

# GMCSF activates NF- $\kappa$ B via direct interaction of the GMCSF receptor with I $\kappa$ B kinase $\beta$

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**Granulocyte-macrophage colony-stimulating factor (GMCSF) has a central role in proliferation and differentiation of hematopoietic cells. Furthermore, it influences the proliferation and migration of endothelial cells. GMCSF elicits these functions by activating a receptor consisting of a ligand-specific  $\alpha$ -chain and a  $\beta$ -chain, which is common for GMCSF, interleukin-3 (IL-3), and IL-5. It is known that various signaling molecules such as Janus kinase 2 or transcription factors of the signal transducer and activator of transcription (STAT) family bind to the**

**common  $\beta$ -chain and initiate signaling cascades. However,  $\alpha$ -chain-specific signal transduction adapters have to be postulated given that IL-3, IL-5, and GMCSF induce partly distinct biologic responses. Using a yeast 2-hybrid system, we identified the  $\alpha$ -chain of the GMCSF receptor (GMR $\alpha$ ) as putative interaction partner of I $\kappa$ B kinase  $\beta$ , one of the central signaling kinases activating the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B). Using endogenous protein levels of endothelial cell extracts, we could verify the interaction by coimmunoprecipitation experi-**

**ments. Fluorescence resonance energy transfer (FRET) microscopy confirmed the direct interaction of CFP-IKK $\beta$  and YFP-GMR $\alpha$  in living cells. Functional studies demonstrated GMCSF-dependent activation of I $\kappa$ B kinase activity in endothelial cells, degradation of I $\kappa$ B, and activation of NF- $\kappa$ B. Further biologic studies using GMCSF-dependent TF-1 cells indicated that GMCSF-triggered activation of NF- $\kappa$ B is important for cell survival and proliferation. (Blood. 2003;102:192-199)**

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## Introduction

Granulocyte-macrophage colony-stimulating factor (GMCSF) is a cytokine that was initially described by its ability to induce colonies of granulocytes and macrophages from myeloid progenitor cells. It is involved in proliferation, maturation, and differentiation of these cells and acts via specific receptors on the cell surface.<sup>1-3</sup> The GMCSF receptor (GMR) belongs to the class 1 subgroup of the cytokine receptor superfamily and is not only expressed on cells of the granulocyte-macrophage lineage but also on endothelial cells, where it was postulated to have a role in proliferation and migration.<sup>4,5</sup> It is composed of 2 glycoprotein subunits: a 60- to 80-kDa  $\alpha$ -chain, which by itself exhibits only low-affinity binding of the ligand, and a 120- to 140-kDa  $\beta$ -chain, which does not bind GMCSF itself but is essential for high-affinity binding in conjunction with the  $\alpha$ -chain.<sup>6-8</sup> Recent data indicate that 2  $\beta$ -chains associate with 1 or 2  $\alpha$ -chains<sup>9,10</sup> and that this ternary GMCSF receptor complex is already preformed in the absence of ligand.<sup>11</sup> While the  $\alpha$ -chain is specific for GMCSF, the  $\beta$ -chain is common for the receptors of GMCSF, interleukin-3 (IL-3), and IL-5, which explains some of the overlapping effects of these 3 cytokines. However, an important contribution of the  $\alpha$ -chains of these receptors to the intracellular signaling was postulated, and some important differences have been reported for the effects of IL-3, IL-5, and GMCSF.<sup>1,3,12-16</sup> For all 3 receptors the  $\alpha$ -chains exhibit rather short cytoplasmic domains as compared with the  $\beta$ -chain, which made it difficult to identify signaling partners for the ligand-specific  $\alpha$ -chains. Recently, an adapter

molecule was characterized as interaction partner of the IL-5 receptor  $\alpha$ -chain,<sup>17</sup> and it could be shown that it is essential for IL-5-mediated signaling to the Sox4 transcription factor, proving the important role of the ligand-specific  $\alpha$ -chain for certain signaling pathways. Similarly, a recent yeast 2-hybrid screening for interaction partners of the GMCSF receptor  $\alpha$ -chain identified a molecule termed GRAP (GMCSF receptor  $\alpha$  subunit-associated protein).<sup>18</sup> However, a contribution of this protein to GMCSF-mediated signaling could not be demonstrated and its role remained enigmatic. In contrast to the so far unsolved question of the function of the GMCSF receptor  $\alpha$ -chain in signal transduction, the role of the common  $\beta$ -chain was clearly demonstrated and characterized in detail. It is known that the common  $\beta$ -chain ( $\beta_c$ ) is able to interact with various receptor-associated proteins that are important for the signaling downstream of the receptor.<sup>3,19,20</sup> One prominent interaction partner is Janus kinase 2 (JAK2), a tyrosine kinase that binds to the common  $\beta$ -chain after stimulation with IL-3, IL-5, or GMCSF followed by transphosphorylation and activation. Subsequently, activated JAK2 phosphorylates tyrosine residues of  $\beta$ -chains and generates binding sites for Src-homology 2 (SH2) domains of other proteins such as members of the signal transducer and activator of transcription (STAT) family. JAK2 also phosphorylates STAT proteins themselves, which leads to their dimerization, activation, and translocation to the nucleus, where they act as transcription factors for responsive genes. In parallel, additional signaling pathways can be activated, such as the

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Ras–Raf–extracellular signal-regulated kinase (Ras–Raf–ERK) pathway, which is important for triggering the cell cycle and is activated by binding of the adapter proteins Shc and Grb2 to the common  $\beta$ -chain. Another signaling pathway triggered by the  $\beta$ -chain is that emerging from phosphatidylinositol-3 kinase (PI-3K), which has a role in regulation of apoptosis and cell survival.<sup>3,21</sup> Moreover, some evidence indicated that also the nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway can be activated by GMCSF,<sup>22–27</sup> although the molecular mechanism for this signal transduction could not be clearly elucidated. Most of the signals that activate NF- $\kappa$ B act on a crucial kinase complex, the I $\kappa$ B kinase signalosome, consisting of the 3 main components IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$ , which upon activation phosphorylate I $\kappa$ B, the inhibitor of NF- $\kappa$ B, leading to its ubiquitination and degradation.<sup>28–31</sup> I $\kappa$ B kinases can be activated by a variety of upstream signaling kinases, such as NIK (NF- $\kappa$ B inducing kinase), MEKK1 (mitogen-activated protein kinase [MAPK]/ERK kinase kinase 1), RIP (TNF receptor interacting protein), or TAK1 (transforming growth factor [TGF]- $\beta$  activated kinase 1), which themselves can be activated by adapter proteins after binding to cell surface receptors following their oligomerization. Furthermore, it was shown that I $\kappa$ B kinases can be directly recruited to the tumor necrosis factor (TNF) receptor complex leading to their activation.<sup>32,33</sup>

In our group, we focused our interest on the elucidation of signaling pathways involving the central I $\kappa$ B kinase, IKK $\beta$ . Using the C-terminal part of IKK $\beta$  as bait in a yeast 2-hybrid screening, we identified the ligand-specific  $\alpha$ -chain of the GMCSF receptor as interaction partner and we could confirm this interaction and its biologic role in GMCSF-triggered NF- $\kappa$ B activation. Thus, we provide evidence that IKK $\beta$  has an important role in GMCSF receptor-mediated signaling.

## Materials and methods

### Materials

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described.<sup>34</sup> For stimulation, recombinant human GMCSF was used at a final concentration of 100 ng/mL. GMCSF was kindly provided by F. Kalthoff (Novartis Research Institute, Vienna, Austria). TF-1 cells (DMSZ, Braunschweig, Germany), a human GMCSF-dependent myeloid leukemic cell line, were cultured at 37°C, 5% CO<sub>2</sub>, in RPMI 1640 plus 10% fetal calf serum (FCS) (Invitrogen, Karlsruhe, Germany) and 2 ng/mL GMCSF. HeLa cells were cultured in Dulbecco modified Eagle medium (DMEM), 10% FCS containing 2 mM glutamine. The ECFP chimera of IKK $\beta$  was cloned from IKK $\beta$ -EYFP<sup>35</sup> by replacing EYFP using *AgeI* and *XbaI*. The EYFP fusion construct of the GMCSF receptor  $\alpha$ -chain (GMR $\alpha$ ) was generated by cloning the GMR $\alpha$  coding sequence without stop codon into pEYFP-N1 (Clontech, Palo Alto, CA).

### Yeast 2-hybrid screen

The yeast 2-hybrid screening was performed with components of the Matchmaker 2-Hybrid System 2 (Clontech). The C-terminal domain of IKK $\beta$  (amino acids 466 to 756; comprising the leucine zipper and helix-loop-helix [HLH] domains) was cloned by polymerase chain reaction (PCR) into pAS2-1 (Clontech), and the resulting construct was verified by sequencing. The PJ69 reporter yeast strain was transformed with the bait construct using the method of Gietz et al,<sup>36,37</sup> and autoactivation was excluded by transformation with an empty library vector. The 2-hybrid screen was performed using a library from phytohemagglutinin (PHA)-stimulated leukocytes ( $3 \times 10^6$  independent clones, Clontech, cat. no. HL4021AB) with bacterial RNA as carrier nucleotides as described by Brondyk and Macara,<sup>38</sup> resulting in about  $2 \times 10^6$  transformants. For the primary selection, colonies were grown on media lacking leucine, trypto-

phan, and adenine (selecting for the presence of both bait and prey plasmids and for the interaction between bait and prey proteins). Positive clones were transferred to quadruplicate selection plates (also lacking histidine) and were tested for  $\beta$ -galactosidase activity. Yeast DNA was prepared by the method of Liang and Richardson,<sup>39</sup> followed by electrotransformation into HB101 bacteria and sequence analysis of lacZ-positive interaction partners.

### Coimmunoprecipitation

HUVECs were stimulated with 100 ng/mL GMCSF for 10 minutes, washed with phosphate-buffered saline (PBS), and lysed with PBS/0.5% Nonidet P-40 (NP-40) containing Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) for 20 minutes at 4°C. Cell extracts were precleared under rotating conditions with protein A–Sepharose (Amersham Pharmacia Biotech, United Kingdom) for 1 hour at 4°C. The supernatants were incubated with anti-IKK $\beta$  (Santa Cruz Biotechnology, Santa Cruz, CA; polyclonal antirabbit, 1  $\mu$ g/mL) or an unrelated antibody as negative control (anti-myc, Upstate Biotechnology, MA; polyclonal antirabbit 1  $\mu$ g/mL) for 1 hour at 4°C followed by addition of fresh protein A–Sepharose and immunoprecipitation for 1 hour at 4°C. Alternatively, IKK signalosomes were immunoprecipitated with agarose-conjugated anti-IKK $\alpha$  antibodies (M-280, Santa Cruz Biotechnology). Beads were washed 4 times with cold PBS, and bound proteins were eluted by heating to 95°C with sodium dodecyl sulfate (SDS) sample buffer. Proteins were resolved on 10% SDS–polyacrylamide gels and blotted on nitrocellulose followed by immunodetection with antibodies against GMR $\alpha$  (Santa Cruz Biotechnology; monoclonal antimouse, 1  $\mu$ g/mL).

### In vitro kinase assays

HUVECs ( $7 \times 10^6$  cells) were incubated with 100 ng/mL GMCSF for different periods of time, washed with ice-cold PBS, and lysed in 1 mL lysis buffer containing 20 mM Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 7.5), 150 mM NaCl, 25 mM  $\beta$ -glycerophosphate, 2 mM EDTA (ethylenediaminetetraacetic acid), 2 mM pyrophosphate, 1 mM orthovanadate, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM NaF, and protease inhibitors. The cell extracts were immunoprecipitated at 4°C for 2 hours with anti-IKK $\alpha$  agarose (Santa Cruz Biotechnology), which precipitates both IKK $\alpha$  and IKK $\beta$ . Agarose beads were washed 3 times with PBS and once with kinase buffer containing 20 mM Tris–HCl (pH 7.5), 20 mM  $\beta$ -glycerophosphate, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M orthovanadate, 50 mM NaCl, 1 mM DTT, 50  $\mu$ M adenosine triphosphate (ATP), and 1 mM NaF. After washing of the beads, 1  $\mu$ g glutathione-S-transferase (GST)–I $\kappa$ B $\alpha$  was added as substrate (or GST–I $\kappa$ B $\alpha$  mutant with Ser32 and Ser36 mutated to alanine, as negative control). Then, 10  $\mu$ L kinase buffer plus 10  $\mu$ Ci (0.37 MBq) [<sup>32</sup>P- $\gamma$ ]ATP (Amersham Biosciences, United Kingdom) per sample was preincubated for 10 minutes at 37°C, subsequently mixed with the beads and the substrate, and incubated at 37°C for 1 hour. Proteins were eluted with SDS buffer at 95°C and separated on a 12% SDS–polyacrylamide gel. The gel was fixed with methanol/acetic acid (10%, respectively), dried, and exposed on a phosphor screen followed by quantification with PhosphorImager equipment (Molecular Dynamics, Germany).

### Western blot detection of I $\kappa$ B $\alpha$ degradation

TF-1 cells were starved by cultivation in the absence of serum and GMCSF for 24 hours, followed by incubation in the presence of GMCSF (2 ng/mL) for different periods of time. Similarly, HUVECs were stimulated for different time periods by addition of GMCSF (100 ng/mL). After incubation, cells were washed with cold PBS, lysed in cold PBS containing 0.5% NP-40, and centrifuged for 15 minutes, 14 000 rpm at 4°C to remove nuclei. Cytoplasmic proteins were separated on a 12% SDS–polyacrylamide gel and blotted on nitrocellulose membrane. Endogenous I $\kappa$ B $\alpha$  was detected with anti-I $\kappa$ B $\alpha$  antibodies (Santa Cruz Biotechnology, clone C-21) using a concentration of 0.4  $\mu$ g/mL.

### Reporter gene assays

HUVECs were transiently transfected with a luciferase reporter construct containing 5 NF- $\kappa$ B promoter sites and a  $\beta$ -galactosidase construct

comprising a ubiquitin promoter (as normalization control) using LipofectAMINE Plus (Life Technologies) as transfection reagent. A total of  $10^6$  cells were transfected with 1.5  $\mu\text{g}$  DNA, 4  $\mu\text{L}$  LipofectAMINE, and 8  $\mu\text{L}$  Plus Reagent. Twenty-four hours after transfection, cells were stimulated with GMCSF or TNF- $\alpha$  for different periods of time, and luciferase activity was determined as described<sup>40</sup> and normalized to  $\beta$ -galactosidase activity determined by colorimetric detection with chlorophenol red  $\beta$ -D-galactopyranosid as substrate.<sup>35</sup>

### Flow analysis

For cell cycle analysis via propidium iodide staining, TF-1 cells were cultured in the absence of GMCSF for 24 hours, followed by incubation in the presence or absence of 2 ng/mL GMCSF, TNF- $\alpha$ , or the NF- $\kappa$ B inhibitor BAY 11-7082 (5  $\mu\text{M}$ ; Santos et al<sup>41</sup>) for another 24 hours. After stimulation, cells were pelleted by centrifugation, resuspended in PBS, fixed by suspension in ethanol (absolute ethanol precooled to  $-20^\circ\text{C}$ , resulting in a final concentration of 70%), incubated on ice for 15 minutes, and again centrifuged. The cell pellet was stained with 0.5 mL of a solution containing 50  $\mu\text{g}/\text{mL}$  propidium iodide, 0.1 mg/mL RNase A, and 0.5  $\mu\text{L}/\text{mL}$  Triton X-100 in PBS for 40 minutes at  $37^\circ\text{C}$ . The propidium iodide fluorescence of DNA revealing the cell cycle profile as well as apoptotic cells (sub- $G_0/G_1$  population) was determined by flow analysis using FACSsort equipment (Becton Dickinson, CA). The alternative detection of apoptotic cells by staining with annexin V-fluorescein isothiocyanate (FITC) (BD Biosciences, Palo Alto, CA) was performed as recommended by the manufacturer.

### Electrophoretic mobility shift assay (EMSA)

HUVECs ( $1.1 \times 10^7$  cells) were incubated with 100 ng/mL GMCSF for 0, 15, 30, and 45 minutes. Nuclear extracts were prepared essentially as described.<sup>42</sup> Protein concentrations of the nuclear extracts were measured with Coomassie staining reagent (BioRad), and 5  $\mu\text{g}$  protein was used for the EMSA. Oligonucleotides with a binding site for NF- $\kappa$ B were labeled with <sup>32</sup>P using Klenow enzyme to a specific activity of about  $10^5$  cpm/ng and incubated for 20 minutes at room temperature with nuclear abstracts in the absence or presence of a  $50 \times$  molar excess of unlabeled competitor DNA sequence (1 ng of oligonucleotide per sample). Protein-DNA complexes were resolved by native polyacrylamide gel electrophoresis (PAGE) using a 10% separation gel (pH 8.8) and a 4% stacking gel (pH 7.0).

The gel was fixed, dried, and exposed to a phosphor screen, followed by analysis on PhosphorImager equipment (Molecular Dynamics).

### Fluorescence microscopy

HeLa cells were seeded on 23-mm round glass coverslips in 6-well plates (400 000 cells per well) and transfected with IKK $\beta$ -ECFP, GMR $\alpha$ -EYFP, and untagged GMR $\beta$  using the calcium precipitation method.

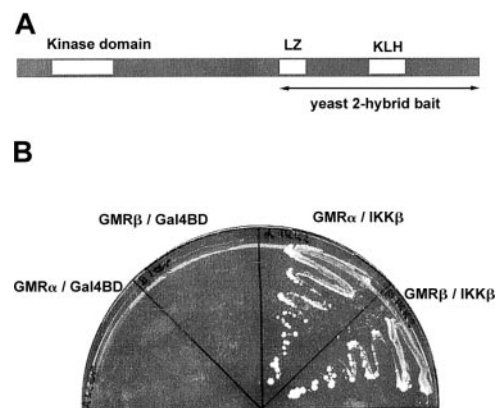
One day after transfection, coverslips were mounted on a perfusion chamber and living cells were imaged by fluorescence microscopy as described<sup>35,43,44</sup> using a Nikon Diaphot inverted microscope equipped with a cooled CCD camera (Kappa, Bad Gleichen, Germany) and filter sets that discriminate between ECFP and EYFP fluorescence (Omega Optical, VT). GMCSF was added to the cells at a concentration of 100 ng/mL, and the images were taken after different time points. Fluorescence resonance energy transfer (FRET) microscopy was performed by monitoring the increase in donor (ECFP) fluorescence after photobleaching of the acceptor. For that purpose, cells were examined with an oil immersion objective (60 $\times$  or 100 $\times$ ) and images were taken with the donor filter at reduced excitation light (using a 90% neutral density filter to prevent donor bleaching) followed by photodestruction of the FRET acceptor (EYFP) with the appropriate filter set under full excitation energy (100 W Mercury lamp) for about 45 to 60 seconds. Subsequently, the neutral density filter was again included in the excitation light path, and another image was taken with the ECFP filter set under the same camera setting as the first one. An increase in the donor fluorescence intensity was visualized by calculating a ratio image of the ECFP image before and after acceptor photobleaching

using NIH-Image software or the Windows-equivalent ScionImage (Scion, MD).

## Results

Because nearly all signaling pathways that trigger activation of NF- $\kappa$ B converge at the level of the I $\kappa$ B kinase complex with IKK $\beta$  being the most important component of it, we aimed to identify binding partners of this kinase by yeast 2-hybrid screening using the C-terminal half of IKK $\beta$ . This part of the protein contains a leucine zipper and an HLH motif representing potential interaction domains (Figure 1A). Screening a library from activated lymphocytes resulted in about 2 million transformants from which 78 colonies grew on triple selection plates (lacking leucine, tryptophan, and adenine). Further increasing the stringency by plating on media that also lacked histidine resulted in 14 clearly positive clones. One of the strongest interaction candidates was identified as a C-terminal fragment of the GMCSF receptor  $\alpha_1$ -chain. This fragment included the short cytoplasmic tail of the  $\alpha_1$ -chain as well as the transmembrane domain and part of the extracellular moiety of the receptor. To exclude a potential artificial interaction mediated by the transmembrane or extracellular regions, we generated a yeast 2-hybrid construct containing only the short cytoplasmic tail comprising 54 amino acids linked to the Gal4 activation domain and tested this construct for interaction with the IKK $\beta$  Gal4-binding domain protein as compared with the empty Gal4-binding domain vector. By that means, we could verify that IKK $\beta$  is capable of interacting specifically with the cytoplasmic domain of the ligand-specific  $\alpha$ -chain of the GMCSF receptor in the yeast system (Figure 1B). Subsequently, we generated a Gal4 activation domain construct comprising the cytosolic domain of the common  $\beta$ -chain. A yeast 2-hybrid test of this construct together with the IKK $\beta$  bait or an empty bait vector indicated that the  $\beta$ -chain of the GMCSF receptor is able to interact with IKK $\beta$  as well (Figure 1B). These data would indicate that IKK $\beta$  is able to associate with the functional GMCSF receptor containing both  $\alpha$ - and  $\beta$ -chains.

After identification of the IKK $\beta$ -GMR interaction in the yeast 2-hybrid system, we aimed to verify this association for endogenous levels of interaction partners in mammalian cells. For that purpose we used endothelial cells, which were reported to contain



**Figure 1. Interaction between IKK $\beta$  and the GMCSF receptor in the yeast 2-hybrid system.** (A) Schematic drawing of the IKK $\beta$  protein: the part that was used as bait in the yeast 2-hybrid screening is indicated as well as leucine zipper (LZ) and helix-loop-helix (HLH) motifs. (B) Interaction between the cytoplasmic domains of GMR $\alpha$  or GMR $\beta$  and IKK $\beta$  in the yeast 2-hybrid system. Control transformations contained GMR $\alpha$  or GMR $\beta$  combined with empty Gal4-binding domain vector (Gal4BD) lacking IKK $\beta$ .

both  $\alpha$ - and  $\beta$ -chains of the GMR.<sup>4,5,45</sup> Because we assumed that the interaction might be ligand dependent, we added GMCSF to part of the samples and immunoprecipitated the IKK signalosome with anti-IKK $\alpha$ , anti-IKK $\beta$  antibodies or negative control antibodies, washed the beads extensively, and performed SDS-PAGE and immunoblotting using an antibody against the  $\alpha$ -chain of the GMCSF receptor. This experiment proved that endogenous levels of the GMCSF receptor can be coprecipitated with both IKK $\alpha$  and IKK $\beta$ . In these coimmunoprecipitation experiments we observed a basal ligand-independent association of IKK proteins with the GMCSF receptor. However, the interaction appeared to be slightly enhanced in the presence of GMCSF (Figure 2A).

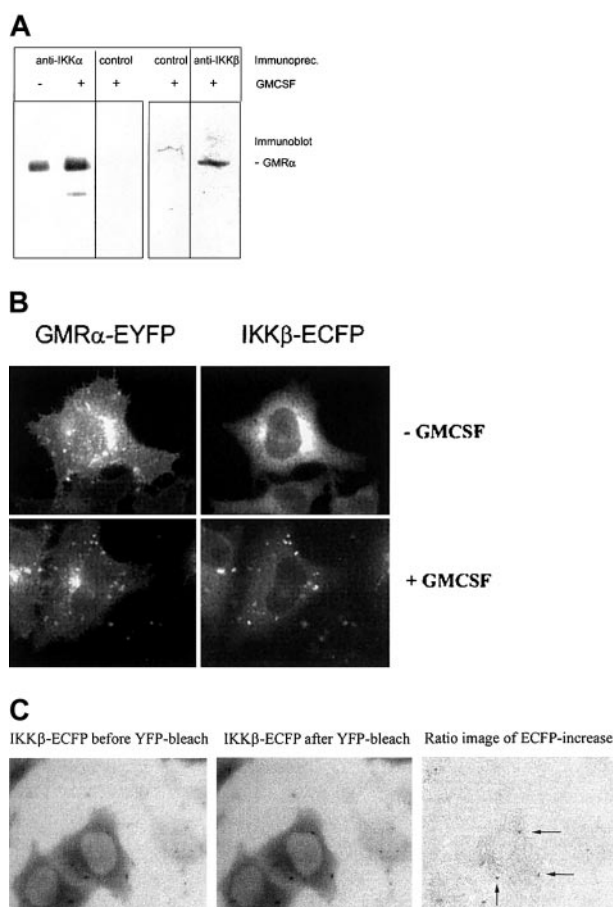
Our next aim was to visualize and characterize the interaction between the GMCSF receptor and the IKK complex in living cells using ECFP and EYFP fusion proteins of IKK $\beta$  and GMR $\alpha$ , respectively. Expression of GMR $\alpha$ -EYFP in various mammalian

cells (293, HeLa, HUVECs) resulted in fluorescence at the cell surface and in intracellular compartments of the secretory pathway, indicating functional incorporation of the fusion protein into the endoplasmic reticulum and correct transport to the cytoplasmic membrane. This pattern was very similar to that observed for an EYFP chimera of the TNF- $\alpha$ -receptor 2, which was functional *in vivo* (data not shown). Coexpression of IKK $\beta$ -ECFP did not significantly change the distribution of GMR $\alpha$ -EYFP and revealed a predominant cytosolic localization of the IKK $\beta$ -ECFP chimera. However, after addition of GMCSF, we observed a faint recruitment of IKK $\beta$  to the cell surface pointing to ligand-enhanced association, and after some time (about 15 minutes) we noted the occurrence of vesicular structures, where IKK $\beta$ -ECFP and GMR $\alpha$ -EYFP clearly colocalized (Figure 2B). Because it is known that GMCSF induces the internalization of its receptor,<sup>46</sup> we assume that these vesicles represent endosomes, where the GMCSF receptor and IKK $\beta$  bound to the cytoplasmic tail of it are concentrated.

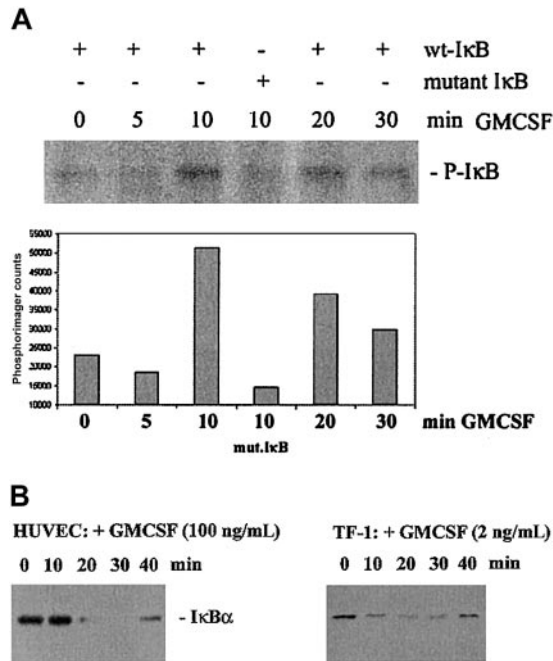
In addition to colocalization studies, we used the ECFP and EYFP fusion proteins of IKK $\beta$  and GMR $\alpha$  for testing the interaction by means of FRET microscopy: bleaching of EYFP, the energy acceptor, resulted in a distinct increase of the IKK $\beta$ -ECFP fluorescence exactly at the sites of colocalization (Figure 2C). This phenomenon is known as donor recovery after acceptor photobleaching and is a clear indication for an energy transfer due to close proximity of appropriate fluorophores. It occurs in the case of an interaction between ECFP and EYFP chimeras.<sup>47</sup>

The direct interaction between the cytoplasmic domains of the GMCSF receptor and IKK $\beta$  and the fact that this association was enhanced in the presence of ligand prompted us to investigate whether IKK $\beta$  is activated by the interaction. For that purpose, we performed *in vitro* kinase assays using endogenous I $\kappa$ B kinases that were immunoprecipitated from endothelial cells at different time points after GMCSF addition. Administration of GMCSF clearly resulted in a specific increase in I $\kappa$ B phosphorylating activity with a peak approximately after 10 minutes (Figure 3A). The kinetics of IKK $\beta$  activation are very similar to those induced by TNF- $\alpha$ , where the peak activity was reported to be in the range of 10 minutes.<sup>48,49</sup> We next aimed to verify whether this GMCSF-induced up-regulation of IKK activity also results in degradation of I $\kappa$ B $\alpha$ . Primary endothelial cells were treated for different periods of time with GMCSF, and I $\kappa$ B $\alpha$  was determined in cell extracts by immunoblotting. A clear down-regulation of I $\kappa$ B $\alpha$  immunoreactivity was observed about 20 to 30 minutes after addition of GMCSF, followed by a subsequent resynthesis, which is typical after activation of NF- $\kappa$ B. Similar results were obtained for the GMCSF-dependent TF-1 cell line after withdrawal of GMCSF for 24 hours, readdition of GMCSF for different periods of time, and analysis of I $\kappa$ B $\alpha$  in cell extracts (Figure 3B). The GMCSF-dependent degradation of I $\kappa$ B $\alpha$  in HUVECs and TF-1 cells pointed to a liberation and activation of NF- $\kappa$ B, which could be confirmed by electrophoretic mobility shift assays showing an increase in NF- $\kappa$ B-specific binding activity in nuclear extracts of GMCSF-treated endothelial cells (Figure 4A).

Subsequently, we investigated the activation of NF- $\kappa$ B using reporter gene assays with NF- $\kappa$ B-dependent luciferase reporter constructs. In this case, a clear up-regulation of luciferase activity was detected about 24 hours after addition of GMCSF, while TNF- $\alpha$  led to induction of luciferase already at earlier time points (Figure 4B). Taken together, these data indicate that GMCSF is able to activate the transcription factor NF- $\kappa$ B via direct binding of I $\kappa$ B kinases to the GMCSF receptor and induction of the kinase

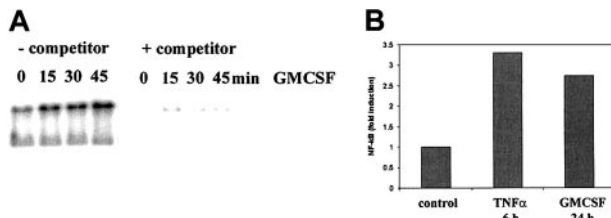


**Figure 2. Interaction between IKKs and the GMCSF receptor in mammalian cells.** (A) Coimmunoprecipitation of endogenous proteins: HUVECs were incubated in the presence or absence of GMCSF for 10 minutes, followed by preparation of cell extracts, immunoprecipitation with anti-IKK $\alpha$  agarose, anti-IKK $\beta$  or control antibodies, and immunoblotting with anti-GMR $\alpha$  antibodies. Equal amounts of proteins were applied. (B) Colocalization of GMR $\alpha$ -EYFP and IKK $\beta$ -ECFP after addition of GMCSF. HeLa cells were transfected with the indicated fluorescent protein chimeras and investigated by fluorescence microscopy using filter sets discriminating between CFP and YFP fluorescence. Representative cells before (- GMCSF) and 20 minutes after addition of GMCSF (+GMCSF) are shown. (C) FRET microscopy demonstrates interaction between GMR $\alpha$ -EYFP and IKK $\beta$ -ECFP. HeLa cells expressing the fluorescent fusion proteins were treated with GMCSF and subjected to FRET microscopy as described in "Materials and methods." Specific bleaching of the acceptor fluorophore (EYFP) resulted in a significant increase of the donor fluorescence (ECFP) exactly at the sites of colocalization. A ratio image of the CFP fluorescence before and after YFP photobleaching of representative cells (20 minutes after GMCSF addition) visualizes the fluorescence resonance energy transfer as indicated by arrows. Images were captured using a  $\times 60$  oil immersion objective.

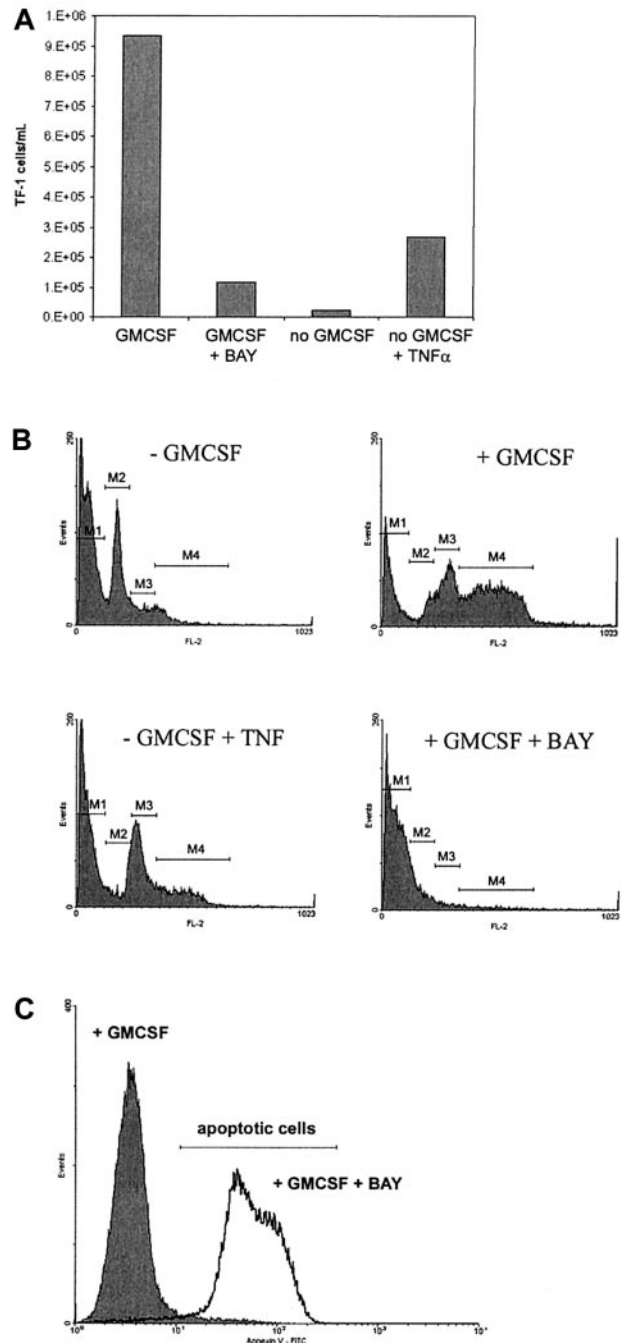


**Figure 3. Activation of IKK activity by GMCSF.** (A) HUVECs were treated with GMCSF for the indicated time periods, followed by immunoprecipitation of endogenous IκB kinases with anti-IKK agarose. In vitro kinase assays were done with recombinant wild-type GST-IκBα or mutant IκBα (with Ser32 and Ser36 mutated to alanine) as described in "Materials and methods." Radioactively labeled IκBα was resolved by SDS-PAGE, detected with PhosphorImager equipment, and quantified as indicated in the lower panel. (B) Degradation of IκBα: HUVECs or TF-1 cells (GMCSF-starved for 24 hours) were treated with GMCSF as indicated, followed by preparation of cell extracts, SDS-PAGE, and immunoblotting for detection of endogenous IκBα.

activity. The activation of the NF-κB signaling pathway by GMCSF implies that it might have a role in biologic functions that are exerted by this cytokine apart from the role of the JAK2-STAT signaling pathway. To answer this question, we investigated the effect of the specific NF-κB inhibitor BAY 11-7082 on proliferation and apoptosis of the GMCSF-dependent cell line TF-1. Addition of BAY 11-7082 to the culture medium strongly reduced the cell numbers in the presence of GMCSF, similar to the values obtained in the absence of the cytokine (Figure 5A), indicating that NF-κB plays a role in survival or proliferation of the cells. Addition of TNF-α in the absence of GMCSF could restore the cell numbers to some extent, implying that activation of the NF-κB pathway by



**Figure 4. Activation of NF-κB activity by GMCSF.** (A) HUVECs were incubated in the presence of GMCSF as indicated, followed by preparation of nuclear extracts and electrophoretic mobility shift assay with <sup>32</sup>P-labeled NF-κB binding oligonucleotides in the absence or presence of an excess of unlabeled competitor oligonucleotides. (B) HUVECs were transfected with an NF-κB-dependent luciferase reporter construct containing 5 tandem repeats of the NF-κB binding site and an NF-κB-independent β-galactosidase vector as normalization control. One day after transfection, GMCSF or TNF-α was added and cell extracts were prepared after the time points indicated, followed by measurement of luciferase and β-galactosidase activity. The stimulation of NF-κB activity as compared with the vector control is given. Data represent the averages of 2 independent experiments.



**Figure 5. Functional role of the GMCSF-mediated NF-κB activation.** (A) Effect of NF-κB inhibition or GMCSF withdrawal on the proliferation of TF-1 cells. Cells were incubated for 3 days in the presence or absence of GMCSF (2 ng/mL), in the presence of GMCSF in the absence or presence of the NF-κB inhibitor Bay 11-7082 (BAY; 5 μM), or in the absence of GMCSF but in the presence of TNF-α (200 U/mL). Cell numbers were recorded by flow analytic counting of cells at defined flow rates. (B) Cell cycle analysis of TF-1 cells. Cells were GMCSF-starved for 24 hours followed by readdition of GMCSF in the absence or presence of the NF-κB inhibitor Bay 11-7082. Alternatively, cells were further incubated in the absence of GMCSF with or without addition of TNF-α. After 24 hours, cells were fixed and permeabilized, followed by propidium iodide staining in the presence of RNase to label DNA. Cells containing less than the diploid DNA content (M<sub>1</sub>; sub-G<sub>0</sub>/G<sub>1</sub>) represent apoptotic cells. The positions of G<sub>0</sub>/G<sub>1</sub> (M<sub>2</sub>), S-phase (M<sub>3</sub>), as well as G<sub>2</sub>/M-phase cells (M<sub>4</sub>) are indicated. (C) Apoptosis of TF-1 cells after inhibition of NF-κB. Cells were incubated in the presence of GMCSF with or without addition of the NF-κB inhibitor Bay 11-7082 for 27 hours. Apoptotic cells were detected by flow analysis after binding of FITC-labeled annexin V.

alternate pathways is at least in part functional with respect to cell survival. For a clearer discrimination between effects on cell proliferation versus those on apoptosis, we performed cell cycle analysis by propidium iodide staining of permeabilized cells. TF-1 cells were cultured for 24 hours in the absence of GMCSF to stop cell cycle progression and accumulate the cells in the G<sub>0</sub> phase. Subsequently, cells were further cultured in the absence or presence of GMCSF, TNF- $\alpha$ , or the NF- $\kappa$ B inhibitor BAY 11-7082 for another 24 hours. As assessed by the number of cells in the sub-G<sub>0</sub>/G<sub>1</sub> region representing apoptotic cells with fragmented DNA, GMCSF withdrawal for 48 hours induced a significant increase in apoptosis (Figure 5B). In contrast, readdition of GMCSF after 24 hours caused a distinct release from the G<sub>0</sub>/G<sub>1</sub> phase and a clear increase of cells in S and G<sub>2</sub>/M phase. Similarly, some release from the G<sub>0</sub>/G<sub>1</sub> stage was observed for cells treated with TNF- $\alpha$ . Cultivation of TF-1 cells in the presence of GMCSF and the NF- $\kappa$ B inhibitor resulted in a cell cycle profile that indicated nearly complete apoptosis of the cells. To confirm this observation, we applied a different method of detecting apoptotic cells, using annexin V staining and flow analysis, which identifies apoptotic cells based on the binding of fluorescently tagged annexin V to phosphatidylserine residues, which occur on the outer face of the membrane at a rather early stage of apoptosis.<sup>50</sup> Cultivation of TF-1 cells in the presence of BAY 11-7082 and GMCSF resulted in a complete shift of the annexin V staining and thus occurrence of apoptotic cells as compared with control cells incubated in the absence of the NF- $\kappa$ B inhibitor (Figure 5C). These data indicate that GMCSF-mediated activation of the NF- $\kappa$ B signaling pathway is crucial for cell survival. Moreover, NF- $\kappa$ B might also have some direct effects on cell proliferation given that activation of the NF- $\kappa$ B pathway by TNF- $\alpha$  also resulted in some induction of proliferation—an effect that might also involve autocrine mechanisms via GMCSF.<sup>51</sup>

## Discussion

The transcription factor NF- $\kappa$ B is of fundamental importance in inflammation and immune cell development. The central role of IKK $\beta$  as a converging point in the activation of NF- $\kappa$ B prompted us to search for interaction partners of this enzyme using a yeast 2-hybrid approach. One of the strongest interaction partners that could be identified was the  $\alpha$ -chain and thus the ligand-specific moiety of the GMCSF receptor. The interaction occurred with the C-terminal part of IKK $\beta$  including a leucine zipper and a helix-loop-helix motif, which are known to serve as contact sites in many different protein interactions. The short cytoplasmic domain of the  $\alpha$ -chain comprising only 54 amino acids was sufficient to mediate specific interaction in the yeast system. Further investigation for a potential interaction between IKK $\beta$  and the  $\beta$ -chain of the receptor, which is also part of the IL-3 and the IL-5 receptor, revealed that the cytoplasmic domain of the common  $\beta$ -chain is interacting with IKK $\beta$  as well (Figure 1). This observation indicates that the actual binding surface for IKK $\beta$  is the combination of  $\alpha$ - and  $\beta$ -chains. However, the interaction of IKK $\beta$  with the  $\alpha$ -chain was of particular interest for us because an interaction partner with a clear signaling function could not be identified yet for the ligand-specific  $\alpha$ -chain, whereas signaling molecules are known to interact with the common  $\beta$ -chain, the most prominent ones being JAK2 and STAT proteins. There is only one previous report on an interaction partner of the  $\alpha$ -chain, which describes a protein termed GRAP.<sup>18</sup> However, this protein did not exhibit any enzymatic domain and

showed no clear indication for a role in GMCSF-dependent signaling. Our observation on IKK $\beta$  as an interaction partner of the ligand-specific  $\alpha$ -chain raises the possibility that IKK $\beta$  might be responsible for at least some of the GMCSF-specific effects that are not observed for IL-3 or IL-5.<sup>12-14</sup> Molecular interactions that are identified by yeast 2-hybrid screening have to be verified for mammalian cells in order to exclude false-positive results that might be caused by the heterologous expression and assay system. In many cases, this is achieved by overexpression of tagged proteins in mammalian cells using transient transfection approaches and coimmunoprecipitation experiments. However, overexpression of proteins might also lead to rather artificial interactions due to high levels of transfected proteins. In our case, we could successfully coprecipitate endogenous levels of IKK proteins and GMCSF receptors, proving the physiological significance and importance of the interaction. These studies indicated some constitutive, ligand-independent association of IKK proteins with the GMCSF receptor. However, in coprecipitation experiments the interaction was slightly enhanced after addition of GMCSF (Figure 2A), and our experiments with ECFP and EYFP chimeras of IKK $\beta$  and GMR $\alpha$  in living cells furthermore implied that the association and subsequent colocalization in endocytic structures is favored by binding of GMCSF to the receptor (Figure 2B). Although we observed constitutive association of IKK proteins with the GMCSF receptor in the absence of GMCSF in coimmunoprecipitation experiments, the clear induction of IKK activity after administration of GMCSF (Figure 3A) indicates that a conformational change of the receptor upon ligand binding obviously activates the kinase activity of associated IKK $\beta$ .

Our studies using fluorescence microscopy of ECFP and EYFP fusion proteins in living cells support the notion that binding of GMCSF to its receptor stimulates its internalization<sup>46</sup> into endocytic compartments, where it colocalizes with IKK $\beta$  bound to the cytoplasmic tail of the receptor. As a consequence endosomes might also serve as signaling surfaces apart from the cytoplasmic membrane. Similar models of signaling-active endosomes were postulated for other receptors such as the EGF receptor<sup>52</sup> and imply that the signaling-active surface, which is available for recruitment of adapter proteins or signaling molecules, is much larger than the cell surface. Besides colocalization of IKK $\beta$  and GMR $\alpha$  on endocytic vesicles, we could also demonstrate a direct interaction of these 2 molecules based on fluorescence resonance energy transfer (Figure 2C), which occurs only between ECFP and EYFP tags that are in very close proximity (less than 10 nm). Moreover, this observation also suggests that the interaction is a direct one without any bridging molecule in between, because in the latter case the distance of the fluorophores most likely would be too far for giving a clear FRET signal. This notion is also in line with the data obtained in the yeast system, because it is rather unlikely that endogenous proteins of yeast would be able to mediate an interaction between 2 human signaling proteins that are phylogenetically rather young.

Furthermore, GMCSF is able to activate endogenous levels of I $\kappa$ B kinases, as revealed by *in vitro* kinase assays after immunoprecipitation of IKK proteins from cells following GMCSF addition (Figure 3A). The peak of kinase activity occurred about 10 minutes after addition of GMCSF, which is very similar to the reported kinetics of IKK $\beta$  activation after addition of TNF- $\alpha$ .<sup>48,49</sup> In line with these data, we could also detect a rapid degradation of I $\kappa$ B $\alpha$  followed by resynthesis (Figure 3B), which is typical for this NF- $\kappa$ B-regulated inhibitor of NF- $\kappa$ B activation.<sup>40</sup> Moreover, the resynthesis of I $\kappa$ B $\alpha$  is an indication for a normal activation of

NF- $\kappa$ B, including liberation from its inhibitor, nuclear translocation, binding to corresponding promoter elements, and induction of transcription. This was also confirmed by electrophoretic mobility shift assays proving the induction of NF- $\kappa$ B DNA binding activity rapidly after addition of GMCSF (Figure 4A). However, in many reporter gene assays based on NF- $\kappa$ B–dependent expression of luciferase, we observed a somewhat slower induction as compared with TNF- $\alpha$ . One possible explanation for this observation is that additional stimulatory pathways are activated by TNF- $\alpha$  that are not induced by GMCSF. It was reported that full activation of NF- $\kappa$ B requires not only liberation of the transcription factor from its inhibitor but also the phosphorylation of NF- $\kappa$ B itself and the presence of coactivators such as CBP (for review, see Baldwin<sup>30</sup> and Silverman and Maniatis<sup>53</sup>). These additional activation pathways are triggered by TNF- $\alpha$ –mediated signaling, whereas it is not clear whether they are also activated by the GMCSF receptor. Another possibility is that a rather low induction of NF- $\kappa$ B by GMCSF results in the expression of TNF- $\alpha$  leading to a subsequent autocrine activation of NF- $\kappa$ B with a certain delay.

The observation of a direct interaction between the GMCSF receptor and IKK $\beta$  and the GMCSF-dependent activation of the kinase activity raises the question about the biologic roles of GMCSF-mediated NF- $\kappa$ B activation. Our results on inhibition of the NF- $\kappa$ B pathway in GMCSF-dependent TF-1 cells indicate that a main role of this signaling pathway is to activate the survival mechanisms that are known to be induced by NF- $\kappa$ B.<sup>29,30</sup> Blocking the NF- $\kappa$ B activation pathway with the specific inhibitor BAY 11-7082 in the presence of GMCSF resulted in a clear induction of apoptosis as revealed by staining with annexin V and the occurrence of fragmented DNA (Figure 5B-C). This effect was similar to that of prolonged GMCSF withdrawal or even more pronounced, which might be an indication for some additional proapoptotic effect of BAY 11-7082. Addition of TNF- $\alpha$  instead of GMCSF had some intermediate effect supporting cell survival but could not completely replace GMCSF. This might be due to additional effects of the JAK-STAT pathway that is activated by GMCSF but not TNF- $\alpha$ . Alternatively, proapoptotic signaling mechanisms induced by TNF- $\alpha$  via tumor necrosis factor receptor 1 (TNFR1) might overrule some of the antiapoptotic effects mediated by NF- $\kappa$ B activation.

Apart from activation of the NF- $\kappa$ B pathway and survival mechanisms by GMCSF, the binding of IKK $\beta$  to the activated GMCSF receptor might have additional biologic roles as indicated by the observation that mutation of all tyrosine residues in the common  $\beta$ -chain could not fully prevent signaling pathways that lead to activation of STAT5.<sup>54</sup> This implies that the tyrosine kinase JAK2 is not the only important signaling molecule in this context, and it is in line with reports that phosphorylation of serine residues (which might be achieved by the serine kinase IKK $\beta$ ) has an important additional role in GMCSF-mediated signaling.<sup>55</sup> This concept is strengthened by 2 recent reports that support a functional link between the IKK complex and the JAK-STAT pathway based on the effect of vitamin C on both NF- $\kappa$ B and STAT5 activation. One report demonstrated that vitamin C inhibits TNF- $\alpha$ –induced NF- $\kappa$ B activation via IKK $\beta$ ,<sup>56</sup> whereas the other report could prove that vitamin C suppresses GMCSF-mediated STAT5 phosphorylation,<sup>57</sup> which might be explained by a contribution of IKK $\beta$  to activation of JAK2 and STAT5.

In conclusion, our data suggest IKK $\beta$  as an important interaction partner of the GMCSF receptor including both the ligand-specific  $\alpha$ -chain and the common  $\beta$ -chain, which transfers the signal of ligand binding into an activation of the kinase activity of IKK $\beta$  resulting in activation of the NF- $\kappa$ B pathway. Furthermore, binding of IKK $\beta$  to the GMCSF receptor and its activation might explain some of the reported signaling crosstalk between the NF- $\kappa$ B and the JAK2-STAT pathway.<sup>26,58</sup> This crosstalk might include reciprocal phosphorylation events leading to mutual regulation of these important pathways—a model that has to be further investigated in the future.

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