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NF-κB Regulates VCAM-1 Expression on Fibroblast-Like Synoviocytes

Ping Li,* Iñaki Sanz, † Regis J. O’Keefe, ‡ and Edward M. Schwarz‡

Expression of VCAM-1 on synovial fibroblasts is a clinical hallmark of rheumatoid arthritis. The interaction of VCAM-1 and its integrin receptor very late Ag-4 is believed to be critically involved in the recruitment and retention of immune cells in the inflamed joints. To study the regulation of VCAM-1 in synovial fibroblasts, fibroblast-like synoviocytes (FLS) were isolated from the knee joints of normal mice and passaged repeatedly to obtain a homogeneous cell population. We have found that VCAM-1 is constitutively expressed on mouse FLS (mFLS) and that its surface expression is further increased after exposure to TNF-α. Nuclear translocation of transcription factor NF-κB including P50/P50 homodimer and P65/P50 heterodimer was activated by TNF-α treatment. In mFLS stably expressing a dominant-negative mutant of the inhibitory protein I-κBα (m-IκB), which does not undergo proteolytic degradation, NF-κB remains in the cytosol and its activation in response to TNF-α is abolished. VCAM-1 protein expression after TNF-α stimulation was blocked in cells expressing the m-IκB. This effect is likely due to the loss of NF-κB-mediated transcription of VCAM-1, because the 5-fold increase in mRNA levels in response to TNF-α is absent in the mutant cells. To confirm these findings, we transfected mFLS with an adenoviral vector containing the m-IκB transgene. VCAM-1 expression was also blocked by m-IκB in this system, whereas cells transduced with a control adenoviral vector remained responsive to TNF-α. These results indicate that NF-κB mediates TNF-α-induced VCAM-1 expression on FLS. *Department of Microbiology and Immunology, †Immunology/Rheumatology Unit, Department of Medicine, and ‡Department of Orthopaedics, University of Rochester Medical Center, Rochester, NY 14642

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; I-κB, inhibitory protein that dissociates from NF-κB; mIκB, dominant-negative mutant I-κBα; mFLS, mouse FLS; EGFP, enhanced green fluorescence protein; MOI, multiplicity of infection.

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Rheumatoid arthritis (RA) has a prevalence of 1–2% and is one of the more common causes of chronic morbidity among people over 65 years of age (1, 2). Disease progression involves the thickening of the synovial lining due to the proliferation of fibroblast-like synoviocytes (FLS) and infiltration by inflammatory cells. This proliferative mass, or pannus, invades and destroys articular cartilage and bone, leading to irreversible destruction of joint structure and function. The established paradigm concerning the cytokine network in RA is that TNF-α is a critical effector in the proinflammatory cytokine cascade (3, 4). Several lines of evidence support this model: 1) TNF-α is expressed at high levels in inflamed synovium (5–7); 2) cultured synoviocytes produce TNF-α for long periods of time (1); 3) addition of anti-TNF-α Abs inhibits the production of other proinflammatory cytokines including IL-1, IL-6, IL-8, and GM-CSF (8); 4) TNF-α can induce joint inflammation and proliferation of FLS (9), trigger cartilage destruction and bone resorption by inducing collagenase synthesis in FLS (10, 11), inhibit proteoglycan synthesis by articular chondrocytes (12, 13), and stimulate bone resorption in vitro (14); 5) TNF-α transgenic mice develop a chronic erosive arthritis (15); 6) virtually all animal models of arthritis are ameliorated by anti-TNF-α (1); and, most importantly, 7) several human clinical trials of anti-TNF-α therapy for RA have shown beneficial results (16, 17). Thus, TNF-α is critically involved in RA pathogenesis.

A second hypothesis has been proposed to explain how the overexpression of TNF-α in joints leads to the infiltration and accumulation of the blood-derived cells seen in RA. In this model, TNF-α up-regulates the expression of adhesion molecules including VCAM-1, ICAM-1, platelet endothelial cell adhesion molecule-1, P-selectin, and E-selectin on the surface of FLS (18–21). These molecules then interact with their cognate receptors on the infiltrating cells, functionally retaining those cells to sustain the inflammatory process. Additionally, it has been shown that these adhesion molecules are also present in soluble forms and further exacerbate disease progression by inducing angiogenesis (22, 23).

Perhaps the best-studied example of this is the interaction of VCAM-1 and very late Ag-4 (24). VCAM-1 is a 90- to 110-kDa glycoprotein that was first identified on the surface of endothelial cells after exposure to inflammatory cytokines such as TNF-α and IL-1β (25). Increased VCAM-1 expression has been demonstrated in the synovial lining from RA patients by immunohistochimical staining (18). FLS derived from this region express VCAM-1 constitutively, and its expression level can be further increased by exposure to TNF-α, IL-1β, IL-4, and IFN-γ (18). In these studies, Firestein and colleagues (18) showed that the α4β1 integrin very late Ag-4 expressed on T cells directly interacts with the cytokine-induced VCAM-1 on FLS and that this interaction is responsible for the increase in T cell binding to FLS after cytokine stimulation.

At present, the precise cellular pathway by which TNF-α induces surface expression of VCAM-1 on FLS is unknown. One such pathway, which is under intense investigation as a target for therapeutic intervention for RA, is the transcription factor NF-κB (26–28). It has been shown that NF-κB activity is up-regulated in joint tissues from RA patients compared with normal or osteoarthritis patients (29) and that inhibiting NF-κB in these cells by...
overexpressing I-κBα (inhibitory protein that binds NF-κB) blocks cytokine and metalloproteinase production (30). Furthermore, studies designed to block NF-κB signaling in a streptococcal cell wall-induced arthritis model in rats with NF-κB oligonucleotide decoys has been shown to inhibit the severity of the joint disease and to prevent reoccurrence (31). Because NF-κB is a global regulator of many genes involved in inflammatory diseases, it is considered as a target with tremendous potential for pharmacological manipulation.

Regulation of VCAM-1 by NF-κB was first implicated in the studies of VCAM-1 promoter in HUVEC, which demonstrate the importance of two adjacent NF-κB binding sites in VCAM-1 promoter responsiveness to TNF-α (32). These findings were cell type specific because the same NF-κB sites failed to provide a response to TNF-α in Jurkat T cells.

To test the hypothesis that VCAM-1 induction by TNF-α in FLS is dependent upon NF-κB activation, we utilized a dominant-negative mutant I-κBα (mIκB), which specifically inhibits NF-κB signaling (33, 34). We demonstrate that in primary FLS, NF-κB signaling is required for the induction of VCAM-1, as evidenced by the fact that five independently isolated stable mIκB transfectants failed to induce VCAM-1 expression in response to TNF-α. Similar results were obtained by transducing normal mouse FLS (mFLS) with a recombinant adeno virus expressing mIκB. Thus, we conclude that TNF-α/NF-κB signaling plays an important role in the regulation of VCAM-1 expression in synovial fibroblasts and that this pathway is a legitimate target for therapeutic intervention.

Materials and Methods

Synoviocyte culture

mFLS were isolated from the knees of five adult CBA × B6 mice as previously described (35). Each mFLS culture was derived from one mouse. After careful removal of the skin and muscle, the tissue of the knee joints was minced, incubated with 1 mg/ml of collagenase (Sigma, St. Louis, MO) in serum-free RPMI 1640 (Life Technologies, Rockville, MD) for 2 h at 37°C, filtered through nylon mesh, and washed extensively. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified 5% CO2 atmosphere. After overnight culture, nonadherent cells were removed, and adherent cells were cultured in DMEM-10% FBS. The cells were passaged by replating at a 1:5 dilution when the cultures reached confluence.

Stable transfection

We generated mFLS cells stably expressing the mIκB by infecting the primary cultures with the L(mIκB)/SN retroviral vector (34) and selecting for G418 (500 μg/ml) resistance over a 2-wk period as done previously (36). After this selection, all of the drug-resistant cells in the plate were analyzed as a pool.

FACS analysis

Flow cytometry was performed essentially as we have previously described (37), using a FACS Calibur cytometer and the Cell Quest plotting program (Becton Dickinson, Franklin Lakes, NJ). Briefly, mFLS (5 × 107) were cultured in DMEM-10% FBS with and without murine TNF-α (Endogen, Woburn, MA). The cells were then harvested in PBS containing 5 mM EDTA at 4°C and stained with biotin-labeled Abs specific for CD11b or VCAM-1, and PE-conjugated streptavidin (PharMingen, San Diego, CA). Isotype-matched mAbs were used as negative controls. A total of 105 cells were analyzed from each sample. The enhanced green fluorescence protein (EGFP) analysis was done without any staining or additional manipulations. Mean fluorescence is equal to (2X/n), where X is the channel value for the nth event. The variable n is the number of events used in the calculation.

Western blotting

mFLS (5 × 106) were grown in 15-cm tissue culture dishes and stimulated for 0, 5, 15, 30, and 60 min with 10 ng/ml of TNF-α. Cytoplasmic and nuclear protein extracts were prepared as previously described (37), and 10 μg of the cytoplasmic extracts was assayed by SDS-PAGE. After transfer to a nylon membrane, the blots were probed with anti-IκB Abs (Santa Cruz Biotechnology, Santa Cruz, CA), HRP-conjugated goat anti-rabbit polyclonal Abs (Amersham, Arlington Heights, IL) were used as secondary Abs. The immune complexes were detected using ECL + (Amersham). To detect mIκB expression in mFLS infected with rAd-CMV-mIκB at multiplicities of infection (MOI) 100, 10, 1.0, 0.1, 0.01, and 0.001, 20 μg of cytoplasmic extracts were used for analysis and detected with ECL.

Gel-shift and supershift assay

Nuclear protein extracts prepared as above were analyzed by EMSA as previously described (38). Briefly, 10 μg of nuclear extracts from each sample were incubated with 1 μg of Poly dl-dC (Pharmacia, Piscataway, NJ) and 2 ng of 32P end-labeled oligonucleotide probe containing the Igκ B binding site for NF-κB (Santa Cruz Biotechnology). The cold oligonucleotide competition control was performed with labeled and unlabeled oligonucleotides at a ratio of 1:25. Supershifts were performed with antisera specific for the NF-κB family members as previously described (37).

Immunoprecipitation-Western blotting

Control and mutant mFLS were cultured in DMEM-10% FBS with and without TNF-α (10 ng/ml; 12 h). Then cells were scraped, washed, and lysed in RIPA buffer (20 mM Tris, 100 mM NaCl, 0.2% Triton X-100, 0.2% Nonidet P-40, and 0.2% deoxycholate) with 0.5% SDS using a 25-gauge syringe as previously described (33). Immunoprecipitation was performed with 150 μg of total cell lysates in 1 ml of RIPA buffer (without SDS), 50 μl of protein A-Sepharose beads, and 1 μg of anti-VCAM-1 Abs (Santa Cruz Biotechnology). After incubation at 4°C overnight, the beads were boiled in 2× SDS sample buffer. The eluted samples were then assayed by Western blotting with the anti-VCAM-1 Abs as used for immunoprecipitation. Peptide competition control was performed with anti-VCAM-1 Ab and a 5-fold (by weight) excess of blocking peptide.

Northern blotting

Total RNA was extracted from control and mutant mFLS after the indicated stimulation using the RNAeasy Kit (Qiagen, Valencia, CA). Ten micrograms of the total RNA was run on a 1.2% agarose gel containing 17.5% formaldehyde and transferred to a Gene Screen Plus membrane (New England Nuclear, Boston, MA). The RNA was UV cross-linked to the membrane. Prehybridization was performed in QuickHyb solution (Stratagene, La Jolla, CA) for 20 min at 68°C. A mouse cDNA probe for VCAM-1 was prepared from a VCAM-1 cDNA clone (AA270461) obtained from American Type Culture Collection (Manassas, VA), and the murine GAPDH probe was provided as a gift of Dr. H. Ruffner (The Salk Institute, La Jolla, CA). The probes were labeled with 32P deoxyctydilate 5′-triphosphate using a random priming kit (Life Technologies) and hybridized to the blots overnight at 68°C. The blot was exposed to X-OMAT AR film (Kodak, Rochester, NY) for autoradiography. The radioactivity was quantitated by Phosphomager using ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Recombinant adenosine production and assay

The rAd-CMV-EGFP and rAd-CMV-mIκB stocks were gifts from Dr. I. Verma (The Salk Institute). These viruses were grown up as we have previously described (39). The viruses were propagated in 293 T cells and purified by ultracentrifugation through a CsCl gradient. Physical particle titers of virus stocks were determined by protein concentration, and the infectious titers were determined by infecting mFLS (5 × 106) at different MOI, incubating overnight, and quantitating the transduced cells 24 h later.

The EGFP expression was assayed by fluorescence microscopy, and the intensity of fluorescence was quantitated with FACS as described above. To analyze mIκB expression, cytoplasmic extracts were assayed by Western blotting as described above.

Results

Constitutive and TNF-α-induced VCAM-1 expression on mFLS

To examine the direct role of TNF-α/NF-κB signaling in the regulation of VCAM-1, we utilized a primary mFLS culture system. Primary cells were isolated by enzymatic digestion of knee joints from normal adult CBA × B6 mice. Initially, two cell populations could be detected by flow cytometry based on size and shape (Fig. 1a), likely representing monocytes and fibroblasts (Fig. 1b). After
nine consecutive passages, a homogeneous cell population characteristic of mFLS was obtained (Fig. 1c) that was 1% CD11b-positive (Fig. 1d). VCAM-1 expression, including TNF-α responsiveness, was determined in this cell population (Fig. 2). Although VCAM-1 was constitutively expressed, its surface levels are significantly increased \((p < 0.05)\) after exposure to 10 ng/ml of TNF-α for 12 h (Fig. 2A). The response was dose- and time-dependent (Fig. 2, B and C) with maximal level detected after stimulation with 100 ng/ml for 12 h. This pattern of VCAM-1 expression was similar to that previously observed in human FLS obtained from RA patients (18). Although other inflammatory cytokines induce VCAM-1 on human FLS, isolated mFLS were unresponsive to 100 ng/ml of IL-1 (data not shown). Interestingly, because constitutive VCAM-1 expression is a unique feature of fibroblast-like synoviocytes, these data further demonstrated that the cells isolated from the mice were indeed synoviocytes.

**Induction of P65/P50 and P50/P50 NF-κB DNA binding activities in mFLS stimulated by TNF-α**

To examine NF-κB response to TNF-α stimulation in mFLS, cytoplasmic and nuclear extracts were prepared (Fig. 3). Western blot analysis with anti-IκBα Abs (Fig. 3A) demonstrated that the cellular IκBα protein was targeted for degradation shortly after TNF-α treatment, with the majority of IκBα protein degraded by 15 min. IκBα protein levels were increased by 30 min and were recovered to basal levels 1 h after stimulation. Nuclear extracts from the same cultures were analyzed by gel-shift with \(^{32}\)P end-labeled oligonucleotides containing a NF-κB consensus sequence to detect NF-κB binding activity (Fig. 3B). Before stimulation, a low level of NF-κB binding activity was detected, which was increased 5 min after TNF-α treatment. The kinetics of NF-κB activation are consistent with rapid IκBα turnover. Supershift assays were performed with Abs to each of the NF-κB protein family members (Fig. 3C). The Ab control lanes contained the same samples used in Fig. 3B, lanes 1 and 3. The lower and upper bands denoted by arrows were different NF-κB DNA binding complexes. In both resting and stimulated mFLS, the lower complex was supershifted only by anti-P50 Abs. While in the stimulated mFLS, the upper one was retarded both by anti-P50 and anti-P65 Abs. These experiments revealed that the predominant NF-κB DNA binding complex in unstimulated mFLS was a P50/P50 homodimer and that after TNF-α treatment this binding activity was enhanced along with the induction of P65/P50 heterodimer.

**Inhibition of TNF-α-mediated VCAM-1 expression on mFLS stably expressing mIκB**

To examine the role of TNF-α/NF-κB signaling in the regulation of VCAM-1 expression, mFLS were infected with a retrovirus containing a dominant-negative IκBα (mIκB) (Fig. 4). The effect of TNF-α on VCAM-1 expression was determined by FACS analysis (Fig. 4A). In resting mFLS, mIκB had little effect on VCAM-1 expression, but in TNF-α-stimulated mFLS, mIκB significantly reduced VCAM-1 expression. These results suggest that TNF-α stimulation induces VCAM-1 expression through NF-κB-dependent mechanisms.

**Figure 1.** Generation of primary mFLS cultures. Primary mFLS were isolated from knee joints of adult mice and passaged as described in Materials and Methods. To characterize their phenotype, the cells were analyzed by FACS without passage (a and b) or after nine consecutive passages (c and d). The relative size and shape of the cells were analyzed by forward and side light scatter (a and c), and surface CD11b expression was detected with anti-CD11b Abs as described in Materials and Methods (b and d).

**Figure 2.** Effect of TNF-α on VCAM-1 expression in mFLS. A, mFLS were incubated in culture media with or without TNF-α (10 ng/ml) for 12 h, and then VCAM-1 surface expression was determined by FACS, as described in Materials Methods, using specific Abs and an irrelevant IgG control as indicated. B, The dose response of VCAM-1 expression was determined 12 h after TNF-α stimulation. C, The time course of VCAM-1 expression was performed by stimulating the cells with TNF-α (10 ng/ml). B and C, Results shown are the average of the mean fluorescence intensity of the stained cells ± SEM \((n = 3; * p < 0.05; Student’s t test)\).
expressing mI-kB, and five pools of stable transformants were independently isolated by G418 selection. A control cell pool was generated with a retrovirus containing an alkaline phosphatase reporter gene. Each transformant represented pools of infected cells to avoid artifacts arising from clonal variation. Inspection of I-kB/NF-kB signaling in the mutant mFLS revealed identical results to what we have observed with other cells (34, 40). In the mI-kB expressing mFLS treated with TNF-α, endogenous I-kBα was degraded, whereas the overproduced mI-kB protein levels were unchanged, resulting in the loss of NF-kB gel shift activity in all five mutant cell pools tested (data not shown). This inhibition was specific for NF-kB because the same extracts did not affect binding to AP-1 or Oct-1 probes (data not shown).

The role of NF-kB signaling in VCAM-1 expression was determined by treating both control and mI-kB-expressing cells with TNF-α. Wild-type and mutant cells were cultured in the presence of 10 ng/ml of TNF-α and analyzed 12 h later for VCAM-1 expression by FACS (Fig. 4, A and B). TNF-α-stimulated VCAM-1 expression was significantly blocked in mutant cells ($p < 0.0001$). As an internal control we found that both wild-type and mutant cell populations constitutively expressed CD29 at a high level and that its surface expression was not altered by retroviral infection or TNF-α treatment (data not shown). Inhibition of NF-kB activity by mI-kB has been shown to sensitize cells to TNF-α-mediated apoptosis (34, 36), but these effects are very modest at 12 h. Although we did observe TNF-α-induced cytotoxicity in the mI-kB transfectants, the cell population gated for VCAM-1 in the FACS analysis was negative for Annexin V staining (data not shown). Thus, it is unlikely that the loss of responsiveness to TNF-α was due to apoptosis of TNF-α-treated mutant clones. Furthermore, the pattern of VCAM-1 expression on control cells expressing alkaline phosphatase was similar to that on wild-type mFLS (Fig. 2). This indicated that random integration of the retroviral vector into the cell genome had no effect on the VCAM-1 expression. Thus, the inhibition of TNF-α-mediated VCAM-1 expression was specific for mI-kB.

To confirm results obtained by FACS, we performed immunoprecipitation-Western blotting to analyze the effect of mI-kB on the total cellular level of VCAM-1 (Fig. 4C). There was a marked increase in VCAM-1 expression in mFLS after TNF-α stimulation, and this induction was predominantly dependent on NF-kB signaling, as shown by the fact that the mutant cells displayed only a slight increase in VCAM-1 expression.

**Regulation of VCAM-1 mRNA levels by NF-kB**

VCAM-1 mRNA expression in response to TNF-α was examined in wild-type and mutant cells to elucidate the regulatory role of NF-kB (Fig. 5). VCAM-1 mRNA levels were increased by 5-fold in control mFLS after 4 h of TNF-α stimulation, whereas only a 1.5-fold increase could be detected in the mutant cells. These findings demonstrate that VCAM-1 mRNA expression is directly regulated by NF-kB.

**Inhibition of VCAM-1 expression on mFLS transduced by rAd-CMV-mI-kB**

To confirm our results with the mutant clones stably expressing mI-kB, mFLS were transduced with recombinant adenoviruses.
The transduction efficiency of mFLS was determined using recombinant adenovirus expressing either EGFP (rAd-CMV-EGFP) or mIκB (rAd-CMV-mIκB) and was analyzed 24 h after infection at various MOI. In the case of Ad-CMV-EGFP, the transduction efficiency was determined by fluorescence microscopy and quantitated by FACS (Fig. 6A). Cytoplasmic extracts from mFLS transduced with rAd-CMV-mIκB at different MOI were assayed by Western blotting with anti-IκBα Abs (Fig. 6B). Both experiments showed that mFLS are amenable to transduction with 100% efficiency occurring at an MOI between 1 and 10 in that a nonlinear increase in target gene product is detected at this MOI. Additionally, we interpret the FACS data from the experiment performed with an MOI of 1 to indicate that these cells can be transduced by more than one virus.

These viruses were then used to determine the effect of the mIκB on VCAM-1 expression in mFLS (Fig. 7). Similarly to our previous experiments, TNF-α-induced VCAM-1 expression was significantly blocked (p < 0.005) by the overexpression of mIκB (MOI = 10), whereas infection by the control virus had no effect on VCAM-1 expression. This result confirms the data obtained with retroviral transformants stably expressing mIκB.

Discussion

Experimental and clinical evidence continues to mount supporting the hypothesis that RA is an inflammatory disease whereby the pathology is both triggered and sustained by the inappropriate expression of cytokines, which orchestrate the destruction of the joint. Proinflammatory cytokines, which are overexpressed in RA joints (7, 41), have been shown to induce a variety of disease genes, including other cytokines, proteases, oxygenases, and adhesion molecules, as well as to stimulate cell proliferation (4, 42). Among those proinflammatory cytokines, TNF-α has received the greatest attention because of its dominance in the pathogenesis of RA (3, 4). The mechanism through which TNF-α mediates the cellular infiltration of the joint and the development of the pannus remains uncertain. A general hypothesis to explain this is that TNF-α stimulation of FLS leads to the up-regulation of adhesion.

FIGURE 4. Inhibition of TNF-α-induced VCAM-1 expression on stable transformants expressing mIκB. A, VCAM-1 expression on control cells and a representative mutant cell (mIκB#3) were analyzed by FACS as described in Fig. 2. The quantitation of VCAM-1 surface expression is shown in B, where the open bars indicate no treatment and filled bars represent mFLS treated with 10 ng/ml of TNF-α. The data represent the average of three independent experiments ± SEM. *, p < 0.0001, as compared with control cells stimulated with TNF-α (Student’s t test). C, VCAM-1 expression in control cells and mIκB#3 analyzed by immunoprecipitation-Western blotting. mFLS were cultured without TNF-α (−) or with 10 ng/ml of TNF-α (+) for 12 h, and VCAM-1 was immunoprecipitated from cell lysates and assayed by Western blotting. The peptide competition control (lane 5) was performed on the same extract as was used in lane 2. These data are representative of three independent experiments.
molecules like VCAM-1 on the surface of these cells. This results in the recruitment and retention of leukocytes in the joint through interactions with cognate integrin receptors (18–21). The inflammatory process is further exacerbated by soluble forms of these adhesion molecules, which have been shown to induce angiogenesis (22, 23). Thus, the cellular pathways involved in the induction of VCAM-1 by TNF-α in FLS become important issues. In this study we explore the possibility that this response is primarily controlled by the transcription factor NF-κB.

Two NF-κB binding sites that are responsive to TNF-α in endothelial cells have been identified in the promoter of VCAM-1 (32). Using a molecular approach, we have selectively inhibited this pathway and examined the role of NF-κB in TNF-α-mediated VCAM-1 expression in mFLS. Primary mFLS constitutively expressed VCAM-1 and responded to TNF-α in a dose- and time-dependent manner (Fig. 2). As previously reported in other fibroblasts (34, 36), TNF-α rapidly induced NF-κB signaling in mFLS, and IκBα degradation was associated with the increase in NF-κB DNA binding activity. IκBα levels dropped immediately after TNF-α stimulation, reaching their lowest levels at 15 min before resynthesis of the protein returns it back to the original level 60 min after stimulation (Fig. 3A). NF-κB DNA binding activity directly correlates with IκBα levels, in that the peak activity is detected 15 min after stimulation and returns to basal levels at 60 min (Fig. 3B). Surprisingly, the major NF-κB complex induced by TNF-α stimulation is a p50 (NF-κB1) homodimer, although a p50/p65 (Rel A) heterodimer is also induced (Fig. 3C). Because IκBα does not efficiently bind to p50 homodimer, it is likely that this inducible NF-κB complex is the result of p105 precursor processing (43–45). Collectively, the data suggest the importance of NF-κB in TNF-α-mediated expression of VCAM-1.

**FIGURE 5.** Effect of mIκB on VCAM-1 mRNA expression in mFLS. mFLS were stimulated with TNF-α (10 ng/ml) for the time indicated, and total RNA was extracted and analyzed by Northern blotting as described in Materials and Methods. The blot was then rehybridized with probes specific for GAPDH as a loading control.

**FIGURE 6.** Transduction efficiency of mFLS with recombinant adenovirus. A, mFLS were infected with control rAd-CMV-EGFP at different MOI as indicated. EGFP expression was assayed by fluorescence microscopy and FACS. The two peaks in the histogram of the MOI = 1 sample represent mFLS infected with one (arrow) or multiple (arrowhead) recombinant viruses. B, Western blotting of protein extracts from mFLS infected with rAd-CMV-mIκB was performed as described in Materials and Methods.
Consistent with these findings, inhibition of NF-κB blocked TNF-α-mediated effects. After infection with the ml-κB retrovirus and selection for G418 resistance, pools of mFLS defective in NF-κB signaling were readily obtained and analyzed for VCAM-1 expression by FACS and immunoprecipitation-Western blotting. Although ml-κB had virtually no effect on constitutive VCAM-1 expression, the response to TNF-α was significantly inhibited (p < 0.0001; Fig. 4). To show that these effects are likely due to the loss of NF-κB-mediated transcription in the ml-κB-expressing mFLS, we demonstrated that the 5-fold increase in VCAM-1 mRNA levels 1 h after TNF-α stimulation is lost in the mutant cells (Fig. 5). The finding that VCAM-1 mRNA levels remain elevated for several hours after induction is indicative of the stability of this transcript and offers an explanation of how surface levels of VCAM-1 peak 12 h after TNF-α stimulation, assuming that continuous translation of this mRNA occurs.

To confirm that these findings were not a result of clonal selection, we utilized recombinant adenovirus vectors. Using an MOI that was sufficient to transduce the entire culture (Fig. 6), we again found evidence that NF-κB signaling is required for VCAM-1 induction in mFLS after TNF-α stimulation (Fig. 7). The loss of VCAM-1 expression in the rAd-CMV-mlkB-infected cells was not due to effects of the viral vector because no expression was lost in the rAd-CMV-EGFP-infected cells. Recently, Miagkov et al. (31) used a similar I-κB adenoviral vector to block arthritis in a rat model. They concluded that the mechanism by which the disease was inhibited was via apoptosis of the synovial cells. Based on our findings here, it is likely that at least some of the anti-inflammatory effects of these vectors are mediated by their ability to inhibit NF-κB signaling.

**FIGURE 7.** Inhibition of VCAM-1 expression in mFLS transduced by rAd-CMV-mlkB. Uninfected mFLS (no virus), mFLS infected with control rAd-CMV-EGFP, or rAd-CMV-mlkB at MOI = 10 were incubated for 24 h to recover from the infection and incubated with and without TNF-α (10 ng/ml) for 12 h. VCAM-1 expression was then analyzed by FACS as described in Fig. 2. A, The broken histogram indicates nontreated mFLS, and the solid histogram represents mFLS stimulated with TNF-α. B, VCAM-1 expression was quantitated and presented as an average of three independent experiments ± SEM, where the open bars represent no TNF-α treatment and the filled bars represent mFLS incubated with TNF-α. *p < 0.005, as compared with rAd-CMV-EGFP-infected cells stimulated with TNF-α (Student’s t test).
effects seen in those animals were due to the inhibition of VCAM-1 expression.

In conclusion, these experiments provide the first evidence that NF-κB directly mediates the induction of VCAM-1 in mFLS by TNF-α. These findings provide future rationale for the use of pharmacological agents or gene delivery systems that inhibit NF-κB as treatments for RA.

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