The IKK-2/IκBα/NF-κB Pathway Plays a Key Role in the Regulation of CCR3 and eotaxin-1 in Fibroblasts

A CRITICAL LINK TO DERMATITIS IN IκBα-DEFICIENT MICE*

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Tumor necrosis factor (TNF)-α-induced phosphorylation of the IκB proteins by the IκB kinase (IKK) complex containing IKK-2 and subsequent degradation of the IκB proteins are prerequisites for NF-κB activation, resulting in the stimulation of a variety of pro-inflammatory target genes. The C-C chemokine eotaxin-1 is a potent chemoattractant for eosinophils and Th2 lymphocytes, may play an important role in the pathogenesis of atopic dermatitis, and acts via binding to its receptor CCR3. To investigate the role of NF-κB signaling in the regulation of these genes, we stably expressed a transdominant mutant of IκBα and a constitutively active mutant of IKK-2 in mouse NIH3T3 fibroblasts. The transdominant IκBα mutant completely inhibited TNF-α-mediated induction of both eotaxin-1 and CCR3, whereas expression of constitutively active IKK-2 was sufficient to drive almost full expression of these two genes in the absence of TNF-α. Moreover, we observed elevated expression levels of CCR3 and eotaxin-1 protein levels in the skin of IκBα-deficient mice characterized by a widespread dermatitis. Finally, using dermal fibroblasts derived from IκBα-deficient mice, we observed elevated basal expression, enhanced inducibility by TNF-α, and attenuated down-regulation upon TNF-α withdrawal of both CCR3 and eotaxin-1 mRNA levels. These results demonstrate that the IKK-2/IκBα/NF-κB pathway plays a critical role for CCR3 and eotaxin-1 expression in fibroblasts and suggests a critical link to the pathogenesis of atopic dermatitis.

The NF-κB1 transcription factor family is the most critical regulator of immediate transcriptional responses in inflammatory situations. Rel family members (p65/RelA, RelB, c-Rel, p50, and p52) form homo- or heterodimeric complexes with each other that constitute the NF-κB complex (1, 2). The critical role of NF-κB family members for distinct cellular functions, such as cell proliferation, cytokine gene expression, or protection from apoptosis, has been revealed by gene knockout experiments (2). In addition, there is increasing evidence for a role of NF-κB transcription factors in different pathophysiological processes, including atherosclerosis and cancer (3). In resting cells, NF-κB is inactive because of association with inhibitor IκB proteins that mask the nuclear localization sequence of NF-κB, thereby retaining it in the cytoplasm and preventing DNA binding. Several IκB proteins are involved in the control of NF-κB activity, three of these, i.e. IκBα, IκBβ, and IκBε, act in a stimulus-dependent manner. Upon inflammatory activation, IκB is phosphorylated in its N-terminal domain; subsequently it becomes ubiquitinylated and finally degraded by the proteasome. This allows nuclear translocation of NF-κB and binding to cognate DNA motifs in the promoter region of target genes, which subsequently initiates transcription. The critical step in NF-κB activation is the phosphorylation of IκBα by a large multisubunit kinase complex consisting of IκB kinases (IKK) 1/α and 2/β as well as an additional essential protein, NEMO/IKKγ (reviewed in Ref. 2). NEMO represents the regulatory component of the IKK complex, whereas IKK1 and IKK2 act as catalytic subunits. Both IKKs can phosphorylate all three IκB proteins (α, β, and ε) to a similar extent; however, from gene knockout experiments it became clear that IKK2 plays the dominant role in signal-induced phosphorylation/degradation of IκB proteins (reviewed in Ref. 2). IκB degradation and subsequently NF-κB activity can be induced in many cell types by different stimuli. Several parallel signal transduction pathways appear to exist, all of which ultimately result in IKK activation and IκB degradation (Ref. 4 and references therein). Among the best understood signaling pathways are the ones for the inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1 (5).

Chemokines are a large family of small proteins involved in the activation and recruitment of specific cell populations during disease (reviewed in Ref. 6). Eotaxin-1 is a potent eosinophil chemoattractant belonging to the class of C-C chemokines (7). The protein is potent in inducing eosinophil accumulation in vivo (7, 8). Eotaxin-1 knockout mice demonstrate that eotaxin-1 enhances the magnitude of the early eosinophil recruitment after allergen challenge in models of asthma, even though the suppression of eosinophil accumulation in challenged/sensitized mice was only partial (reviewed in Ref. 9). Eotaxin-1 expression was found to be restricted to a few cell types, including eosinophils, bronchial epithelial cells, and dermal fibroblasts (reviewed in Refs. 9 and 10). Its expression has been found to be enhanced in these cell types in asthmatics, and increased expression is associated with disease severity.
Moreover, eotaxin-1 expression in epithelial cells was found to be increased in atopic dermatitis, as well as in other inflammatory conditions (14). The expression of eotaxin-1 can be induced by TNF-α in epithelial cell lines, such as A549 cells (15), and by IL-1α and TNF-α in fibroblasts (16, 17). In contrast to most other eosinophil chemotactants of the CC-chemokine family that generally act on several receptors, eotaxin-1 only signals via one specific chemokine receptor, namely the G protein-coupled receptor CCR3 (reviewed in Ref. 18). CCR3 is prominently expressed on eosinophils, basophils, Th2-type lymphocytes, and fibroblasts (reviewed in Refs. 10 and 12). Based on these findings, an analysis of the regulation of CCR3 and eotaxin-1 expression in fibroblasts, in particular in dermal fibroblasts, is likely to yield information relevant to the pathogenesis of allergic inflammation such as atopic dermatitis.

Although very little is known on how CCR3 is regulated at the transcriptional level, there is increasing evidence that NF-κB may be involved in the regulation of eotaxin-1 expression. Firstly, NF-κB elements are present in the eotaxin-1 promoter in both humans and mice (17, 19). Second, mouse knockouts lacking the p50 subunit of NF-κB show no eotaxin-1 induction in response to ovalbumin challenge (20). Third, eotaxin-1 promoter activity was increased by TNF-α, and an NF-κB binding site was shown to be critical for this induction in the airway epithelial cell line BEAS-2B (21). Finally, an NF-κB binding site in the eotaxin-1 promoter was shown to be critical for the induction of eotaxin-1 by IL-1β in A549 airway epithelial cells (22). Even though these observations point toward an important role of NF-κB in eotaxin-1 regulation, neither of these reports has demonstrated whether or not this signaling pathway is essential in a stimulus-dependent manner and/or in an in vivo situation.

Here, we have investigated the role of NF-κB signaling for the regulation of CCR3 and eotaxin-1, two key regulators of atopic inflammatory responses. Upon exogenous expression of CA IKK-2 and TD IkBα in the fibroblast cell line NIH3T3, we show that NF-κB signaling is critical for the induction of CCR3 and eotaxin-1 in response to TNF-α. Using IκBα-deficient mice, we provide evidence that CCR3 and eotaxin-1 are physiological targets of NF-κB signaling in vivo and for their up-regulation in IκBα-deficient skin that may contribute to the skin pathology resembling atopic dermatitis.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—**NIH3T3 (a kind gift from Dr. Garin-Chesa, Boehringer Ingelheim Pharma KG), mouse embryonic fibroblasts, mouse dermal fibroblasts (isolation described below), and dnNX amphotropic retrovirus producer cells (a kind gift from G. Nolan, Stanford, CA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (PAN Systems, Aidenbach, Germany), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C, 5% CO2. For stimulation experiments human recombinant TNF-α (a gift from Dr. Adolf, Boehringer Ingelheim) was dissolved in a buffer containing 10 mM sodium phosphate, pH 7, 200 mM sodium chloride, and 2 mg/ml bovine serum albumin and used at the indicated concentrations. At least 12 h prior to stimulation, the cells were held in starvation medium consisting of Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Stable Transformation of NIH3T3 with 3xIkBα Luciferase Reporter and Luciferase Activity Assay—**For generation of stable transfectants of the NIH3T3 cell line, cells were electroporated (Bio-Rad gene pulser) with 20 μg of the 3xIkBα luc reporter plasmid together with 1 μg of a pB5.puro vector (confering resistance to puromycin) at 250 microfarad and 450 V. After electroporation, the cells were immediately resuspended in medium and seeded in 10-cm tissue culture plates. Cell clones with an integrated reporter gene were selected in medium containing 6 μg/ml puromycin with selection starting 48 h after electroporation. After 10–14 days single clones were picked and expanded. For measurement of luciferase activity cells were harvested, and luciferase activity was determined using the Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany).

**Retroviral Vectors and Stable Producer Cell Lines—**The pCFSG-EGZ retroviral vector used for infection has been described earlier (23). All cDNAs were inserted into blunt EcoRI/BamHI sites. Mutant IκBα was provided by Patrick Evans (Micromed, Munich, Germany) and the mutant IκK-2 cDNA by Alain Israel (Institut Pasteur, Paris, France). Sequences of retroviral vectors were confirmed by DNA sequencing. dnNX producer cells plated at a density of 1 × 106/10-cm plate were transfected using the calcium phosphate precipitation method with 10 μg of plasmid DNA as described (24). 24 h later, transfection efficiency was determined by monitoring green fluorescent protein expression by fluorescence microscopy (Improvision, Heidelberg, Germany). Transfection efficiencies usually ranged between 70 and 80%. 24 h after transfection, 1 mg/ml zeocin (Invitrogen) was added to the cells, which were then grown in the presence of this agent for another 2 weeks until all the cells were positive for green fluorescent protein.

**Retroviral Infection of NIH3T3 3xIkBα with Supernatant from dnNX Producer Cells—**One day before infection, NIH3T3 cells were seeded in six-well plates at a density of 2 × 105 cells/well, and the dnNX cells were seeded at a density of 3 × 105/10-cm plate. At the day of infection, dnNX cell supernatant was obtained and filtered through a 0.45-μm filter, and 5 μg/ml polybrene (Sigma) was added to the filtrate. Thereafter, medium was removed from NIH3T3 cells and replaced by dnNX cell supernatant. Culture plates were centrifuged at 1000 × g for 3 h, and supernatants then removed and replaced by conventional Dulbecco's modified Eagle’s medium. 48 h later the efficiency of infection was monitored by fluorescence microscopy as described above (infection efficiencies of NIH3T3 cells ranged between 80 and 90% depending on the retrovirus used), and selection with zeocin (1000 μg/ml) was started.

**Western Blot Analysis and Electrophoretic Mobility Shift Assay—**Preparation of whole cell extracts was performed as described earlier (25). For Western blot analysis, 50 μg of protein extracts/lane were separated on 12.5% polyacrylamide gels and transferred onto polyvinylidine difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 7.5% dry milk in PBS containing 0.2% Tween 20. For subsequent washes, 0.2% Tween 20 in PBS was used. The membranes were labeled with affinity-purified rabbit antisera against IκBα or IκK-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, the membranes were stained with horseradish peroxidase-coupled secondary antibody anti-rabbit IgG antibody (Dianova, Hamburg, Germany) that was visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Inc.). As a loading control, the membrane was incubated with a mouse anti-GAPDH antibody (40 μg/ml, 60 min at 56 °C) and, after extensive washing with PBS containing 0.2% Tween 20, labeled with rabbit polyclonal antibody against p65 (Santa Cruz Biotechnology). After incubation with secondary antibody anti-rabbit IgG antibody and washing with PBS containing 0.2% Tween 20, chemiluminescent substrate was added, and the membrane was subjected to autoradiography (as described above). Electrophoretic mobility shift assays were performed essentially as described before (25).

**Semiquantitative RT-PCR—**Total RNA was extracted, and semiquantitative RT-PCR was carried out as described earlier (10). Mouse CCR3 was amplified with primers 5'-CAA CCT GGC AAC TAT TCG CCT G-3' (sense) and 5'-GCA AAC AGA CCA TGG AGC AGT C-3' (antisense; 37 cycles); mouse eotaxin-1 was amplified with primers 5'-CAT CAG ATG ACC CCT GAA AGC-3' (sense) and 5'-TCC CCG AGA GCA GCT CTG AGG A-3' (antisense; 37 cycles); mouse EF-1α was amplified with primers 5'-AGT TTA AGG AGG AGT CTG CT-3' (sense) and 5'-CAA TCA GGA CAC CAG ACT C-3' (antisense; 23 cycles); all primers were obtained from MWG Biotech (Ebersberg, Germany). IκBα-specific primers were a kind gift from A. Beg (Columbia University, New York, NY) and were used for genotyping of IκBα knockout mice and for amplification of IκBα cDNA.
Role of NF-κB for CCR3 and eotaxin-1 Expression

Results

Retroviral Transduction of NIH3T3 Cells with Dominant Interfering Mutants of the IKK/1xBo Pathway—Some earlier reports had suggested a role of NF-κB in the regulation of eotaxin-1 expression in airway epithelial cells (21). We wanted to determine whether eotaxin-1 and CCR3 are inducibly expressed in fibroblasts and what role, if any, NF-κB signaling plays in such a scenario. To analyze CCR3 and eotaxin-1 inducibility, we chose primary mouse embryo fibroblasts that were treated for 4 or 24 h with TNF-α. RT-PCR analysis revealed that the expression of both genes was up-regulated upon stimulation with TNF-α and that stimulation for 24 h resulted in no further induction compared with stimulation for 4 h (Fig. 1A). Similarly, CCR3 and eotaxin-1 were up-regulated in the mouse fibroblast cell line NIH3T3 upon addition of TNF-α. Although stimulation with TNF-α for 1 h resulted in only a weak induction of these two genes, a 4-h stimulation period resulted in high expression levels of CCR3 and eotaxin-1 (Fig. 1B). These results provided a first hint that NF-κB signaling may be involved in the regulation of these two genes in fibroblasts.

To correlate all subsequent modulations of the NF-κB signaling pathway directly to NF-κB function, we engineered NIH3T3 fibroblasts that stably express a luciferase gene under the control of multimerized NF-κB sites (Fig. 2A). Treatment of individual stably transfected clones with TNF-α resulted in a strong up-regulation of luciferase activity, and the best clone (clone 12) showed an almost 250-fold induction upon addition of TNF-α (Fig. 2B). This clone was then infected with retroviruses expressing either a TD 1xBo protein (serines 32 and 36 are mutated to alanine), a constitutively active (CA) IKK-2 protein (two serines in the activation loop are mutated to glutamic acid residues), or an empty vector control (Fig. 2C). Stably infected cells could be visualized by immunofluorescence microscopy as the retroviruses coexpress enhanced green fluorescent protein. As shown in Fig. 2D, the infection rate of NIH3T3 clone 12 cells with these three constructs was close to 90% even prior to selection. Expression of TD-1xBo and CA-IKK-2 was controlled by Western immunoblotting (Fig. 2E). This analysis revealed strong overexpression of the mutant proteins as compared with the endogenous counterparts. In the presence of high levels of exogenous TD-1xBo, expression of endogenous 1xBo was barely detectable. This is most likely due to reduction in NF-κB activity, resulting in a decrease in 1xBo synthesis. High levels of CA-IKK2 also resulted in decreased levels of endogenous 1xBo. This can be explained by constitutive stimulation of IxBo phospho-rylation, resulting in its degradation. Moreover, overexpression of CA-IKK-2, but not of TD-1xBo, resulted in slightly elevated levels of endogenous IKK-1 (Fig. 2E).

Dominant Interfering Mutants in IKK-2 and 1xBo Show That Activation of NF-κB Is Critical for Inducible CCR3 and eotaxin-1 Expression in NIH3T3 Fibroblasts—The consequences of TD-1xBo and CA-IKK-2 expression on NF-κB activity were analyzed by luciferase assays and electrophoretic mobility shift assay. Although NIH3T3 clone 12 cells stably infected with an empty vector showed a more than 100-fold induction of luciferase activity upon stimulation with TNF-α, cells infected with TD-1xBo displayed no significant luciferase activity, regardless of whether TNF-α was present or absent (Fig. 3A). In contrast, cells infected with CA-IKK-2 exhibited a high level of luciferase activity already in the absence of TNF-α (25-fold higher than empty vector-infected cells), which could not be further elevated upon addition of TNF-α. Similar results were obtained by monitoring DNA binding activity of NF-κB in these cells (Fig. 3B). In the presence of TD-1xBo, no detectable NF-κB DNA binding activity could be induced by TNF-α. In contrast, the CA-IKK-2 expressing cells showed already considerable NF-κB DNA binding activity in the absence of TNF-α, which was, however, further increased at the 4-h time point. We then asked whether this NF-κB modulation affected the inducible expression of CCR3 and eotaxin-1 in NIH3T3 fibroblasts. RT-PCR analyses revealed that infection with the control vector did not affect the expression/induction of the transcripts of these two genes (Fig. 3C). In contrast, in cells infected with TD-1xBo protein, expression could not be induced by treatment of the cells with TNF-α. Importantly, cells expressing the CA-IKK-2 protein showed a high expression level of these two genes in the absence of TNF-α. After 4 h of TNF-α treatment, the inducible expression level was slightly increased further. These results demonstrate that activation of NF-κB is critical for the induction of CCR3 and eotaxin-1 gene expression in fibroblasts and that selective activation of this pathway already partially activates expression of these genes.

Elevated Basal Expression, Enhanced Inducibility, and Attenuated Down-regulation of CCR3 and eotaxin-1 in Fibroblasts Isolated from IxBo-deficient Mice—Given the critical role that NF-κB plays for CCR3 and eotaxin-1 expression, we asked whether expression of these genes is affected in dermal fibroblasts of mice lacking the IxBo protein. Mice bearing a homozygous null mutation in the gene coding for IxBo die about 7–8 days after birth because of a massive myeloproliferative disorder (26). Fig. 4A shows the genotyping of 7-day-old IxBo wild-type, heterozygous, and homozygous mutant mice (obtained by A. Beg, Columbia University). As already described (26, 27), IxBo-homozygous null pups at day 7 are significantly smaller than their littermates and show a widespread dermatitis characterized by xerosis, scaling plaques, and lichenification (Fig. 4, B and C). Next we analyzed skin sections (stained with hematoxylin-eosin) of 7-day-old wild-type (+/+), heterozygous (+/−), and homozygous null (−/−) animals. The wild-type skin (Fig. 5A) displays the normal aspect of a 7-day-old murine skin with an intact epidermis comprising morphologically nor-
mal keratinocytes, a pronounced granular zone, and an ortho-
kertotic basket-woven stratum corneum. The dermis is well
demarcated and reveals a texture of loosely woven collagen
fibers and an intense infiltrate of numerous fibroblasts and
histiocytes, as well as some lymphocytes and plasma cells, yet
hardly any granulocytes. The zone of the subcutaneous fat
tissue as well as the underlying muscle fibers are morphologi-
cally normal. The dermis and subcutaneous fat tissue present
with a high amount of normally differentiated hair follicles and
sebaceous gland units. Although skin from 7-day-old +/- pups

FIG. 2. Retroviral transduction of
NIH3T3 fibroblasts with dominant
interfering mutants of the IKK/IkB/NF-
κB pathway. A, schematic representation
of the 3xκB.luc reporter stably
transfected into NIH3T3 fibroblasts. B,
upon stable transfection, individual
clones were assayed for their luciferase
activity in the absence or upon stimula-
tion with 40 ng/ml TNF-α for 4 h (left
panel). R.L.U., relative light units. The
c fold induction upon TNF-α-stimulation is
indicated in the right panel. Among the
c clones showing the best response, clone 12
developed the highest inducibility (246×)
upon TNF-α addition and was selected for
further studies. C, schematic representa-
tion of the retrovirus used for the expres-
sion of TD-IκBα or CA IKK-2 mutants
(Modulators). IRES, internal ribosome
entry site; LTR, long terminal repeat;
Zeo, zeocin. As a control, a retrovirus
without a modulator insert was used.
NIH3T3 clone 12 cells were infected with
parental vector or retroviruses expressing
developing the interferon-κB mutants as
described under “Experimental Proce-
dures.” D, 48 h after infection, stably
infected cells were visualized by immuno-
fluorescence microscopy for enhanced
green fluorescent protein expression. E,
to determine the expression levels of dom-
ninant interfering mutants compared with
their endogenously expressed wild-type
counterparts, infected NIH3T3 clone 12
cells were stimulated with TNF-α (40 ng/
ml) for 2 h, and whole cell lysates were
prepared for Western blot analysis, using
an IKK- and IκBα-specific antibody si-
multaneously for visualization. Protein
bands of CA-IKK-2, endogenous IKK-1,
TD-IκBα, and endogenous IκBα are indi-
cated. The blot was subsequently stripped
and reprobed with a p65/RelA antibody,
to monitor equal loading (lower panel).
Role of NF-κB for CCR3 and eotaxin-1 Expression

Fig. 3. Activation of NF-κB is critical for CCR3 and eotaxin-1 expression in NIH3T3 fibroblasts. A, NIH3T3 clone 12 cells stably transfected with 3xIkBa.luc and subsequently stably infected with either the empty vector control, TD-IkBα, or CA-IKK2 were stimulated with 40 ng/ml TNF-α for 8 h, and luciferase activity was determined. Luciferase activity is calculated from three independent measurements and indicated as the mean in relative light units (R.L.U.). The fold induction in the presence of TNF-α is indicated above the bars indicating activity. B, whole cell lysates (6 μg) from NIH3T3 clone 12 cells stably infected with the indicated constructs and treated as indicated were incubated with a IkBa-specific probe. The positions of the induced NF-κB complexes are indicated. The lower band indicates a nonspecific binding complex and can be used as an internal loading control. All lanes were loaded equally, except the far right lane (IKK-2, 4 h, control), which was underloaded, resulting in an underrepresentation of the NF-κB binding activity. C, time dependence of CCR3 and eotaxin-1 transcript accumulation in NIH3T3 clone 12 cells stably infected with empty vector control, TD-IkBα, or CA-IKK2. Total RNA was extracted from cells either unstimulated or stimulated with 40 ng/ml TNF-α for 1 or 4 h. 100 ng of total RNA was subjected to RT-PCR analysis using gene-specific primers as described under “Experimental Procedures.” Lane 10 shows a control with conditions as in lane 9 lacking cDNA because of omission of reverse transcriptase. CCR3-, eotaxin-1, and EF-1α-specific amplification products are indicated.

Fig. 4. Phenotype of mice bearing a homozygous mutation in the gene coding for IkBa. A, PCR analysis of tail DNA preparations from offspring of heterozygous matings. The three genotypic categories are indicated as wild-type (+/+), heterozygous (+/−), and homozygous (−/−). The targeted locus results in a 180-bp fragment. B and C, phenotype of IkBa−/− animals. IkBa-homozygous null pups at day 7 are characterized by a widespread dermatitis with marked scaling (arrows, B and C) and thickened skin with increased markings, referred to as “lichenification” (indicated by dotted oval in C). (Fig 5B) shows a variable slight increase in infiltrating leukocytes (that has not been described previously), no major difference in terms of the epidermal, dermal, and subcutaneous architecture was noted. In contrast, a skin cross-section from an IkBa-deficient pup (Fig 5C) differs notably from both the wild-type and heterozygous skin. Overall, there is a striking degree of architectural disorganization in particular with regard to the dermal and subcutaneous fat layer; the epidermal granular zone is less pronounced and the pattern of cornification is more stratified than basket-woven. The cutaneous/subcutaneous border is hardly discernible, and the subcutaneous fat tissue appears remarkably reduced, whereas the muscle bundles are well developed. The dermal connective tissue is interspersed by a dense infiltrate comprising fibroblasts, lym-
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A primary dermal fibroblasts from IκBα wild-type (+/+) and mutant (−/−) animals were isolated and expanded. The cells were treated with 40 ng/ml TNF-α for the indicated time periods. After 4 h, TNF-α was washed away thoroughly, medium without TNF-α was added, and incubation was continued for the indicated time periods. Total RNA was extracted and subjected to RT-PCR analysis using gene-specific primers as described under “Experimental Procedures.” B-E, immunohistochemistry of cryostat skin cross-sections of 7-day-old wild-type (B and D) and homozygous IκBα mutant (C and E) pups. CCR3 and Eotaxin-1 protein levels were determined by incubation with polyclonal antibodies against CCR3 and Eotaxin-1 and by performing a routine avidin-biotin-immunoperoxidase staining procedure. Note increased staining of CCR3 in IκBα −/− skin, in particular in suprabasal layers of the epidermis (arrow, C) but also in dermal fibroblasts (arrow, C), whereas only one layer of basal keratinocytes expresses CCR3 in the skin cross-section from a control (B). D and E, increased expression of Eotaxin-1 on keratinocytes and dermal fibroblasts from IκBα-deficient mice (−/−, arrows, E). Scale bar, 25 μm.

**DISCUSSION**

The activation of eotaxin-1 and its receptor CCR3 is thought to be a critical step in the onset of inflammatory reactions associated with allergic asthma or atopic dermatitis. Here we have analyzed the contribution of the IKK-2/IκBα/NF-κB signaling pathway to CCR3 and eotaxin-1 expression upon inflammatory stimulation in fibroblasts, a cell type central to inflammatory conditions. We used a retroviral transduction approach that allowed the expression of dominant interfering mutants of components of the NF-κB signaling pathway. The consequences of this modulation of NF-κB activity on the expression of endogenous CCR3 and eotaxin-1 in NIH3T3 fibroblasts was analyzed. Using this approach, we were able to demonstrate that the inhibition of NF-κB signaling by expression of a transdominant mutant of IκBα results in an almost complete block-ade of TNF-α-induced expression of CCR3 and eotaxin-1. In contrast, a constitutively active version of IKK-2 by itself was sufficient to induce maximal expression of these genes in the absence of TNF-α. Interestingly, IκBα mutant skin cells, including fibroblasts, exhibited elevated levels of CCR3 and eotaxin-1 protein levels, and also the analysis of dermal fibroblasts derived ex vivo from these IκBα-deficient pups demonstrated a critical role of NF-κB signaling in the regulation of these two genes.
NF-κB has been previously implicated in allergic inflammation. The majority of proteins encoded by NF-κB target genes participate in the host immune responses. These include a large number of cytokines and chemokines, as well as receptors required for leukocyte adhesion and migration (29). There has been suggestive evidence that NF-κB regulates the chemokine eotaxin-1, a central mediator in recruiting of eosinophils in allergic inflammation. Mochizuki et al. (30) reported that TNF-α, an inducer of NF-κB signaling, stimulated eotaxin-1 expression in fibroblasts. We have found a similar effect of TNF-α in the induction of eotaxin-1 mRNA in NIH3T3 cells, mouse embryonic fibroblasts, and mouse dermal fibroblasts. Yang et al. (20) have shown that mice deficient in the p50 subunit of NF-κB protein do not mount eosinophilic lung inflammation and that eotaxin-1 expression was inhibited compared with that in wild-type mice. These data are in agreement with our findings indicating an important role for NF-κB in the regulation of eotaxin-1. Furthermore, in vitro studies using reporter constructs have suggested that overlapping elements for NF-κB and Stat6 within the eotaxin-1 promoter mediate the transcriptional induction by TNF-α and IL-4, respectively, in airway epithelial cells (21). Our data confirm and extend these findings and demonstrate in vivo roles for IKK-2, IκBα, and NF-κB in the TNF-α-induced regulation of eotaxin-1. In contrast to eotaxin-1, very little has been reported on the mechanisms of CCR3 regulation so far. Analysis of the CCR3 gene revealed a complex 5′ exon organization and a broadly active promoter with eosinophil-selective elements. The CCR3 promoter also appears to contain putative NF-κB binding sites, which, however, have not yet been further analyzed (31). Although further studies are required to demonstrate whether CCR3 is a direct target gene of NF-κB, our results show a functional requirement of IKK-2/IKκBα/NF-κB signaling in the regulation of CCR3 expression. To our knowledge, this is the first demonstration that NF-κB signaling is critical for TNF-α-mediated induction of CCR3 expression in fibroblasts and provides additional evidence for a role of NF-κB in allergic inflammation.

IκBα is the major ubiquitous cytoplasmic inhibitor that is critical for regulating the rapid transient nuclear induction of NF-κB. Although the embryonic development of mice lacking IκBα appears to be normal, IκBα−/− mice die 7–10 days postnatally, afflicted by severe widespread inflammatory dermatitis and granulocytosis (26, 27). Coincident with this phenotype, the expression of certain proinflammatory cytokines and factors associated with granulocyte recruitment, adherence, and activation such as TNF-α, granulocyte-colony stimulating factor, murine macrophage inflammatory protein-2, and the adhesion molecule vascular cell adhesion molecule-1 is enhanced. However, not all genes known to be induced by NF-κB are up-regulated in IκBα−/− cells. Our results clearly provide evidence that regulation of CCR3 and eotaxin-1 by NF-κB occurs at least in part via repression by IκBα, because expression levels of these two target genes are elevated in the skin of IκBα−/− mice. Furthermore, dermal fibroblasts isolated from these mice show elevated basal expression, enhanced inducibility, and attenuated down-regulation of CCR3 and eotaxin-1 expression. Interestingly, despite the absence of IκBα in these knockout mice, changes in the constitutive nuclear levels of NF-κB are cell type-dependent. For example, whereas an increase in constitutively nuclear p50/relA and p50 homodimers was observed in IκBα−/− thymocytes and splenocytes, the levels of constitutive NF-κB complexes were unchanged in IκBα−/− embryonic fibroblasts (26). Our observation of an elevated basal level of the two NF-κB target genes in dermal fibroblasts of IκBα−/− mice argues that IκBα plays a critical role in regulating the cytoplasmic retention of NF-κB in unstimulated dermal fibroblasts. This has not been observed in embryonic fibroblasts derived from these mice (27). Furthermore, we detected a prolonged post-induction repression of CCR3 and eotaxin-1 in dermal fibroblasts derived from lckBα−/− mice upon removal of TNF-α. Because attenuated down-regulation of NF-κB signaling has also been observed in mouse embryonic fibroblasts of IκBα−/− mice (27), the requirement for IκBα in termination of the NF-κB response appears to be a more general mechanism. Recent data indicate that activation of IKK-2, rather than IKK-1, participates in the primary pathway by which proinflammatory stimuli induce NF-κB function. IKK-2 has been shown to play a central role in IL-1- and TNF-α-mediated NF-κB activation and expression of proinflammatory genes in several cell types (reviewed in Ref. 32). Our results indicate that IKK-2 is also a critical regulator of proinflammatory gene expression in fibroblasts. Activation of NF-κB leads to the induction of multiple genes, encoding at least 27 different cytokines and chemokines, receptors involved in immune recognition, proteins involved in antigen presentation, and receptors required for leukocyte adhesion and migration (reviewed in Ref. 33). Thus, NF-κB activation is assumed to lie at the heart of many inflammatory diseases, such as rheumatoid arthritis, asthma (20), and inflammatory bowel disease (reviewed in Ref. 32). In addition, NF-κB regulation may be involved in the pathogenesis of diseases such as atherosclerosis and Alzheimer’s disease, in which the inflammatory response is at least partially involved (reviewed in Ref. 33). Several lines of evidence suggest that NF-κB activation of cytokine genes is an important contributor to the pathogenesis of atopic asthma, which is characterized by the infiltration of eosinophils and lymphocytes into the sites of inflammation (34). Many recent in vivo and in vitro studies have implicated eotaxin-1 in this process (7, 35, 36). Recently it has been demonstrated that eotaxin-1 and CCR3 protein expression is significantly enhanced in lesional skin of patients suffering from atopic dermatitis (AD) (13). Eotaxin-1 is a potent chemoattractant and activator not only of eosinophils and basophils but also for Th2 lymphocytes (37), which are associated with the initial phase of inflammation in AD (38). The suggestion that NF-κB dysregulation may be a critical factor in mediating susceptibility to AD is supported by the findings that RelB-deficient mice show a phenotype and histopathological changes resembling AD (39), accompanied by increased mRNA levels of eotaxin-1 and CCR3 in lesional skin. It should be noted, however, that the basis of the inflammatory pathology in relB−/− mice may be due to the absence of certain thymic and splenic dendritic cell populations that account for the inability to delete autoreactive T cells (40). These T cells may, in a feedback cycle, stimulate resident cells, e.g., fibroblasts, to release chemokines and therefore increase leukocyte accumulation in the affected tissue (39).

Our results suggest a critical role of NF-κB signaling in the pathogenesis of AD for the following reasons. First, the regulation of eotaxin-1 and CCR3 by the IKK-2/IκBα/NF-κB pathway is important at the mRNA level in fibroblasts. Second, IκBα-deficient mice are afflicted by a severe widespread dermatitis (Refs. 26 and 27 and our results) that revealed several histopathological parallels to AD in humans. Coincident with this phenotype, the expression of CCR3 and eotaxin-1 in dermal fibroblasts isolated from lesional IκBα−/− skin was enhanced and prolonged in response to the activation signal TNF-α, and moreover, Eotaxin-1 and CCR3 protein expression in lesional skin from these animals was markedly induced. It remains to be elucidated whether fibroblasts in patients with AD show altered p50/relA/NF-κB activity. However, the ele-
vated expression levels of Eotaxin-1 and CCR3 in lB-deficient mice offers a likely explanation for the presence of infiltrating leukocytes in the skin of these pups and their skin pathology resembling AD. A detailed characterization of NF-κB signaling in AD will be essential to develop specific therapeutic strategies for atopic diseases such as asthma and AD.

Acknowledgments—We thank Dr. Bernd Baumann for helpful discussions and critical reading of the manuscript, Dragon Marinovic and Tatjana Samardzic for assistance with immunofluorescence, Dr. Amer Beg for the lB mutant mice, and U. Leschik, C. Pantic, and E. Peschke for excellent technical assistance.

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The IKK-2/IκBα/NF-κB Pathway Plays a Key Role in the Regulation of CCR3 and eotaxin-1 in Fibroblasts: A CRITICAL LINK TO DERMATITIS IN IκBα-DEFICIENT MICE
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J. Biol. Chem. 2002, 277:1268-1275.
doi: 10.1074/jbc.M109358200 originally published online November 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109358200

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