two-step recombinant PCR was as follows; first step PCR was constructed as two separate reactions. Second step PCR reactions were set up using 10 μl of each of the first step PCR reactions, 2.5 μl of each of the second step antisense primer, 1 μl of dH2O. The second step PCR reaction was set up using 10 μl of each of the first step PCR reactions, 2.5 μl of optimized Pfu polymerase buffer (Promega), 0.4 μM dNTP mixture, and 2 units of Pfu polymerase in a total reaction volume of 25 μl. Reaction was set up in the same manner using h40XbaIR primer and the appropriate antisense primer for the given mutant as listed in Table I. In all reactions we used 1 μg of hCD40wt/pCDNA3 template, 100 ng of h40HindIIIF primer, and 100 ng of appropriate antisense primer, 2.5 μl of optimized Pfu polymerase buffer (Promega), 0.4 mM dNTP mixture, and 2 units of Pfu polymerase in a total reaction volume of 25 μl. Reaction was set up in the same manner using h40XbaIR primer and the appropriate sense primer for the given mutant as listed in Table I.
reaction volume of 25 μl. The PCR program was set up as described for first step reactions, with annealing temperature as shown in Table I for each mutant. A total number of 15 cycles was used, followed by addition of 2% β-Mercaptoethanol and 1% bovine serum albumin. PCR products were digested with 1 U of DpnI per reaction and, after mixing, was incubated for a further 20 min. For supershift assays, reactions were incubated for a further 16 h in the presence of 2 μg of anti p50/p65 or anti-Jun antiserum (Santa Cruz Biotechnology, Inc.) and, after mixing, was incubated for 20 min. For supershift assays, reactions were incubated for a further 16 h in the presence of 2 μg of anti p50/p65 or anti-Jun antiserum (Santa Cruz Biotechnology, Inc.). EMSA and supershift reaction mixtures were then resolved on a 1% agarose gel. Gels were run at a 100 V for 1.5 h prior to transfer onto nitrocellulose as previously described (27). Following blockade of nonspecific protein binding, nitrocellulose blots were incubated for 1 h with primary antibodies (diluted in TBS/Trition 20 (0.075%) containing 3% Marvel and 3% bovine serum albumin. Rabbit polyclonal antibodies recognizing JunD, JunB, and c-Jun (Santa Cruz Biotechnology, Inc.) were used at a final concentration of 1 μg/ml. Blots were then washed three times in TBS/Trition 20 prior to incubation for 1 h with sheep anti-rabbit horseradish peroxidase antibody (1:2000). After extensive washing in TBS/Trition 20 the blots were processed to distilled water for detection of antigen using the ECL system (Amersham Biosciences, Buckinghamshire, United Kingdom).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—mRNA was extracted from 1 × 10^6 FSDC cells which were either left untreated or were treated with 30 μg/ml anti-CD40 mAb 3/23 for 24, 48, and 72 h using the “Quickprep micro mRNA Purification kit” (Amersham Biosciences) as per the manufacturer’s instructions. 5 μg of mRNA obtained was used to generate first strand cDNA using random hexamer primer (dN18) and a first strand cDNA synthesis system (Amersham Biosciences) as per the manufacturer’s instructions. PCR amplification of mouse IL-6, IL-12 (p35 and p40), IL-4, IFNγ, TRAF2, TRAF6, and β-actin cDNAs was carried out using specific oligonucleotide primers selected within the coding regions of the genes. IL-6 primers were 5′-actagctgctggactacaatgc-3′ (sense) and 5′-gccctagaagtggagactg-3′ (antisense) designed to produce a 739-bp product; IL-12p40 primers were 5′-gacaggctggttgtcagga-3′ (sense) and 5′-gcttgctgtgctgaaactg-3′ (antisense) designed to produce a 1007-bp product; IFNγ primers were 5′-gccatgtgctgctgaaactg-3′ (sense) and 5′-ctctgctgagctggtc-3′ (antisense) (antisense) designed to produce a 472-bp product; TRAF6 primers were 5′-gaagaagtctggaagag-3′ (sense) and 5′-caagaagcagctgcatc-3′ (antisense) designed to produce a 500-bp product. PCRs were composed of 1 μl of cDNA template, 100 ng each of sense and antisense oligonucleotide primers, 2.5 μl of optimized TaqPCR buffer (Promega), 0.4 mM dNTP mixture, and 2 units of Taq polymerase in a total reaction volume of 25 μl. Following an initial 5-min incubation at 94 °C, PCRs were performed using 1 min annealing step (at 50 °C for IL-6, 59 °C for IL-12p25, 57.5 °C for IL-12p40, 54 °C for IL-10, 56 °C for IFNγ and IL-4, 55.7 °C for TRAF2, 55.4 °C for TRAF6, and 55 °C for β-actin), followed by a 2-min elongation step at 72 °C, and a 45-s denaturation step at 94 °C. A total number of 27, 30, 35, 35, 35, 35, 35, and 27 PCR cycles were carried out for detection of IL-6, IL-12p25, IL-12p40, IFNγ, IL-4, TRAF2, TRAF6, and β-actin, respectively, followed by a final elongation reaction for 10 min at 72 °C. PCR products were separated by electrophoresis at 80 V for 60 min through a 1% agarose gel and were detected by ethidium bromide staining. Expected sizes of specific PCR products (620, 739, 1007, 488, 400, 472, 768, and 500 bp for IL-6, IL-12p25, IL-12p40, IFNγ, IL-4, TRAF2, TRAF6, and β-actin, respectively) were verified by reference to a 1-kilobase DNA ladder.

CD40-Induced Interleukin-6 Gene Transcription in Dendritic Cells

**RESULTS**

**CD40-stimulated Induction of Cytokine mRNAs in Primary Immature Murine Bone Marrow-Derived DCs and FSDCs**—To circumvent problems associated with heterogeneity of isolated primary DCs and to generate sufficient cell numbers to perform an in-depth analysis of CD40 regulation of IL-6 gene transcription in DCs we used an established cell line. FSDC were originally generated by retroviral-mediated immortalization of DC progenitors from fetal mouse skin and resemble immature BMDC in terms of their morphology, function, and antigenic phenotype (28). Prior to use of FSDC for gene transcription studies we established by fluorescence-activated cell sorter analysis that FSDC express surface CD40 (data not shown). We then determined the relative ability of primary immature murine BMDC and FSDC to express a variety of cytokine transcripts in response to stimulation for 24 h with an anti-mouse CD40 mAb (9/23). Fig. 1 shows RT-PCR analysis of IL-6, IL-12 (p35 and p40), IFNγ, and IL-4 in unstimulated (−) and anti-CD40 stimulated (+) BMDC or FSDC. The data show a similar profile for cytokine mRNA expression in BMDC and FSDC with both cells undergoing a strong induction of IL-6 and both the p35 and p40 subunits of IL-12, by contrast neither of the two cell cultures displayed expression of IFNγ or IL-4. These data suggest that FSDC are a good model for studying the transcriptional induction of IL-6 gene expression by engagement of CD40.

**Requirement for AP1, NF-κB, and NF-IL-6 Sites for CD40 Induction of IL-6 Promoter Activity in FSDC—FSDC were transfected with wild type and mutant IL-6-promoter-luciferase constructs together with a control TK-renilla-luciferase vector to normalize data for differences in transfection efficiency. The mutant IL-6 promoter constructs lacked binding sites for AP1, NF-κB, NF-IL-6, or NF-κB+NF-IL-6 and have...
recently been described elsewhere (26, 27). The IL-6 promoter had a weak basal level of transcription that was induced by 20.5-fold in cells stimulated for 24 h with anti-CD40 (fig 2, A and B). These basal and inducible promoter activities therefore reflect the IL-6 mRNA expression data presented in Fig. 1. Mutation of the NF-κB site in the IL-6 promoter reduced both basal and CD40-induced transcription such that fold induction in response to anti-CD40 was just 6.4-fold (Fig. 2, A and B). Disruption of NF-IL-6 binding to the promoter mainly affected basal transcription, with the induction in response to CD40 at 24.5-fold being higher than for the wild type promoter. However, a double mutation that perturbed both NF-κB and NF-IL-6 binding almost completely abolished the ability of anti-CD40 to induce promoter activity (1.5-fold). These data indicate that NF-κB and NF-IL-6 may act in synergy to regulate CD40 signaling at the IL-6 promoter. Mutagenesis of the AP1 site in the IL-6 promoter had a minor effect on basal transcription but reduced anti-CD40-inducible promoter activity to 11-fold, which is almost a 50% reduction of transcription relative to the wild type promoter. Induction of high level IL-6 gene transcription in FSDC by engagement of surface CD40 therefore requires all three transcription factor binding motifs in the IL-6 promoter.

Induction of NF-κB by Anti-CD40—To confirm a role for NF-κB in the regulation of CD40-inducible IL-6 gene transcription, FSDC were co-transfected with 100 ng of pRLTK and 1 μg of either wild type pIL6-Luc651 or mutated pIL6-Luc651 constructs carrying mutation in the AP1, NF-κB, and NF-IL6 sites. The transfected cultures were split into two flasks. 24 h later, one culture flask was incubated with 30 μg/ml anti-CD40 mAb 3/23 for a further 24 h, while the remaining flask was left untreated. Samples treated with 3/23 are depicted in black solid boxes, whereas untreated samples are in white. Luciferase activities were normalized to pRLTK activity and expressed as the mean ± S.E. of three independent transfection experiments. Statistical analysis was performed by Student’s t test. *, **, and *** denote p < 0.05, 0.01, and 0.005, respectively. B, fold induction of the sample incubated with anti-CD40 mAb 3/23 versus the non-treated sample for each of the pIL6-Luc651 constructs.
IκBD displayed a reduction in both the basal and anti-CD40 induced IL-6 promoter activities relative to cells co-transfected with the control empty vector (fig 3A). When fold induction by anti-CD40 was determined we found that overexpression of IκBD caused a diminution of inducible IL-6 promoter activity from 21.6-fold (control) to 7.7-fold (Fig. 3B). This level of inhibition was similar to the drop in fold-induction observed when the NF-κB site of the IL-6 promoter was disrupted (Fig. 2B).

Activation and DNA binding of NF-κB are therefore required for induction of IL-6 gene transcription in CD40-stimulated FSDC. To determine whether there is a similar requirement in primary DCs we determined the effects of the NF-κB inhibitor MG132 (36) on CD40 induced IL-6 mRNA expression in BDMC. As shown in the representative gel in Fig. 3C inhibition of NF-κB activation by MG132 completely blocked CD40 induced elevation of IL-6 transcript.

We next determined if anti-CD40 treatment of FSDC induced NF-κB DNA binding activities using a double stranded oligonucleotide carrying the NF-κB binding sequence from the IL-6 promoter as an EMSA probe (fig 4A). Unstimulated FSDC (lanes 3, 5, 7, 9, and 11) expressed two NF-κB DNA binding complexes, a weak low mobility complex (complex 2) and a more intense higher mobility interaction (complex 1). Both interactions were found to be sequence specific (data not shown) and were induced following a 24-h exposure of FSDC to anti-CD40 (compare lanes 2 and 3), however, the induction of complex 2 was more impressive than complex 1. For reasons not yet known the mobility of complex 1 was occasionally slightly altered in CD40-stimulated cells. To determine the protein components of the two CD40-inducible NF-κB complexes we performed supershift/antibody interference experiments. Complex 2 was reactive with both anti-p50 (lane 4) and anti-p65 (lane 6) antisera, moreover incubation of pre-formed protein-DNA complexes with a combination of the two antibodies (lane 8) resulted in almost total interference of complex 2. By contrast, complex 1 was not reactive with either the p50 or p65 antisera either alone or in combination. From these data we conclude that the major inducible NF-κB DNA binding activity of anti-CD40 stimulated FSDC is the classic p50:p65 heterodimer. Previous studies have shown that the NF-κB

**Fig. 3. Induction of the IL-6 promoter requires activation of NF-κB.**

A, FSDCs were transfected with 100 ng of pRLTK, 1 μg of wild type pIL6-Luc651, and 2 μg of IκBα trans-dominant negative (IκBD) expression vector or pcDNA3 as control. The transfected cultures were split into two flasks. 24 h after transfection, one flask was incubated with 30 μg/ml anti-CD40 mAb 3/23 for 24 h while the remaining flask was left untreated. Luciferase activity was determined 48 h after transfection. Luciferase activities were normalized to pRLTK activity and expressed as the mean ± S.E. of three independent transfection experiments. Samples treated with 3/23 are depicted in black solid boxes, whereas untreated samples are in white. Statistical analysis was performed by Student's t test. *** denotes p < 0.005. B, fold induction of the samples incubated with anti-CD40 mAb 3/23 versus the non-treated samples as shown in Fig. 3A. C, mRNA was isolated from BMDCs which were either untreated (−) or incubated for 24 h with 30 μg/ml anti-CD40 mAb 3/23 together with either 5 μM MG132 dissolved in MeSO or MeSO alone. The mRNA was used to obtain first strand cDNA, which was used as a template in RT-PCR. cDNA species encoding murine IL-6 and β-actin were amplified over 32 and 27 cycles in RT-PCR reactions using protocols described under Materials and Methods. The gels shown are representative of at least two independent experiments. A 1-kilobase DNA ladder was run alongside the PCR products to confirm correct sizes of the amplified cDNA fragments.
DNA-binding site in the IL-6 promoter overlaps with a sequence that acts as a binding site for the transcriptional repressor CBF1 (also designated RBP-J) (37, 38). Since this overlapping sequence was included in our NF-κB EMSA probe, we therefore investigated the possibility that NF-κB complex 1 may be due to binding of CBF1. As precise nucleotides required for binding of p50:p65 and CBF1 have previously been identified we carried out EMSA using double stranded oligonucleotide probes carrying point mutations that disrupt binding of either p50:p65 or CBF1 (Fig. 4B). Loss of a nucleotide required for p50:p65 binding resulted in disrupted assembly of complex 2 but had no effect on complex 1, this confirms that p50:p65 heterodimers are the protein components of complex 2. In contrast, point mutations that prevent CBF1 binding had no effect on assembly of complex 2 but resulted in a loss of complex 1 formation. Confirmation that CBF1 was the protein component of complex 1 was obtained from supershift/experiments in which we showed that an antisera raised against CBF1 could specifically interfere with complex 1 (Fig. 4C).

**AP1 Is Induced by Anti-CD40 and Is Required for Stimulation of IL-6 Promoter Activity**—As mutagenesis of the AP1 site of the IL-6 promoter resulted in roughly a 50% drop in anti-CD40 stimulated transcription (Fig. 2, A and B) it was of interest to determine whether engagement of surface CD40 induces AP1 DNA binding activity in FSDC. EMSA analysis of protein-DNA complex formation was carried out using a double stranded oligonucleotide probe carrying the AP1-binding site from the IL-6 promoter. Fig. 5A shows that unstimulated FSDC express a weak AP1 DNA binding activity that was strongly induced in cultures treated for 16 h with anti-CD40. Ability of a 100-fold excess of unlabeled AP1 sites to compete for binding and lack of competition by a 100-fold excess of nonspecific (SP1 site) double stranded oligonucleotides confirmed that the AP1 protein-DNA complexes in FSDC were

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**Fig. 4.** EMSA analysis of NF-κB and CBF1 in anti-CD40 stimulated FSDC. **A**, nuclear extracts from control or FSDC cells treated with anti-mCD40 mAb 3/23 for 24 h were isolated and 2 μg used in EMSA with NF-κB double stranded oligonucleotide probe. Two (1 and 2) specific DNA-protein complexes were assembled and are denoted by arrows. Supershift analysis was performed on control and treated samples using antisera recognizing p50 and p65 or JunB as a control. **B**, 2 μg of nuclear extracts from FSDC cells treated with anti-mCD40 mAb 3/23 for 24 h (as obtained in A) were incubated with double stranded NF-κB oligonucleotide probe, or NF-κB probe lacking p50/p65 or CBF1-binding sites. The nucleotide substitutions introduced into the wild type NF-κB oligonucleotide to generate mutant oligonucleotides lacking p50/p65 or CBF1-binding sites are shown below the EMSA gel. **C**, 2 μg of nuclear extracts from FSDC cells treated with anti-mCD40 mAb 3/23 for 24 h (as obtained in A) were incubated with double stranded NF-κB oligonucleotide probe and supershift analysis performed using antisera recognizing p50, p65, CBF1, or JunB as a control.
specific and saturable (Fig. 5A). As transcriptionally active AP1 complexes must contain a Jun (c-Jun, JunB, or JunD) factor either in homodimeric (Jun:Jun) or heterodimeric (Jun in partnership with a Fos family protein) forms, we used antisera recognizing c-Jun, JunD, JunB, c-Fos, or Sp1 as a control. Supershift complexes are shown for extracts incubated with JunB and JunD antisera. Asterisks placed to the left of the supershift complexes are included to aid identification of these species. C, immunoblot analysis of JunD, JunB, and c-Jun protein expression was performed on crude cytoplasmic and nuclear extracts from control and FSDCs treated with 3/23 mAb for 16 h. All gels are representative of three independent experiments. D, FSDCs were transfected with 100 ng of pRLTK, 1 μg of wild type pIL6-Luc651, and 2 μg of JunD dominant negative expression vector pRSV-JunD or pRSV as control. The transfected cultures were split into two flasks and after 24 h one flask was incubated with 30 μg/ml anti-CD40 mAb 3/23 while the remaining flask was left untreated. Luciferase activity was determined 48 h after transfection. Luciferase activities were normalized to pRLTK activity and expressed as the mean ± S.E. of three independent transfection experiments. Samples treated with 3/23 are depicted in black solid boxes, whereas untreated samples are in white. Statistical analysis was performed by Student’s t test. ** denotes p < 0.01.

**FIG. 5. Induction of AP-1 activity by CD40 ligation.** A, nuclear extracts from control or FSDC cells treated with anti-mCD40 mAb 3/23 for 16 h were isolated and 10 μg used in EMSA with AP1 double stranded oligonucleotide probe in presence of 100 nM excess of unlabeled nonspecific oligonucleotide (Sp1) or unlabeled specific AP1 oligonucleotide. B, supershift analysis was performed on 3/23 mAb treated FSDC samples using antisera recognizing c-Jun, JunD, JunB, c-Fos, or Sp1 as a control. Supershift complexes are shown for extracts incubated with JunB and JunD antisera. Asterisks placed to the left of the supershift complexes are included to aid identification of these species. C, immunoblot analysis of JunD, JunB, and c-Jun protein expression was performed on crude cytoplasmic and nuclear extracts from control and FSDCs treated with 3/23 mAb for 16 h. All gels are representative of three independent experiments. D, FSDCs were transfected with 100 ng of pRLTK, 1 μg of wild type pIL6-Luc651, and 2 μg of JunD dominant negative expression vector pRSV-JunD or pRSV as control. The transfected cultures were split into two flasks and after 24 h one flask was incubated with 30 μg/ml anti-CD40 mAb 3/23 while the remaining flask was left untreated. Luciferase activity was determined 48 h after transfection. Luciferase activities were normalized to pRLTK activity and expressed as the mean ± S.E. of three independent transfection experiments. Samples treated with 3/23 are depicted in black solid boxes, whereas untreated samples are in white. Statistical analysis was performed by Student’s t test. ** denotes p < 0.01.

**CD40 Induces Interleukin-6 Gene Transcription in Dendritic Cells**
transfected into FSDC cells along with 100 ng of pRLTK and 2 μg of empty vector pcDNA3 or pcDNA3-derived expression vectors carrying cDNA cassettes for wild type hCD40, truncated hCD40 containing three remaining intracellular residues (hCD40KKV), hCD40 mutants carrying point mutations (hCD40T254A, hCD40T254E, and hCD40T254S), hCD40 with a point mutation and a deletion of carboxyl-terminal 15 amino acids (hCD40T254Δ15), hCD40 with 15 carboxyl-terminal amino acids deleted (hCD40Δ262), or a fusion protein made up of hCD450 extracellular and transmembrane domains and hCD22 intracellular domain. The transfected cultures were split into two flasks, one flask was treated with 300 ng/ml anti-human CD40 mAb LOB7.6 for 24 h and the other was left untreated. 24 h later, cells were harvested and luciferase assays performed. Samples treated with LOB7.6 are depicted in black solid boxes, whereas untreated samples are in white. Luciferase activities were normalized to pRLTK activity and expressed as the mean ± S.E. of five independent transfection experiments.

FIG. 6 Requirement for specific amino acid sequences in the cytoplasmic domain of CD40. A, 1 μg of wild type pH6-651Luc was transfected into FSDC cells along with 100 ng of pRLTK and 2 μg of empty vector pcDNA3 or pcDNA3-derived expression vectors carrying cDNA cassettes for wild type hCD40, truncated hCD40 containing three remaining intracellular residues (hCD40KKV), hCD40 mutants carrying point mutations (hCD40T254A, hCD40T254E, and hCD40T254S), hCD40 with a point mutation and a deletion of carboxyl-terminal 15 amino acids (hCD40T254Δ15), hCD40 with 15 carboxyl-terminal amino acids deleted (hCD40Δ262), or a fusion protein made up of hCD450 extracellular and transmembrane domains and hCD22 intracellular domain. The transfected cultures were split into two flasks, one flask was treated with 300 ng/ml anti-human CD40 mAb LOB7.6 for 24 h and the other was left untreated. 24 h later, cells were harvested and luciferase assays performed. Samples treated with LOB7.6 are depicted in black solid boxes, whereas untreated samples are in white. Luciferase activities were normalized to pRLTK activity and expressed as the mean ± S.E. of five independent transfection experiments.

Antisera confirmed specificity of the results obtained using the Jun and Fos antisera.

As changes in AP1 activity can be regulated by either transcriptional or post-translational events it was of interest to establish if anti-CD40 treatment of FSDC alters the expression of Jun proteins. Fig. 5C shows a Western blot analysis of Jun protein expression in the cytoplasmic and nuclear fractions of unstimulated (−) and anti-CD40 stimulated (+) FSDC. All three Jun factors were expressed in both the cytoplasm and nucleus of FSDC. The cytoplasmic pool of the Jun factors was unchanged upon stimulation with anti-CD40, by contrast nuclear levels of c-Jun were slightly elevated while nuclear JunB expression was strongly induced. Confirmation of the requirement for Jun activity in the IL-6 promoter response to anti-CD40 treatment was obtained by co-transfection of FSDC with the IL-6 promoter and an expression vector (RSV-JunD) for a dominant negative JunD protein. The mutant JunD protein expressed from this vector lacks a functional transactivation domain (27). As the different Jun proteins are able to interact with each other to form functional AP1 binding dimers, over-expression of the dominant negative JunD will sequester c-Jun, JunB, and JunD proteins into functionally inactive dimers. Fig. 5D shows that co-transfection of RSV-JunD was without effect on basal levels of IL-6 promoter activity but profoundly inhibited CD40-induced transcription.

Structural Requirements in the Cytoplasmic Domain of CD40 Required for Induction of IL-6 Promoter Activity—Previous studies in B and T cell lines have determined specific amino acid sequences in the cytoplasmic tail of CD40 that are required for activation of intracellular signaling in response to engagement of the receptor (2, 14–21). To determine the structural features of the CD40 cytoplasmic domain required for induction of IL-6 gene transcription in FSDC we employed the following strategy. A panel of wild type and mutant (carrying deletions or point mutations in the cytoplasmic domain) human CD40 expression vectors were co-transfected into FSDC with the IL-6 promoter-luciferase vector. The transfected culture was then exposed for 24 h to LOB7.6, an anti-CD40 monoclonal antibody that specifically recognizes human CD40 (hCD40) and lacks species cross-reactivity. Fluorescence-activated cell sorter detection of transfected hCD40 was used to confirm that the different expression vectors generated quantitatively similar levels of immunoreactive cell surface hCD40 (data not shown). FSDC transfected with wild type hCD40 displayed a 5.3-fold induction of IL-6 promoter activity in response to LOB7.6 (Fig. 6A). However, in contrast cells either transfected with the promoter alone or with mutant CD40 expression vectors producing proteins that either lacked a cytoplasmic tail (hCD40KKV) or had the cytoplasmic domain from CD22 in place of the CD40 cytoplasmic domain were unable to induce IL-6 transcription in response to LOB7.6. These data show, as expected, that the cytoplasmic domain of CD40 is critical for signaling. Mutation of threonine 254 to a residue that is not a target for phosphorylation (hCD40T254E and hCD40T254A) dramatically reduced LOB7.6 stimulation of IL-6 promoter activity. However, hCD40 carrying a serine substitution at this
residue retained an ability to induce IL-6 promoter activity in response to LOB7.6. Phosphorylated threonine 254 has been reported to be critical for recruitment of TRAF2, -3, and -5 in response to engagement of CD40 (15–18), our data therefore suggest a role for these adaptor proteins in the induction of IL-6 gene transcription. Construct hCD40Δ262 lacks 15 amino acids (262–277) at the COOH-terminal end of hCD40, which includes critical binding sites for TRAF3 (Q263), TRAF5, and Jak3 but is not critical for TRAF2 binding (15, 16, 18, 39). This construct was able to support LOB7.6-induced IL-6 promoter activity albeit at a reduced level (60%) relative to the induction obtained with wild type hCD40. A construct in which both threonine 254 is mutated and the final 15 amino acids are deleted (hCD40T254AΔ262) was as expected unable to support LOB7.6 induction of IL-6 gene transcription. These data suggest that neither TRAF3, TRAF5, nor Jak3 play an essential role in anti-CD40 stimulation of IL-6 gene transcription in FSDC and indicate that TRAF2 is likely to be a major signal transducer of this response.

Lee and colleagues (16) recently reported that TRAF2 mediates the induction of ICAM-1 in CD40 stimulated B cells via activation of NF-κB and Jun-N-terminal kinase 1. To determine whether TRAF2 binding is also required for induction of NF-κB by engagement of hCD40 in FSDC we co-transfected the panel of hCD40 expression vectors together with an IκB-α promoter-luciferase reporter construct. Activity of the IκB-α promoter is critically dependent on binding of NF-κB, forming a negative feedback mechanism for NF-κB activation in cells (40). Measurement of IκB-α promoter activity can therefore be used as a sensitive and specific monitor of NF-κB activation. Overexpression of wild type hCD40, hCD40T254S, and hCD40Δ262 gave rise to an elevation of basal IκB-α promoter activity, all three constructs also supported a strong induction of promoter activity in cells stimulated for 24 h with LOB7.6 (Fig. 6B). By contrast, FSDC transfected with the remaining mutant hCD40 constructs that disrupt TRAF2 binding in addition to TRAF3, TRAF5, and Jak3 displayed low basal levels of IκB-α promoter activity that were not inducible by LOB7.6.

**Inhibition of TRAF2 Signaling Blocks CD40 Activation of IL-6 Gene Transcription—RT-PCR** was used to confirm that FSDC express TRAF2 and TRAF6 transcripts, with no detectable change in expression following anti-CD40 treatment (fig 7A). To help confirm a major role for TRAF2 in anti-CD40 stimulation of IL-6 gene transcription we determined the effects of overexpressing a dominant negative TRAF2 protein on IL-6 promoter activity. Co-transfection of the IL-6 promoter-luciferase reporter construct with an expression vector for a dominant negative TRAF2 resulted in a complete inhibition of anti-CD40 stimulated transcription (Fig. 7B). By contrast overexpression of a dominant negative TRAF6 protein had only a minor effect on the responsiveness of the IL-6 promoter to anti-CD40 treatment (Fig 7C). We were also able to show that dominant negative TRAF2 blocked anti-CD40 induction of IκB-α promoter activity while again dominant TRAF6 had only a minor inhibitory effect (data not shown). These data indicate a critical requirement for TRAF2 mediated signaling events as part of the pathways that stimulate NF-κB activity and IL-6 gene transcription in response to anti-CD40 treatment of FSDC.

**Prolonged Anti-CD40 Stimulation of FSDC Leads to Reduced Basal Expression of IL-6—** In the experiments we have described so far, FSDC were stimulated with anti-CD40 for only 24 h. Since it is possible that DCs may interact with CD40L bearing T cells for longer periods of time it was important to investigate the longevity of the IL-6 response. Fig. 8 shows an RT-PCR analysis of IL-6 mRNA expression in FSDC incubated for 24, 48, and 72 h in the absence and presence of the anti-mouse CD40 monoclonal antibody 3/23. As expected a 24-h stimulation of FSDC with 3/23 gave rise to a strong induction of IL-6 mRNA expression. However, this induction was terminated by 48 h and moreover was accompanied by an almost complete loss of IL-6 transcript relative to its basal level of expression detected in control unstimulated cells. After 72 h stimulation there was a slight but incomplete recovery of basal IL-6 mRNA expression. We conclude that sustained engagement of surface CD40 leads to a repression of IL-6 gene transcription. To establish a mechanism for this response we used EMSA to determine the time-dependent effects of anti-CD40 stimulation on NF-κB DNA binding activities (Fig. 9A). Maximal induction of the p50:p65 complex was observed at 16 h of stimulation after which there was a modest but steady decline in the intensity of this complex. By contrast the CBF1 protein-DNA interaction remained at an elevated level after 48 h of stimulation. The effects of increased CBF1 expression on IL-6 promoter activity was tested by co-transfecting FSDC with the IL-6 promoter-luciferase reporter and an expression vector for CBF1. As shown in Fig. 9B, overexpression of CBF1 dramatically repressed both basal and anti-CD40 induced IL-6 promoter activity. We therefore suggest that decline in activation of p50:p65 NF-κB coupled with prolonged elevation of CBF1 DNA binding activity may contribute to the reduced basal expression of IL-6 in FSDC stimulated with anti-CD40 for periods in excess of 24 h.

**Signaling Events Downstream of CD40 Are Conserved in DC2.4 and FSDC Cell Lines—** Having determined the intracellular signaling events involved in the induction of IL-6 gene transcription in 3/23-stimulated FSDC it was considered important to verify our findings in a second mouse bone marrow-derived DC cell line DC2.4. RT-PCR analysis of IL-6 expression revealed that although DC2.4 expressed a higher basal level of IL-6 mRNA than FSDC there was a significant induction of expression at 24 h of anti-CD40 stimulation that with further stimulation was reduced to below basal levels as observed with FSDC (data not shown). We then determined the role played by NF-κB (Fig. 10A), AP1 (Fig. 10B), TRAF2 (Fig. 10C), TRAF6 (Fig. 10D), and CBF1 (Fig. 10E) in DC2.4. Treatment of DC2.4 with 3/23 for 24 h resulted in a reproducible 2–3-fold induction of IL-6 promoter activity, which is a weaker response than observed for FSDC. However, the effects of attenuating each of the five signaling molecules were similar between DC2.4 and FSDC. Inhibition of TRAF2 and NF-κB resulted in a total inhibition of 3/23 induced transcription, by contrast expression of dominant negative TRAF6 had only a minor inhibitory effect. Expression of dominant negative JunD significantly reduced 3/23 induced transcription although this effect was not as pronounced as that observed in FSDC. Finally overexpression of CBF1 dramatically repressed basal and anti-CD40 inducible IL-6 promoter activity in DC2.4. These data therefore support the generality of our findings in FSDC for mouse DC lines.

**DISCUSSION**

Despite the emergence of DCs as key cellular players in the immune system, the signalling events that regulate DC function are poorly understood. In the present study we have made a detailed analysis of the molecular events that lead to transcriptional induction of IL-6 in DCs stimulated via the CD40 signalling pathway. We have shown that engagement of CD40 on DCs leads to a powerful, but transient induction of IL-6 mRNA expression. This induction was maximal at 24 h after addition of anti-CD40 to cultures and was followed by a rapid diminution of IL-6 transcript to levels that were less than those found in unstimulated cultures. By investigating the structural requirements at both
the CD40 cytoplasmic tail and the IL-6 promoter, together with establishing changes in the activity of specific transcription factors, we have been able to determine the mechanisms underlying these CD40 stimulated responses in DCs.

The IL-6 promoter was weakly active in FSDC, as expected from the low but detectable level of IL-6 transcript found in both FSDC and primary BMDC. Anti-mouse CD40 mAb 3/23 stimulated a powerful induction of IL-6 promoter activity in FSDC, as did anti-human CD40 mAb when added to FSDC transfected with a human CD40 expression construct. These observations contradict an earlier study using the B cell line CH12.LX in which it was shown that while membrane-bound CD40L could induce IL-6 promoter activity and mRNA expression, anti-CD40 mAb could not (14). Another discrepancy between this latter study and ours is the role of NF-κB, the transcription factor was apparently dispensable for CD40 acti-
vation of IL-6 induction in CH12.LX (14), yet was critical for CD40-induction of IL-6 promoter function in FSDC, DC2.4, and primary bone marrow-derived DCs. The precise reasons for these discrepancies are not known, however, it is possible that there are inherent differences in either the phenotype of the cell lines or the quality of the antibodies used in the two studies. Whatever these differences may be, our study clearly demonstrates that engagement of endogenous mouse CD40 or transfected human CD40 by specific antibodies can induce IL-6 promoter activity in FSDC. This induction was dependent on the presence of intact DNA-binding sites for NF-κB and AP-1. Both basal and anti-CD40 inducible activities of the IL-6 promoter were regulated by NF-κB. Furthermore, we were able to show that blockade of the activation of NF-κB by the proteasome inhibitor MG132 resulted in a total inhibition of anti-CD40 induced IL-6 mRNA expression in primary BMDC. By contrast, AP-1 appeared to be mainly required for inducible IL-6 promoter activity, since mutation of the AP-1 site of the IL-6 promoter or overexpression of a dominant negativeJunD protein only had a significant negative effect on the promoter in cells treated with anti-CD40.

Engagement of endogenous CD40 on FSDC by addition of mAb 3/23 resulted in a transient induction of AP-1 DNA binding activity that was maximal after 16 h of stimulation. A single, but diffuse AP-1 DNA-protein complex was induced by 3/23 and consisted of at least three Jun proteins together with at least one Fos family protein, c-Fos. Examination of Jun protein expression revealed that the major alteration in 3/23 stimulated cells was elevated nuclear expression of JunB. Although we did not detect any significant changes in the expression of c-Jun or JunD, the presence of both these proteins in the nucleus of FSDC, together with our ability to detect them in the anti-CD40-induced AP-1 complex indicates that post-translational events probably regulate their activity in FSDC. Of relevance to this idea, it is well established that engagement of CD40 results in the activation of the Jun N-terminal kinases (JNKs), which in turn are able to attenuate c-Jun and JunD activities (2).

![Image](53x480 to 293x645)

**Fig. 8.** Induction of IL-6 gene transcription by anti-CD40 is a transient response. mRNA was isolated from control or FSDCs treated with anti-mCD40 mAb 3/23 for 24, 48, or 72 h. mRNA was used to obtain first strand cDNA which was used as a template in RT-PCR reactions using protocols described under Materials and Methods. Utilizing this method, cDNA species encoding murine IL-6 and β-actin were amplified. The gels shown are representative of at least two independent experiments. A 1-kilobase DNA ladder was run alongside the PCR products to confirm correct sizes of the amplified cDNA fragments.

![Image](51x101 to 553x379)

**Fig. 9.** Prolonged induction of CBF1 DNA binding activity and repression of IL-6 promoter function. A, 2 μg of nuclear extract isolated from FSDC cells treated with anti-mCD40 mAb 3/23 for 0, 2, 4, 6, 16, 24, and 48 h was used in EMSA with NF-κB double stranded oligonucleotide probe. B, FSDCs were transfected with 100 ng of pRLTK, 1 μg of wild type pIL6-Luc651, and 2 μg of CBF1 expression vector pH282 or empty vector pSG5. The cultures were split into two flasks and 24 h after transfection one flask was incubated with 30 μg/ml anti-CD40 mAb 3/23 for 24 h, while the remaining flask was left untreated. The cells were then harvested and luciferase assay performed. Samples treated with 3/23 are depicted in black solid boxes, whereas untreated samples are in white. Luciferase activities were normalized to pRLTK activity and expressed as the mean ± S.E. of three independent transfection experiments. Statistical analysis was performed by Student’s t test. *** denotes p < 0.005.
and Fra1 at either the mRNA or protein level (41–43). However, few studies have investigated CD40 mediated induction of AP-1 activity or Jun and Fos protein family expression in monocytes or DCs. Revy et al. (19) suggested that CD40 stimulation of human monocytes was associated with induction of NF-kB but not AP-1. Hence it is possible that monocytes and DCs differ in their ability to activate AP-1 in response to engagement of CD40, however, confirmation of this idea requires detailed comparative studies that are beyond the scope of the present study. From our data we can conclude that CD40 signaling in FSDC leads to the activation of a variety of Jun (c-Jun, JunB, and JunD) containing AP-1 dimers. We can also conclude that these AP-1 dimers are transcriptionally active and in combination with NF-kB and NF-IL-6 are able to stim-
Activation of NF-κB is a widely documented consequence of CD40 signaling and is known to be required for many CD40-induced changes in gene expression (2). There is also a wide literature concerning the role of TRAFs as mediators of CD40-induced NF-κB activation; however, there is controversy regarding the requirement for specific TRAFs in this response (14–16, 18). Studies in 293 cells have shown that NF-κB and JNK can be activated by overexpression of TRAF2, TRAF5, and TRAF6, by contrast TRAF1, TRAF3, and TRAF4 are unable to activate either NF-κB or JNK when overexpressed (16). Our data suggest that TRAF2 is the critical mediator of anti-CD40 stimulated NF-κB activity and IL-6 gene transcription in FSDC and DC2.4. We were able to rule out an essential role for TRAF3/5 heterodimers and Jak3 signaling by showing that a hCD40 protein lacking the most COOH-terminal 15 amino acids that are critical for binding these proteins (15, 16, 18, 39) had the ability to induce IκB-α and IL-6 promoter activities. By contrast a hCD40 protein carrying a single amino acid substitution T254A (hCD40T254A) that abolishes binding of TRAF2 in addition to TRAF3/5 and Jak3 (16, 18) was unable to induce IκB-α promoter activity. Furthermore, this mutant displayed a markedly reduced stimulation of IL-6 promoter activity in response to anti-CD40 (1.5-fold, compared with a 5-fold response with wild type hCD40). Our data are therefore in agreement with wild type hCD40. We also thank Steve Lyons and Kenneth Rock for their help with obtaining the DC2.4 cell line.

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**CD40 Induces Interleukin-6 Gene Transcription in Dendritic Cells**