

# Heterogeneous Requirement of I $\kappa$ B Kinase 2 for Inflammatory Cytokine and Matrix Metalloproteinase Production in Rheumatoid Arthritis

## Implications for Therapy

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**Objective.** To investigate the potential role of I $\kappa$ B kinase 1 (IKK-1) and IKK-2 in the regulation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation and the expression of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), as well as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), in rheumatoid arthritis (RA).

**Methods.** Recombinant adenoviruses expressing  $\beta$ -galactosidase, dominant-negative IKK-1 and IKK-2, or I $\kappa$ B $\alpha$  were used to infect ex vivo RA synovial membrane cultures and synovial fibroblasts obtained from patients with RA undergoing joint replacement surgery, or human dermal fibroblasts, human umbilical vein endothelial cells (HUVECs), and monocyte-derived macrophages from healthy volunteers. Then, their effect on the spontaneous or stimulus-induced release of inflammatory cytokines, VEGF, and MMPs from RA synovial membrane cells was examined.

**Results.** IKK-2 was not required for lipopolysaccharide (LPS)-induced NF- $\kappa$ B activation or TNF $\alpha$ , IL-6, or IL-8 production in macrophages, but was essential for this process in response to CD40 ligand,

TNF $\alpha$ , and IL-1. In synovial fibroblasts, dermal fibroblasts, and HUVECs, IKK-2 was also required for LPS-induced NF- $\kappa$ B activation and IL-6 or IL-8 production. In RA synovial membrane cells, IKK-2 inhibition had no effect on spontaneous TNF $\alpha$  production but significantly reduced IL-1 $\beta$ , IL-6, IL-8, VEGF, and MMPs 1, 2, 3, and 13.

**Conclusion.** Our study demonstrates that IKK-2 is not essential for TNF $\alpha$  production in RA. However, because IKK-2 regulates the expression of other inflammatory cytokines (IL-1 $\beta$ , IL-6, and IL-8), VEGF, and MMPs 1, 2, 3, and 13, which are involved in the inflammatory, angiogenic, and destructive processes in the RA joint, it may still be a good therapeutic target.

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) plays a central role in the pathogenesis of rheumatoid arthritis (RA) because it is at the apex of inflammatory and destructive processes that operate in the joint. Clinical trials using anti-TNF $\alpha$  blocking biologic agents have demonstrated that TNF $\alpha$  regulates the expression of inflammatory cytokines and chemokines, adhesion molecules, and thus, leukocyte trafficking in the joints, matrix metalloproteinases (MMPs), and joint destruction, as well as vascular endothelial growth factor (VEGF) and angiogenesis (1–5).

Three anti-TNF $\alpha$  blocking agents based on TNF $\alpha$ -neutralizing monoclonal antibodies or soluble TNF receptors (infliximab, adalimumab, and etanercept) have now been approved for human use and many more are under development, but they have some major disadvantages, including their high cost and the inconvenient administration route that requires repeated in-

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jections (6–8). Thus, research is currently under way to look for better means to block TNF $\alpha$  expression by designing small molecule inhibitors. This requires the determination of what regulates or is rate-limiting for TNF $\alpha$  expression in human macrophages, the major cell type producing TNF $\alpha$  in the RA joint. TNF $\alpha$  gene expression is under complex control, with the p38 mitogen-activated protein kinase pathway controlling translation possibly by actions on the 3'-untranslated region (9), and with the 5'-untranslated/promoter region containing binding sites for multiple transcription factors, including nuclear factor  $\kappa$ B (NF- $\kappa$ B), activator protein 1, nuclear factor for the interleukin-6 (IL-6) gene, and nuclear factor of activated T cells (10–12). NF- $\kappa$ B has recently attracted particular attention because of its ability to regulate macrophage TNF $\alpha$  production induced in response to lipopolysaccharide (LPS), ultraviolet light, phorbol myristate acetate, or contact with cytokine-activated T cells (13,14).

NF- $\kappa$ B has been detected by immunohistology in human synovial tissue from RA patients during both early and later stages of disease and in both macrophage- and fibroblast-like synoviocytes (15,16). In particular, macrophage-like synoviocytes that localize in the synovial lining layer and the vascular endothelium have been shown to contain p65 and p50 NF- $\kappa$ B subunits in their nucleus (16). This NF- $\kappa$ B activation is of functional relevance, since we recently showed that it regulates the expression of TNF $\alpha$  as well as other proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, IL-8, MMP-1 (collagenase 1) and MMP-3 (stromelysin 1), without major effects on the expression of antiinflammatory cytokines IL-10, IL-11, and IL-1 receptor antagonist, soluble TNF receptors, or tissue inhibitor of metalloproteinases 1 (17) in ex vivo synovial membrane cultures. In streptococcal cell wall- and pristane-induced arthritis, inhibition of NF- $\kappa$ B through the administration of  $\kappa$ B decoy oligonucleotides or proteasome inhibitors has also been shown to be beneficial (18,19).

However, because NF- $\kappa$ B is involved in normal immune and homeostatic processes, such as the prevention of apoptosis in certain tissues (e.g., liver) (20–22), its prolonged inhibition may have hazards and is unlikely to be a direct therapeutic target. Much more likely and more therapeutically attractive would be the selective inhibition of upstream molecules such as I $\kappa$ B kinase 2 (IKK-2), a kinase previously shown to be essential for TNF $\alpha$ - and IL-1-induced I $\kappa$ B $\alpha$  degradation, NF- $\kappa$ B activation, and cytokine expression (21–28). One study recently demonstrated that IKK-2 is an important kinase required for TNF $\alpha$ - or IL-1-induced NF- $\kappa$ B activation

and expression of IL-6, IL-8, intercellular adhesion molecule 1, and MMP-1 in passaged synoviocytes (29), although the significance of these observations in terms of the treatment of RA is not clear. Passaged synoviocytes obtained from patients with RA do not produce TNF $\alpha$ , are not representative of the rheumatoid synovium because the process of growing them selects for the fibroblast-like cells that probably represent ~10% of the total synovium, and require in vitro stimulation to produce many of the inflammatory mediators seen in vivo.

Another study performed in the rat adjuvant-induced arthritis model demonstrated significant benefit after intraarticular injection of an adenoviral construct encoding a dominant-negative form of IKK-2 (IKK2dn) after disease onset that correlated with a decrease in NF- $\kappa$ B DNA-binding in the nucleus of synovial cells (30). However, that study did not provide the potential mechanisms involved. Moreover, rat adjuvant-induced arthritis is distinguished from RA by its lack of chronicity (31). This suggests differences in disease pathogenesis and makes it difficult to extrapolate observations made in rat adjuvant-induced arthritis to the human situation. Thus, there is a need to further study the role of IKK-2 in systems that more closely resemble human RA.

In this study, we examined the involvement of IKK-2 and IKK-1 in NF- $\kappa$ B activation and TNF $\alpha$  production in primary human macrophages, the main cell type producing TNF $\alpha$  in the RA joint, and in ex vivo RA synovial cultures that consist of the entire population of cells found in vivo (i.e., ~30% T cells, 30–40% macrophages, and fewer fibroblasts, endothelial cells, dendritic cells, plasma cells, and B lymphocytes) (32) and that, in the absence of extrinsic stimulation, spontaneously produce the same inflammatory mediators seen in vivo. We then extended our studies to other primary human cells, such as RA synovial fibroblasts, normal dermal fibroblasts, and human umbilical vein endothelial cells (HUVECs), as well as other molecules involved in inflammatory, angiogenic, or destructive processes in RA, such as IL-1 $\beta$ , IL-6, IL-8, VEGF, and MMPs 1, 3, 9, and 13 (collagenase 3). Our results are the first to demonstrate that IKK-2 is not universally required for TNF $\alpha$  production in the RA synovium, although it is required for the production of other inflammatory cytokines (IL-1 $\beta$ , IL-6, and IL-8), VEGF, or MMPs. This has important implications for the targeting of this kinase in RA.

## MATERIALS AND METHODS

**Reagents.** Human recombinant macrophage colony-stimulating factor (M-CSF), IL-1, and TNF $\alpha$  were gifts from the Genetics Institute (Cambridge, MA), Roche Laboratories (Nutley, NJ), and the Centre of Molecular and Macromolecular Studies (Lodz, Poland), respectively. Soluble noncovalent trimeric CD40 ligand (CD40L) was purchased from Pepro-Tech (London, UK), and *Escherichia coli* LPS and lipid A were obtained from Sigma (Poole, UK). CD40L-transfected and control mouse fibroblast cell lines were obtained from Professor D. Gray (University of Edinburgh, Edinburgh, UK) and Professor A. Schimpl (University of Würzburg, Würzburg, Germany).

**Cells, specimens, and culture.** Primary human dermal fibroblasts, HUVECs, peripheral blood monocytes, RA synovial membrane cells, and rheumatoid synovial fibroblasts from patients undergoing joint replacement surgery were isolated as previously described (32–38). Macrophages were derived from monocytes after differentiation for 2–3 days with 100 ng/ml of M-CSF (35). All cells were cultured in RPMI 1640 containing 5% (volume/volume) fetal bovine serum (FBS) and 100 units/ml of penicillin/streptomycin, except for HUVECs, which were cultured in RPMI 1640 containing 10% (v/v) FBS, 10% (v/v) newborn calf serum, 100 units/ml of penicillin/streptomycin, and 50 units/ml of heparin.

**Adenovirus vectors and their propagation.** Ad0, Ad $\beta$ -gal, AdGFP, and AdIKK1dn were kind gifts of Dr. A. Byrnes (Oxford University, Oxford, UK), Quantum Biotech (Carlsbad, CA), and Dr. M. Karin (University of California, San Diego), respectively. AdIKK2dn and I $\kappa$ B $\alpha$  were produced by us (39). All viruses were E1/E3-deleted, belonged to the Ad5 serotype, and had been previously used in other studies (39–43). Viruses were propagated in HEK 293 cells (American Type Culture Collection, Manassas, VA), purified by ultracentrifugation through 2 CsCl gradients, and viral titers were determined by plaque assay as previously described (44).

**Adenoviral infection of cells.** Dermal fibroblasts, HUVECs, macrophages, and rheumatoid membrane cell cultures were infected in serum-free medium with adenoviruses at a multiplicity of infection (MOI) of 100. After 2 hours, the adenovirus-containing medium was removed and complete medium was added. For passaged synovial fibroblasts, an MOI of 500 was used because these cells are more resistant to adenovirus infection. These MOIs have been previously shown by us and others to result in >90% of these cells expressing the transgene of interest (17,35,41,42,45).

**Western blotting and electrophoretic mobility shift assay (EMSA).** Two days after infection, dermal fibroblasts, synovial fibroblasts, HUVECs, or macrophages were stimulated with either the vehicle control, 20 ng/ml of TNF $\alpha$ , 20 ng/ml of IL-1, 30  $\mu$ g/ml of CD40L, or 1  $\mu$ g/ml (10 ng/ml for macrophages) of LPS for 45 minutes, and cytosolic and nuclear extracts were prepared as previously described (46). For rheumatoid membrane cell cultures, cytosolic and nuclear extracts were collected in the absence of further stimulation. Cytosolic proteins were subsequently separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% (weight/volume) polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane for Western blotting. Antibodies for IKK-2 and I $\kappa$ B $\alpha$  were purchased from Santa

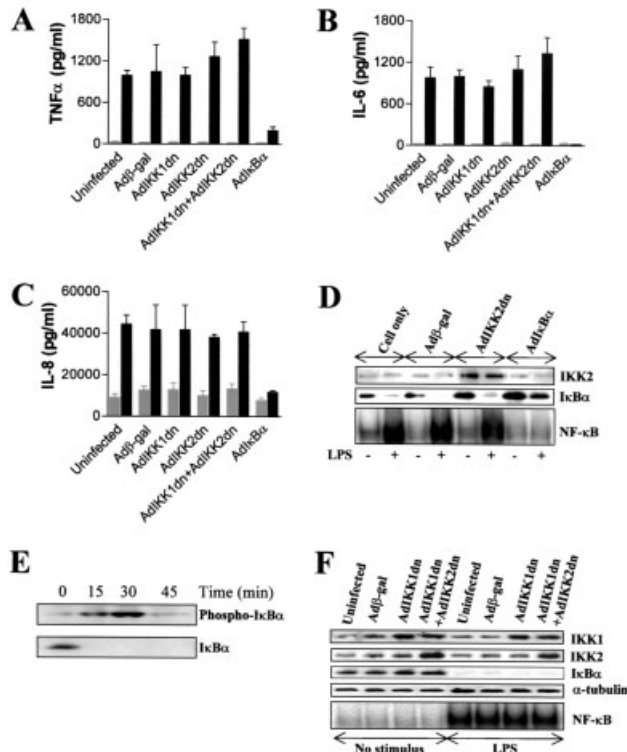
Cruz Biotechnology (Santa Cruz, CA). The antibody recognizing Ser<sup>32–36</sup> phosphorylated I $\kappa$ B $\alpha$  was purchased from Cell Signaling Technology (Hitchin, UK). Nuclear extracts (10  $\mu$ g) were examined for NF- $\kappa$ B DNA-binding activity using EMSA, as previously described (47).

**Analysis of cytokines.** Cells were plated in 96- and 24-well tissue culture plates (Falcon, Oxford, UK) and were left uninfected or were infected with adenovirus. Two days after infection, cells were stimulated for another 24 hours with 20 ng/ml of TNF $\alpha$ , 20 ng/ml of IL-1, 30  $\mu$ g/ml of soluble CD40L, and 1  $\mu$ g/ml of LPS (10 ng/ml for macrophages), and supernatants were collected after 24 hours. In preliminary experiments, these concentrations induced maximal cytokine production in the cell types of interest (data not shown). In some cases, macrophages were cocultured with transfected mouse fibroblasts at a 1:1 ratio for 24 hours. For rheumatoid membrane cell cultures, supernatants were collected after 24–48 hours in the absence of further stimulation. Supernatants were then analyzed for TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 by enzyme-linked immunosorbent assay (ELISA) (PharMingen, Oxford, UK). Absorbance was examined on a spectrophotometric ELISA plate reader (Multiscan Biochromic; Lab-systems, Helsinki, Finland) and analyzed using a DeltaSoft II.4 software program (DeltaSoft, Princeton, NJ). In all cases, viability of the cells was not significantly affected over this time period when examined by the MTT assay (Sigma) (48).

**Statistical analysis.** Mean, SD, and SEM values and statistical significance were calculated using GraphPad version 3 (GraphPad Software, San Diego, CA). For statistical analysis of parametric data, a repeated-measures analysis of variance test with Dunnett's post-test was used.

## RESULTS

**Dispensable role of IKK-2 for LPS-induced NF- $\kappa$ B activation or TNF $\alpha$  production in primary human macrophages.** Although most of the TNF $\alpha$  in the rheumatoid synovium is produced by cells of the macrophage lineage, there are as yet no studies on the role of IKKs in that process, mainly because of the difficulty of transfecting primary monocyte/macrophages with DNA (49). Using recombinant, replication-deficient adenoviruses that efficiently infect primary human macrophages (50), it was possible in this study to examine the role of IKK-2 in TNF $\alpha$  cytokine production. Unexpectedly, we found that AdIKK2dn did not significantly inhibit LPS-induced TNF $\alpha$  production, although AdI $\kappa$ B $\alpha$  did (Figure 1A). Similarly, AdIKK2dn had no effect on LPS-induced IL-6 or IL-8 production (Figures 1B and C). There was also no effect of IKK2dn expression on I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation as examined by EMSA (Figure 1D). Using an antibody that specifically recognizes I $\kappa$ B $\alpha$  phosphorylated in both Ser<sup>32</sup> and Ser<sup>36</sup>, we confirmed that LPS induced I $\kappa$ B $\alpha$  phosphorylation at these residues (Figure 1E). The same results were also obtained when we repeated the experiments with lipid A



**Figure 1.** Role of dominant-negative form of I $\kappa$ B kinase 2 (IKK2dn) in lipopolysaccharide (LPS)-induced nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) production in human macrophages. Macrophage colony-stimulating factor-differentiated macrophages were left uninfected or were infected with adenoviruses encoding  $\beta$ -galactosidase, AdIKK1dn, AdIKK2dn, AdIKK1dn plus AdIKK2dn, or AdI $\kappa$ B $\alpha$ . **A–C**, Cells were stimulated with vehicle control (shaded bars) or 10 ng/ml LPS (solid bars) for 24 hours, and supernatants were collected and examined for the presence of TNF $\alpha$ , interleukin-6 (IL-6), and IL-8 by enzyme-linked immunosorbent assay. Values are the mean and SD cytokine production in triplicate cultures and are representative of 11 independent donors. **D** and **F**, Cells were stimulated with 10 ng/ml of LPS for 45 minutes, and cytosolic or nuclear extracts were obtained and examined for the presence of IKK-1, IKK-2, I $\kappa$ B $\alpha$ , phosphorylated I $\kappa$ B $\alpha$ , or  $\alpha$ -tubulin by Western blotting and for NF- $\kappa$ B DNA-binding activity by electrophoretic mobility shift assay. **E**, Cells were stimulated with 10 ng/ml of LPS and, at 0, 15, 30, and 45 minutes, cytosolic and nuclear extracts were obtained and examined for the presence of I $\kappa$ B $\alpha$  and phosphorylated I $\kappa$ B $\alpha$  (phospho-I $\kappa$ B $\alpha$ ) by Western blotting.

as an alternative Toll-like receptor 4 (TLR-4) ligand (data not shown).

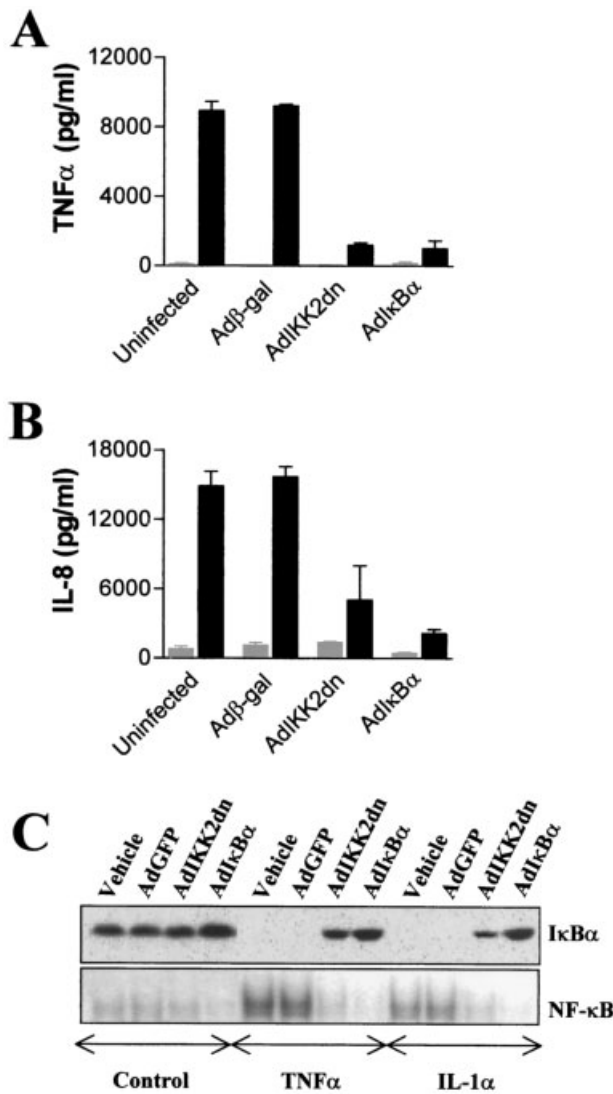
The intriguing observation that LPS induced NF- $\kappa$ B activation and cytokine production in an IKK-2-independent manner in primary human macrophages raised the question of whether IKK-1 was used instead. To address that, we used adenoviral infection to express

IKK1dn or both IKK1dn and IKK2dn in macrophages (Figure 1F). AdIKK1dn, either alone or in combination with AdIKK2dn, had no effect on LPS-induced TNF $\alpha$ , IL-6, or IL-8 production or on I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation (Figures 1A–C and F). These results suggested that in primary human macrophages, TNF $\alpha$ , IL-6, or IL-8 production or I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation in response to LPS can be induced in an IKK-1- or IKK-2-independent manner.

**Stimulus dependence of IKK-2 usage for NF- $\kappa$ B activation and TNF $\alpha$  or other cytokine production.** The observation that IKK-2 is not required for NF- $\kappa$ B activation and cytokine production in macrophages in response to LPS was against the conventional wisdom that inflammatory stimuli activating NF- $\kappa$ B converge at the level of IKK-2 (28). Thus, we tried to determine why there was that paradox. It was possible that IKK2dn was not functioning as a dominant-negative in human macrophages or simply that IKK-2 usage was specific for particular stimuli and not others or was specific for particular cell types. Thus, we examined whether IKK-2 was involved in TNF $\alpha$ , IL-6 or IL-8 production, and/or NF- $\kappa$ B activation in primary human macrophages in response to other inflammatory stimuli, such as CD40L, IL-1, or TNF $\alpha$ . Stimulation of CD40 on human macrophages was achieved using mouse fibroblasts that had been stably transfected with a plasmid encoding CD40L (51). Mouse fibroblasts transfected with an empty plasmid were used as a control (51) and induced no cytokine production (Figure 2A). We found that AdIKK2dn or AdI $\kappa$ B $\alpha$  potently inhibited CD40L-induced TNF $\alpha$  (Figure 2A), IL-6, and IL-8 production (data not shown) in macrophages. CD40L-induced NF- $\kappa$ B activation examined by EMSA was also inhibited (data not shown). Infection with Ad $\beta$ -gal had no significant effect on CD40 signaling (Figure 2A).

We also found that AdIKK2dn blocked I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B DNA-binding activity in response to IL-1 or TNF $\alpha$  (Figure 2C). Neither IL-1 nor TNF $\alpha$  strongly induced cytokine production in human macrophages. However, it was possible to observe that IL-1-induced IL-8 production was markedly inhibited by IKK2dn (Figure 2B) and AdI $\kappa$ B $\alpha$ , but not Ad $\beta$ -gal (Figures 2A and B). These data demonstrate that IKK2dn is a functional inhibitor and that IKK-2 usage is stimulus-specific in this cell type and is essential for CD40L-, TNF $\alpha$ -, and IL-1-induced, but not LPS-induced, NF- $\kappa$ B activation and cytokine production.

**Cell type dependence of IKK-2 usage for NF- $\kappa$ B activation and cytokine production.** We next investigated the role of IKK-2 in inflammatory cytokine pro-

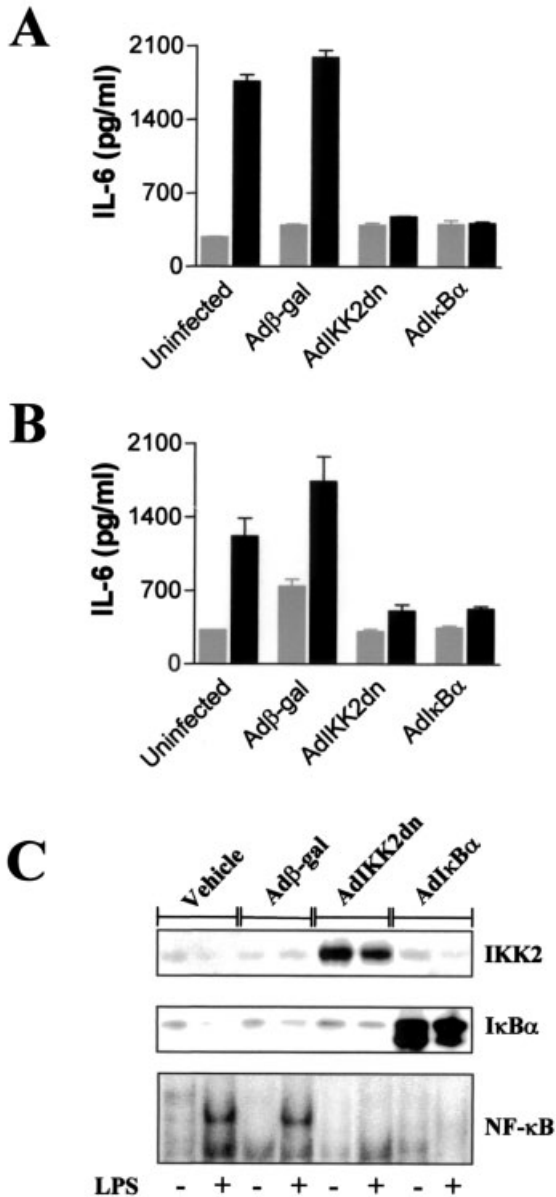


**Figure 2.** Inhibition of IL-1 $\alpha$ -, TNF $\alpha$ -, and CD40 ligand (CD40L)-induced NF- $\kappa$ B activation and cytokine production in human macrophages by IKK2dn. Cells were left uninfected or were infected with Ad $\beta$ -gal, AdIKK2dn, and AdI $\kappa$ B $\alpha$ , and were cultured for another 24 hours. **A**, Cells were stimulated with vehicle control (shaded bars) or 10 ng/ml of LPS (solid bars) for 24 hours, and supernatants were collected and examined for the presence of TNF $\alpha$  by enzyme-linked immunosorbent assay (ELISA). Values are the mean and SD cytokine production in triplicate cultures and are representative of 3 independent donors. **B**, Cells were stimulated with vehicle control (shaded bars) or 20 ng/ml of IL-1 $\alpha$  (solid bars) for 24 hours, and supernatants were collected and examined for the presence of IL-8 by ELISA. Values are the mean and SD cytokine production in triplicate cultures and are representative of 3 independent donors. **C**, Cells were stimulated with vehicle control, 20 ng/ml of IL-1 $\alpha$ , or 20 ng/ml of TNF $\alpha$  for 45 minutes. Cytosolic and nuclear extracts were then obtained and examined for the presence of I $\kappa$ B $\alpha$  by Western blotting, and NF- $\kappa$ B DNA-binding activity was determined by electrophoretic mobility shift assay. See Figure 1 for other definitions.

duction and NF- $\kappa$ B activation in other nonmyeloid cell types to determine whether IKK-2 usage was also cell-type specific. First, we studied RA synovial fibroblasts, which do not make TNF $\alpha$  protein in response to stimulation. Synovial fibroblasts were obtained through the expansion of fibroblasts present in ex vivo RA synovial membrane cultures. As in macrophages, AdIKK2dn and AdI $\kappa$ B $\alpha$  blocked TNF $\alpha$ - and IL-1-induced IL-6 or IL-8 production in synovial fibroblasts (data not shown). However, in contrast to human macrophages, AdIKK2dn and AdI $\kappa$ B $\alpha$  also inhibited LPS-induced IL-6 (Figure 3A) or IL-8 production (data not shown) in these cells. Similar data were obtained in dermal fibroblasts derived from healthy volunteers (data not shown).

We also examined the role of IKK-2 in the activation of endothelial cells. We found that AdIKK2dn and AdI $\kappa$ B $\alpha$  inhibited TNF $\alpha$ - and IL-1-induced IL-6 and IL-8 production in HUVECs, as expected (data not shown). In addition, AdIKK2dn and AdI $\kappa$ B $\alpha$  inhibited LPS-induced IL-6 (Figure 3B) or IL-8 production (data not shown) and LPS-induced I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation (Figure 3C) in these cells. These observations demonstrated that IKK-2 usage is also cell-type specific and essential for LPS-induced IL-6 and IL-8 production, as well as NF- $\kappa$ B activation in primary fibroblasts and endothelial cells. Although fibroblasts and endothelial cells do not produce TNF $\alpha$ , IKK-2 inhibition may be beneficial in other pathologic aspects of RA that involve fibroblast proliferation and activation of the endothelium, which increases leukocyte trafficking to the inflamed joints.

**Necessity of IKK-2 for IL-1 $\beta$ , IL-6, IL-8, and VEGF, but not TNF $\alpha$ , production.** Studies in different human primary cells suggest that there is heterogeneity in the requirement of IKK-2 for NF- $\kappa$ B activation and, more importantly, TNF $\alpha$ , IL-6, or IL-8 production that depends on the cell type and stimulus used. Because the stimulus leading to the expression of molecules involved in inflammatory and destructive processes in RA is not known, we decided to examine whether IKK-2 is involved in TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 production in ex vivo short-term RA synovial membrane cultures. Again, we used recombinant adenoviruses because we have previously shown that this method results in >90% of synovial macrophages, fibroblasts, and T cells expressing the transgene of interest (17). We found that AdGFP infected almost all cells present in RA synovial membrane cultures (Figure 4A), and AdIKK2dn or AdI $\kappa$ B $\alpha$  (used as a positive control) resulted in

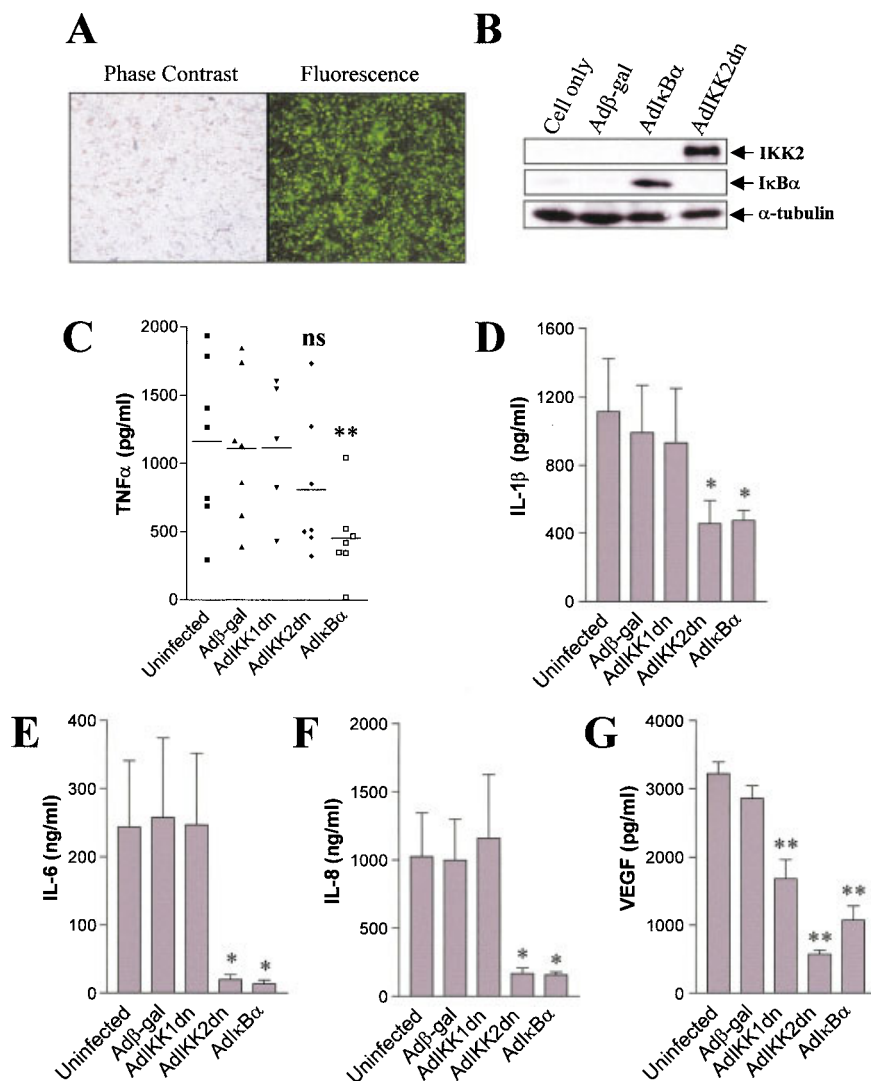


**Figure 3.** Inhibition of LPS-induced NF- $\kappa$ B activation in human umbilical vein endothelial cells (HUVECs) and synovial fibroblasts by IKK2dn. HUVECs and synovial fibroblasts were left uninfected or were infected with Ad $\beta$ -gal, AdIKK2dn, or AdI $\kappa$ B $\alpha$ , and cultured for 24 hours to express the transgene of interest. **A**, HUVECs and **B**, synovial fibroblasts were stimulated with vehicle control (shaded bars) or 1  $\mu$ g/ml of LPS (solid bars) for 24 hours, and supernatants were collected and examined for the presence of IL-6 by enzyme-linked immunosorbent assay. Values are the mean and SD cytokine production in triplicate cultures and are representative of 5 independent donors. **C**, For NF- $\kappa$ B studies, HUVECs were stimulated with 1  $\mu$ g/ml LPS for 45 minutes. Cytosolic and nuclear extracts were obtained and examined for the presence of IKK2dn or I $\kappa$ B $\alpha$  by Western blotting, and NF- $\kappa$ B DNA-binding activity was examined by electrophoretic mobility shift assay. See Figure 1 for other definitions.

high levels of expression of IKK2dn and I $\kappa$ B $\alpha$ , respectively (Figure 4B). In some cases, AdIKK1dn was also used. Two days after infection, the effect of these molecules on spontaneous cytokine production was studied. As we previously reported (17,35) AdI $\kappa$ B $\alpha$  significantly inhibited TNF $\alpha$  ( $61 \pm 10\%$  inhibition;  $P < 0.01$ ), IL-1 $\beta$  ( $58 \pm 5\%$ ;  $P < 0.05$ ), IL-6 ( $96 \pm 3\%$ ;  $P < 0.05$ ), and IL-8 ( $86 \pm 2\%$ ;  $P < 0.05$ ) production, whereas a control Ad $\beta$ -gal virus had no effect (Figures 4C–G). Unexpectedly, however, we found that neither AdIKK2dn nor AdIKK1dn had any significant effect on TNF $\alpha$  production ( $30 \pm 17\%$  [ $P > 0.05$ ] and  $4 \pm 19\%$  [ $P > 0.05$ ], respectively) in RA synovial membrane cultures. In contrast, AdIKK2dn produced inhibition similar to that of AdI $\kappa$ B $\alpha$  on IL-1 $\beta$  ( $59 \pm 12\%$ ;  $P < 0.05$ ), IL-6 ( $92 \pm 3\%$ ;  $P < 0.05$ ), and IL-8 ( $84 \pm 4\%$ ;  $P < 0.05$ ) (Figures 4C–G). This was not due to increased cell death, as demonstrated when cells were examined by the MTT viability test (data not shown) (48).

We also investigated for the first time the expression of the angiogenic cytokine VEGF in the ex vivo RA synovial membrane cultures. We found that VEGF expression was NF- $\kappa$ B dependent and was blocked equally well by both AdI $\kappa$ B $\alpha$  ( $67 \pm 6\%$ ;  $P < 0.01$ ) and AdIKK2dn ( $82 \pm 2\%$ ;  $P < 0.01$ ), whereas Ad $\beta$ -gal had no effect. Interestingly, VEGF expression was also inhibited by AdIKK1dn ( $52 \pm 2\%$ ;  $P < 0.01$ ), but whether this was due to modulatory phosphorylation effects of IKK-1 on certain NF- $\kappa$ B DNA-binding subunits that enhance their transcription activity (24,52,53) or to a requirement of NF- $\kappa$ B2 processing for optimal VEGF expression remains to be determined. These data suggested that although IKK-2 is not required for TNF $\alpha$  production in RA, it is still involved in the expression of other inflammatory and angiogenic cytokines, and thus, is still a potential target.

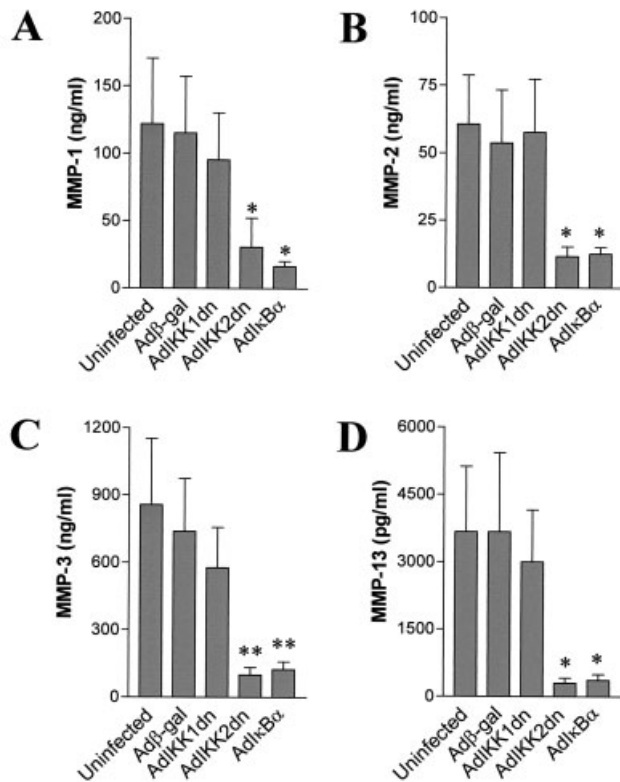
**Necessity of IKK-2 for production of MMPs 1, 2, 3, and 13.** We subsequently investigated the role of IKK-2 in the production of MMPs in the RA synovial membrane. These enzymes play a role in the destructive process of RA by breaking down human cartilage and bone (54–56). We previously showed that MMP-1 and MMP-3 expression in ex vivo RA synovial membranes is NF- $\kappa$ B dependent because AdI $\kappa$ B $\alpha$  down-regulates their expression (17). In this study, we also examined the expression of MMP-2 (gelatinase A) and MMP-13 and found that their expression is also NF- $\kappa$ B dependent (Figures 5B and C). We then looked at the role of IKK-2 in that process and found that AdIKK2dn,



**Figure 4.** Effects of IKK1dn, IKK2dn, and I $\kappa$ B $\alpha$  overexpression in cytokine production in rheumatoid arthritis (RA) synovial membrane cells from patients undergoing joint replacement surgery. RA synovial membrane cells were left uninfected or were infected with AdGFP, Ad $\beta$ -gal, AdIKK1dn, AdIKK2dn, and AdI $\kappa$ B $\alpha$ . Cells were cultured for another 2 days. **A**, After AdGFP infection, cells were examined for the presence of green fluorescence by phase-contrast and fluorescence microscopy. **B**, Cytosolic extracts were obtained and examined for the presence of IKK-2, I $\kappa$ B $\alpha$ , or  $\alpha$ -tubulin by Western blotting. Supernatants were collected and examined for the presence of **C**, TNF $\alpha$ , **D**, IL-1 $\beta$ , **E**, IL-6, **F**, IL-8, and **G**, vascular endothelial growth factor (VEGF) by enzyme-linked immunosorbent assay. Values are the mean and SD cytokine production in triplicate cultures from 7 unrelated patients. For statistical analysis of these parametric data, a repeated-measures analysis of variance test with Dunnett's post-test was used to compare uninfected control cells with recombinant adenovirus-infected cells. NS = not significant; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ . See Figure 1 for other definitions.

but not Ad $\beta$ -gal (control virus) or AdIKK1dn, could block the expression of MMP-1 ( $75 \pm 18\%$ ;  $P < 0.05$ ), MMP-2 ( $81 \pm 6\%$ ;  $P < 0.05$ ), MMP-3 ( $88 \pm 4\%$ ;  $P < 0.01$ ), and MMP-13 ( $92 \pm 3\%$ ;  $P < 0.05$ ) (Figures

5A–D). These data suggested that IKK-2 is also essential for the expression of extracellular matrix-degrading enzymes that are responsible for promoting destructive processes in RA.



**Figure 5.** Effects of IKK1dn, IKK2dn, and IκBα overexpression in matrix metalloproteinase (MMP) production in rheumatoid arthritis (RA) synovial membrane cells from patients undergoing joint replacement surgery. RA synovial membrane cells were left uninfected or were infected with Adβ-gal, AdIKK1dn, AdIKK2dn, and AdIκBα. Cells were cultured for another 2 days. Supernatants were collected and examined for the presence of **A**, MMP-1, **B**, MMP-2, **C**, MMP-3, and **D**, MMP-13 by enzyme-linked immunosorbent assay. Values are the mean and SD cytokine production in triplicate cultures from 5 unrelated patients. For statistical analysis of these parametric data, a repeated-measures analysis of variance test with Dunnett’s post-test was used to compare uninfected control cells with recombinant adenovirus-infected cells. \* = *P* < 0.05; \*\* = *P* < 0.01. See Figure 1 for other definitions.

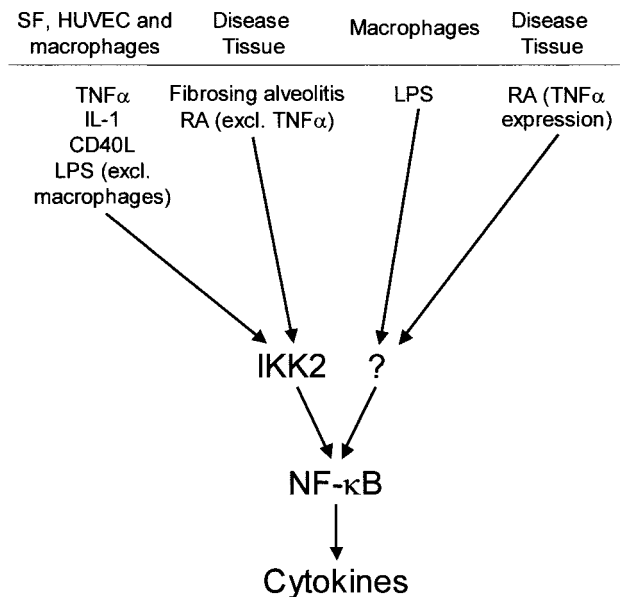
**DISCUSSION**

TNFα blockade is a validated treatment in RA, and extensive research is under way to target molecules that control TNFα expression. Two such molecules are NF-κB and IKK-2, a kinase that activates NF-κB and NF-κB-dependent gene expression in a number of systems. NF-κB has been shown to be activated in RA and to be of functional relevance since it controls the expression of TNFα and other proinflammatory cytokines and MMPs (17,57). However, no such role for IKK-2 has yet been demonstrated. In this study, we

examined the role of IKK-2 in this process and found that IKK-2 is not essential for TNFα production in RA ex vivo synovial membrane cultures from patients undergoing joint replacement surgery. This was unexpected and prompted us to investigate why this is the case.

We found that there is heterogeneity in the requirement of IKK-2 for NF-κB activation and NF-κB-dependent gene expression that depends on the stimulus and cell type used (Figure 6). Thus, in primary human macrophages, the main producers of TNFα in the RA joint, IKK-2 is not required for LPS-induced NF-κB activation and TNFα, IL-6, or IL-8 production, whereas it is still essential for CD40L-, TNFα-, or IL-1-induced NF-κB activation and TNFα, IL-6, or IL-8 production. IKK-1 is not alternatively used instead of IKK-2 to induce NF-κB activation and cytokine production in response to LPS. However, in dermal fibroblasts, RA synovial fibroblasts, and HUVECs, IKK-2 is essential for TNFα-, IL-1-, and LPS-induced NF-κB activation and IL-6 or IL-8 production. This is due to a direct effect of IKK-2 on IκBα degradation and NF-κB activation, and is consistent with other studies that have examined some of these aspects (29,39,58).

In contrast, our findings in primary human macro-



**Figure 6.** Cell type- and stimulus-dependent requirement of IκB kinase 2 (IKK-2) for nuclear factor κB (NF-κB) activation and inflammatory cytokine production in humans. SF = synovial fibroblasts; HUVEC = human umbilical vein endothelial cells; TNFα = tumor necrosis factor α; IL-1 = interleukin-1; CD40L = CD40 ligand; LPS = lipopolysaccharide; excl. = excluding; RA = rheumatoid arthritis.

phages are inconsistent with those of previous studies of THP-1 human monocytic cells that showed a 50% inhibition of an NF- $\kappa$ B reporter gene by IKK2dn. These studies did not examine endogenous NF- $\kappa$ B-dependent promoters, nor did they look at cytokine gene expression induced by LPS (59,60). Moreover, IKK1dn could be equally effective as IKK2dn (60), a result that is inconsistent with evidence from mice deficient in these molecules (21,22,25–27). In addition, our findings suggest significant differences in LPS signaling between macrophages and endothelial cells or fibroblasts. A possible reason for the discrepancy in these data is that, as for nuclear factor  $\kappa$ B-inducing kinase (NIK), there is a different usage of signaling components between transformed cell lines (e.g., THP-1 cells), where cell proliferation is the essential process, and primary cells such as macrophages, which do not divide (42,61,62). However, in the case of IKK-2, the situation appears more subtle, since IKK-2 is also involved in LPS signaling in primary cells, such as HUVECs and synovial fibroblasts. The fact that IKK2dn is expressed and is effective at blocking signaling by IL-1, TNF $\alpha$ , and CD40L would discount questions about the effectiveness of AdIKK2dn as an inhibitory construct.

Differential usage of IKK-2 between macrophages and synovial fibroblasts or HUVECs may be due to the involvement of alternative adapter molecules in signaling through the LPS receptor TLR-4. Two such molecules, MyD88 and Mal/TIRAP, have already been reported to play a role in TLR-4-induced TNF $\alpha$  and IL-6, but not IP-10, production (63,64). It has been suggested that IP-10 production requires the activation of interferon-regulatory factor 3 (65), and this may depend on the adapter molecule TRIF/TICAM-1, another recently identified MyD88 homolog (66,67). Thus, distinct adapter molecules of TLR-4 are likely to engage alternative signaling pathways and alternative kinases involved in I $\kappa$ B $\alpha$  phosphorylation. In addition to IKK-1 and IKK-2, other kinases have been shown to phosphorylate I $\kappa$ B $\alpha$ , including IKK $\epsilon$ /IKK $\zeta$ , p90<sup>rsk</sup>, PKR (double-stranded RNA-dependent kinase), casein kinase II, and the catalytic subunit of DNA-dependent kinase (68–73), although it remains to be determined whether these kinases specifically phosphorylate both Ser<sup>32</sup> and Ser<sup>36</sup> of I $\kappa$ B $\alpha$ . The importance and involvement of these kinases in IKK-2-independent pathways of LPS signaling need to be addressed.

Interestingly, in ex vivo RA synovial membrane cells, we found that although IKK-2 is not required for TNF $\alpha$  production, it is still essential for the production of IL-1 $\beta$ , IL-6, and IL-8. This paralleled the results with

AdI $\kappa$ B $\alpha$  that confirmed our previous observations that NF- $\kappa$ B is required for IL-1 $\beta$ , IL-6, and IL-8 production (17,35). In addition, we found that IKK-2 and NF- $\kappa$ B are essential for the expression of the angiogenic cytokine VEGF and the extracellular matrix-degrading enzymes MMPs 1, 3, 9, and 13. Inhibition was not due to increased cell death of AdIKK2dn-infected cells, a finding that was consistent with our previous work in RA synovial membrane cells using AdI $\kappa$ B $\alpha$  (17,35). On the other hand, IKK-1 was not involved in the release of any of these mediators, with the exception of VEGF. How IKK-1 is involved in VEGF expression in RA synovial membrane cultures is not yet clear, but this may require processing of NF- $\kappa$ B2, since we have evidence that in primary human macrophages that produce NF- $\kappa$ B-dependent VEGF in response to various stimuli (74), NIK is also involved (Andreakos E and Kiriakidis S: unpublished observations).

Because rheumatoid synovial membrane cultures consist of a complex mixture of cells comprising T cells, macrophages, and fibroblasts, these observations may reflect the selective use of IKK-2 for NF- $\kappa$ B activation and NF- $\kappa$ B-dependent gene expression in different cell types and in response to different stimuli. While rheumatoid macrophages are the major source of TNF $\alpha$ , isolated rheumatoid fibroblasts can produce abundant IL-1 $\beta$ , IL-6, IL-8, VEGF, and MMPs (but not TNF $\alpha$ ) in response to cytokines in an IKK-2-dependent manner, as our data and those of other investigators (58) demonstrate. Another, but not exclusive, explanation for this observation could be the ability of IKK2dn to block TNF $\alpha$ -mediated NF- $\kappa$ B activation and NF- $\kappa$ B-dependent gene expression. Such a mechanism is supported by our previous finding that TNF $\alpha$  is at the apex of a proinflammatory network or cascade that operates in the rheumatoid synovium, and that blockade of TNF $\alpha$  activity by neutralizing antibodies results in the downregulation of IL-1 $\beta$ , IL-6, IL-8, and MMP production both in dissociated synovial membrane cultures in vitro (37,38) and in patients with RA in vivo (3).

In contrast to RA, studies that we performed recently in bronchoalveolar lavage cultures from patients with fibrosing alveolitis showed that IKK-2 is required for the production of TNF $\alpha$  as well as IL-6 and IL-8 (75). The constituent cells of this system are just alveolar T cells and macrophages, and TNF $\alpha$  is mainly produced by macrophages. However, it is likely that the primary stimulus inducing TNF $\alpha$  production in patients with fibrosing alveolitis is different from that in patients with RA, and different or alternative signaling mechanisms are used. In terms of RA therapy, these observa-

tions suggest that although IKK-2 is not involved in TNF $\alpha$  production, it still plays a central role in the inflammatory, angiogenic, and destructive processes that operate in the RA synovial joint. This may have the advantage of not compromising the immune system in a major way, since macrophage function is partially maintained. Whether IKK-2 inhibition will be clinically effective can only be resolved by clinical trials, which are eagerly awaited.

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### REFERENCES

- Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* 1996;14:397–440.
- Brennan FM, Browne KA, Green PA, Jaspar JM, Maini RN, Feldmann M. Reduction of serum matrix metalloproteinase 1 and matrix metalloproteinase 3 in rheumatoid arthritis patients following anti-tumor necrosis factor- $\alpha$  (cA2) therapy. *Br J Rheumatol* 1997;36:643–50.
- Charles P, Elliott MJ, Davis D, Potter A, Kalden JR, Antoni C, et al. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF- $\alpha$  therapy in rheumatoid arthritis. *J Immunol* 1999;163:1521–8.
- Paleolog EM, Young S, Stark AC, McCloskey RV, Feldmann M, Maini RN. Modulation of angiogenic vascular endothelial growth factor by tumor necrosis factor  $\alpha$  and interleukin-1 in rheumatoid arthritis. *Arthritis Rheum* 1998;41:1258–65.
- Ballara S, Taylor PC, Reusch P, Marme D, Feldmann M, Maini RN, et al. Raised serum vascular endothelial growth factor levels are associated with destructive change in inflammatory arthritis. *Arthritis Rheum* 2001;44:2055–64.
- Andreaskos E, Foxwell BM, Brennan FM, Maini RN, Feldmann M. Cytokines and anti-cytokine biologicals in autoimmunity: present and future. *Cytokine Growth Factor Rev* 2002;13:299–313.
- Feldmann M. Development of anti-TNF therapy for rheumatoid arthritis. *Nat Rev Immunol* 2002;2:364–71.
- Andreaskos E, Taylor PC, Feldmann M. Monoclonal antibodies in immune and inflammatory diseases. *Curr Opin Biotechnol* 2002;13:615–20.
- Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 1994;372:739–46.
- Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV. Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor  $\alpha$  gene in primary macrophages. *J Exp Med* 1990;171:35–47.
- Goldfeld AE, Strominger JL, Doyle C. Human tumor necrosis factor  $\alpha$  gene regulation in phorbol ester stimulated T and B cell lines. *J Exp Med* 1991;174:73–81.
- McCaffrey PG, Goldfeld AE, Rao A. The role of NFATp in cyclosporin A-sensitive tumor necrosis factor- $\alpha$  gene transcription. *J Biol Chem* 1994;269:30445–50.
- Bondeson J, Browne KA, Brennan FM, Foxwell BM, Feldmann M. Selective regulation of cytokine induction by adenoviral gene transfer of I $\kappa$ B $\alpha$  into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor- $\kappa$ B independent. *J Immunol* 1999;162:2939–45.
- Brennan FM, Hayes AL, Ciesielski CJ, Green P, Foxwell BM, Feldmann M. Evidence that rheumatoid arthritis synovial T cells are similar to cytokine-activated T cells: involvement of phosphatidylinositol 3-kinase and nuclear factor  $\kappa$ B pathways in tumor necrosis factor  $\alpha$  production in rheumatoid arthritis. *Arthritis Rheum* 2002;46:31–41.
- Asahara H, Asanuma M, Ogawa N, Nishibayashi S, Inoue H. High DNA-binding activity of transcription factor NF- $\kappa$ B in synovial membranes of patients with rheumatoid arthritis. *Biochem Mol Biol Int* 1995;37:827–32.
- Marok R, Winyard PG, Coumbe A, Kus ML, Gaffney K, Blades S, et al. Activation of the transcription factor nuclear factor- $\kappa$ B in human inflamed synovial tissue. *Arthritis Rheum* 1996;39:583–91.
- Bondeson J, Foxwell B, Brennan F, Feldmann M. Defining therapeutic targets by using adenovirus: blocking NF- $\kappa$ B inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares anti-inflammatory mediators. *Proc Natl Acad Sci U S A* 1999;96:5668–73.
- Miagkov AV, Kovalenko DV, Brown CE, Didsbury JR, Cogswell JP, Stimpson SA, et al. NF- $\kappa$ B activation provides the potential link between inflammation and hyperplasia in the arthritic joint. *Proc Natl Acad Sci U S A* 1998;95:13859–64.
- Palombella VJ, Conner EM, Fuseler JW, Destree A, Davis JM, Laroux FS, et al. Role of the proteasome and NF- $\kappa$ B in streptococcal cell wall-induced polyarthritis. *Proc Natl Acad Sci U S A* 1998;95:15671–6.
- Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF- $\kappa$ B. *Nature* 1995;376:167–70.
- Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, et al. The IKK $\beta$  subunit of I $\kappa$ B kinase (IKK) is essential for nuclear factor  $\kappa$ B activation and prevention of apoptosis. *J Exp Med* 1999;189:1839–45.
- Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL, et al. Embryonic lethality, liver degeneration, and impaired NF- $\kappa$ B activation in IKK- $\beta$ -deficient mice. *Immunity* 1999;10:421–9.
- DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive I $\kappa$ B kinase that activates the transcription factor NF- $\kappa$ B. *Nature* 1997;388:548–54.
- Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, et al. IKK-1 and IKK-2: cytokine-activated I $\kappa$ B kinases essential for NF- $\kappa$ B activation. *Science* 1997;278:860–6.
- Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, et al. Abnormal morphogenesis but intact IKK activation in mice lacking the IKK $\alpha$  subunit of I $\kappa$ B kinase. *Science* 1999;284:316–20.
- Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, et al. IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev* 1999;13:1322–8.
- Li Q, Van Antwerp D, Mercurio F, Lee KF, Verma IM. Severe liver degeneration in mice lacking the I $\kappa$ B kinase 2 gene. *Science* 1999;284:321–5.
- Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF- $\kappa$ B activity. *Annu Rev Immunol* 2000;18:621–63.
- Aupperle K, Bennett B, Han Z, Boyle D, Manning A, Firestein G. NF- $\kappa$ B regulation by I $\kappa$ B kinase-2 in rheumatoid arthritis synovio-cytes. *J Immunol* 2001;166:2705–11.
- Tak PP, Gerlag DM, Aupperle KR, van de Geest DA, Overbeek M, Bennett BL, et al. Inhibitor of nuclear factor  $\kappa$ B kinase  $\beta$  is a key regulator of synovial inflammation. *Arthritis Rheum* 2001;44:1897–907.
- Williams RO. Rodent models of arthritis: relevance for human disease. *Clin Exp Immunol* 1998;114:330–2.
- Feldmann M, Maini RN. Anti-TNF $\alpha$  therapy of rheumatoid

- arthritis: what have we learned? *Annu Rev Immunol* 2001;19:163–96.
33. Bondeson J, Brennan F, Foxwell B, Feldmann M. Effective adenoviral transfer of I $\kappa$ B $\alpha$  into human fibroblasts and chondrosarcoma cells reveals that the induction of matrix metalloproteinases and proinflammatory cytokines is nuclear factor- $\kappa$ B dependent. *J Rheumatol* 2000;27:2078–89.
  34. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *J Clin Invest* 1973;52:2745–56.
  35. Foxwell B, Browne K, Bondeson J, Clarke C, de Martin R, Brennan F, et al. Efficient adenoviral infection with I $\kappa$ B $\alpha$  reveals that macrophage tumor necrosis factor  $\alpha$  production in rheumatoid arthritis is NF- $\kappa$ B dependent. *Proc Natl Acad Sci U S A* 1998;95:8211–5.
  36. Buchan G, Barrett K, Turner M, Chantry D, Maini RN, Feldmann M. Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 $\alpha$ . *Clin Exp Immunol* 1988;73:449–55.
  37. Brennan FM, Chantry D, Jackson A, Maini R, Feldmann M. Inhibitory effect of TNF $\alpha$  antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet* 1989;2:244–7.
  38. Baker D, Butler D, Scallon BJ, O'Neill JK, Turk JL, Feldmann M. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. *Eur J Immunol* 1994;24:2040–8.
  39. Oitzinger W, Hofer-Warbinek R, Schmid JA, Koshelnick Y, Binder BR, de Martin R. Adenovirus-mediated expression of a mutant I $\kappa$ B kinase 2 inhibits the response of endothelial cells to inflammatory stimuli. *Blood* 2001;97:1611–7.
  40. Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, et al. Activation by IKK $\alpha$  of a second, evolutionary conserved, NF- $\kappa$ B signaling pathway. *Science* 2001;293:1495–9.
  41. Wrighton CJ, Hofer-Warbinek R, Moll T, Eytner R, Bach FH, de Martin R. Inhibition of endothelial cell activation by adenovirus-mediated expression of I $\kappa$ B $\alpha$ , an inhibitor of the transcription factor NF- $\kappa$ B. *J Exp Med* 1996;183:1013–22.
  42. Smith C, Andreakos E, Crawley JB, Brennan FM, Feldmann M, Foxwell BM. NF- $\kappa$ B-inducing kinase is dispensable for activation of NF- $\kappa$ B in inflammatory settings but essential for lymphotoxin  $\beta$  receptor activation of NF- $\kappa$ B in primary human fibroblasts. *J Immunol* 2001;167:5895–903.
  43. Andreakos E, Smith C, Monaco C, Brennan FM, Foxwell BM, Feldmann M. I $\kappa$ B kinase 2 but not NF- $\kappa$ B-inducing kinase is essential for effective DC antigen presentation in the allogeneic mixed lymphocyte reaction. *Blood* 2003;101:983–91.
  44. Graham FL, Prevec L. Methods for construction of adenovirus vectors. *Mol Biotechnol* 1995;3:207–20.
  45. Ciesielski CJ, Andreakos E, Foxwell BM, Feldmann M. TNF $\alpha$ -induced macrophage chemokine secretion is more dependent on NF- $\kappa$ B expression than lipopolysaccharides-induced macrophage chemokine secretion. *Eur J Immunol* 2002;32:2037–45.
  46. Whiteside ST, Visvanathan KV, Goodbourn S. Identification of novel factors that bind to the PRD I region of the human  $\beta$ -interferon promoter. *Nucleic Acids Res* 1992;20:1531–8.
  47. Clarke CJ, Taylor-Fishwick DA, Hales A, Chernajovsky Y, Sugamura K, Feldmann M, et al. Interleukin-4 inhibits kappa light chain expression and NF $\kappa$ B activation but not I $\kappa$ B $\alpha$  degradation in 70Z/3 murine pre-B cells. *Eur J Immunol* 1995;25:2961–6.
  48. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.
  49. Stacey KJ, Ross IL, Hume DA. Electroporation and DNA-dependent cell death in murine macrophages. *Immunol Cell Biol* 1993;71:75–85.
  50. Horwood NJ, Smith C, Andreakos E, Quattrocchi E, Brennan FM, Feldmann M, et al. High-efficiency gene transfer into nontransformed cells: utility for studying gene regulation and analysis of potential therapeutic targets. *Arthritis Res* 2002;4 Suppl 3:S215–S225.
  51. Wöhleben G, Gray D, Schimpl A. In vitro immunization of naive mouse B cells: establishment of IgM secreting hybridomas specific for soluble protein or hapten from B cells cultured on CD40 ligand transfected mouse fibroblasts. *Int Immunol* 1996;8:343–9.
  52. Schmitz ML, Bacher S, Kracht M. I $\kappa$ B-independent control of NF- $\kappa$ B activity by modulatory phosphorylations. *Trends Biochem Sci* 2001;26:186–90.
  53. Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. I $\kappa$ B kinases phosphorylate NF- $\kappa$ B p65 subunit on serine 536 in the transactivation domain. *J Biol Chem* 1999;274:30353–6.
  54. Knauper V, Cowell S, Smith B, Lopez-Otin C, O'Shea M, Morris H, et al. The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction. *J Biol Chem* 1997;272:7608–16.
  55. Matrisian LM. Metalloproteinases and their inhibitors in matrix remodeling. *Trends Genet* 1990;6:121–5.
  56. Cowell S, Knauper V, Stewart ML, D'Ortho MP, Stanton H, Hembry RM, et al. Induction of matrix metalloproteinase activation cascades based on membrane-type 1 matrix metalloproteinase: associated activation of gelatinase A, gelatinase B and collagenase 3. *Biochem J* 1998;331:453–8.
  57. Feldmann M, Andreakos E, Smith C, Bondeson J, Yoshimura S, Kiriakidis S, et al. Is NF- $\kappa$ B a useful therapeutic target in rheumatoid arthritis? *Ann Rheum Dis* 2002;61 Suppl 2:ii13–8.
  58. Aupperle KR, Bennett BL, Boyle DL, Tak PP, Manning AM, Firestein GS. NF- $\kappa$ B regulation by I $\kappa$ B kinase in primary fibroblast-like synoviocytes. *J Immunol* 1999;163:427–33.
  59. O'Connell MA, Bennett BL, Mercurio F, Manning AM, Mackman N. Role of IKK1 and IKK2 in lipopolysaccharide signaling in human monocytic cells. *J Biol Chem* 1998;273:30410–4.
  60. Fischer C, Page S, Weber M, Eisele T, Neumeier D, Brand K. Differential effects of lipopolysaccharide and tumor necrosis factor on monocytic I $\kappa$ B kinase signalsome activation and I $\kappa$ B proteolysis. *J Biol Chem* 1999;274:24625–32.
  61. Matsushima A, Kaisho T, Rennert PD, Nakano H, Kurosawa K, Uchida D, et al. Essential role of nuclear factor (NF)- $\kappa$ B-inducing kinase and inhibitor of  $\kappa$ B (I $\kappa$ B) kinase  $\alpha$  in NF- $\kappa$ B activation through lymphotoxin  $\beta$  receptor, but not through tumor necrosis factor receptor I. *J Exp Med* 2001;193:631–6.
  62. Yin L, Wu L, Wesche H, Arthur CD, White JM, Goeddel DV, et al. Defective lymphotoxin- $\beta$  receptor-induced NF- $\kappa$ B transcriptional activity in NIK-deficient mice. *Science* 2001;291:2162–5.
  63. Horng T, Barton GM, Flavell RA, Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* 2002;420:329–33.
  64. Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 2002;420:324–9.
  65. Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 2001;167:5887–94.
  66. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon- $\beta$  induction. *Nat Immunol* 2003;4:161–7.
  67. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-1 receptor domain-containing

- adapter that preferentially activates the IFN- $\beta$  promoter in the Toll-like receptor signaling. *J Immunol* 2002;169:6668–72.
68. Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue J, Tatsumi Y, et al. IKK-i, a novel lipopolysaccharide-inducible kinase that is related to I $\kappa$ B kinases. *Int Immunol* 1999;11:1357–62.
69. Peters RT, Liao SM, Maniatis T. IKK $\epsilon$  is part of a novel PMA-inducible I $\kappa$ B kinase complex. *Mol Cell* 2000;5:513–22.
70. Schouten GJ, Vertegaal AC, Whiteside ST, Israel A, Toebe M, Dorsman JC, et al. I $\kappa$ B $\alpha$  is a target for the mitogen-activated 90 kDa ribosomal S6 kinase. *EMBO J* 1997;16:3133–44.
71. Kumar A, Haque J, Lacoste J, Hiscott J, Williams BR. Double-stranded RNA-dependent protein kinase activates transcription factor NF- $\kappa$ B by phosphorylating I $\kappa$ B. *Proc Natl Acad Sci U S A* 1994;91:6288–92.
72. Li S, Sedivy JM. Raf-1 protein kinase activates the NF- $\kappa$ B transcription factor by dissociating the cytoplasmic NF- $\kappa$ B-I $\kappa$ B complex. *Proc Natl Acad Sci U S A* 1993;90:9247–51.
73. Liu L, Kwak YT, Bex F, Garcia-Martinez LF, Li XH, Meek K, et al. DNA-dependent protein kinase phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  regulates NF- $\kappa$ B DNA binding properties. *Mol Cell Biol* 1998;18:4221–34.
74. Kiriakidis S, Andreakos E, Monaco C, Foxwell B, Feldmann M, Paleolog E. VEGF expression in human macrophages is NF- $\kappa$ B-dependent: studies using adenoviruses expressing the endogenous NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  and a kinase-defective form of the I $\kappa$ B kinase 2. *J Cell Sci* 2003;116:665–74.
75. Conron M, Andreakos E, Pantelidis P, Smith C, Beynon HL, Dubois RM, et al. Nuclear factor- $\kappa$ B activation in alveolar macrophages requires I $\kappa$ B kinase- $\beta$ , but not nuclear factor- $\kappa$ B inducing kinase. *Am J Respir Crit Care Med* 2002;165:996–1004.