A Novel Role for IκBζ in the Regulation of IFNγ Production

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Abstract

IκBζ is a novel member of the IκB family of NFκB regulators, which modulates NFκB activity in the nucleus, rather than controlling its nuclear translocation. IκBζ is specifically induced by IL-1β and several TLR ligands and positively regulates NFκB-mediated transcription of genes such as IL-6 and NGAL as an NFκB binding co-factor. We recently reported that the IL-1 family cytokines, IL-1β and IL-18, strongly synergize with TNFα for IFNγ production in KG-1 cells, whereas the same cytokines alone have minimal effects on IFNγ production. Given the striking similarities between the IL-1R and IL-18R signaling pathways we hypothesized that a common signaling event or gene product downstream of these receptors is responsible for the observed synergy. We investigated IκBζ protein expression in KG-1 cells upon stimulation with IL-1β, IL-18 and TNFα. Our results demonstrated that IL-1β, as well as IL-1β, induced moderate IκBζ expression in KG-1 cells. However, TNFα synergized with IL-1β and IL-18, whereas by itself it had a minimal effect on IκBζ expression. NFκB inhibition resulted in decreased IL-1β/IL-18/TNFα-stimulated IFNγ release. Moreover, silencing of IκBζ expression led to a specific decrease in IFNγ production. Overall, our data suggests that IκBζ positively regulates NFκB-mediated IFNγ production in KG-1 cells.

Introduction

We previously showed that the Interleukin-1 (IL-1) family members, IL-1β and IL-18, synergize with tumor necrosis factor-α (TNFα) for interferon-γ (IFNγ) production in the human acute myeloid leukemic KG-1 cell line [1]. IL-1β and IL-18 signal via the Interleukin-1 receptor (IL-1R) and IL-18R, respectively, both of which belong to the IL-1R family and the interleukin-1R/Toll-like receptor (IL-1R/TLR) superfamily [2–5]. Members of the IL-1R/TLR family share a cytoplasmic domain known as the Toll/interleukin-1 receptor (TIR) domain and recruit similar adaptor proteins, such as MyD88. Due to these and other similarities, the signaling pathways downstream of IL-1Rs and TLRs lead to similar outcomes, such as the activation of NFκB and MAPKs.

Although the IL-1β and TNFα receptors (IL-1R and TNFR) belong to different families, their signaling pathways utilize similar adaptor molecules, such as TRAFs, and lead to the activation of NFκB and MAPKs [6–8]. Therefore, many of the genes induced by IL-1β and TNFα overlap and the two cytokines lead to similar biological effects. However, induction of certain genes, such as neutrophil gelatinase-associated lipocalin (NGAL/lipocalin-2 [9], human β-defensin 2 (hBD2) [10–14], extracellular matrix metalloprotease 3 (MMP-3) [15] and IL-6 [16–18], is specific for IL-1β. In the same way, expression of other genes, such as complement factor H, is specific for the TNFα signaling pathway [17]. Moreover, expression of the novel member of the IκB family of NFκB regulators, IκBζ, has been shown to be specific to the IL-1R/TLR pathway (e.g. upon IL-1α/β, LPS stimulation), not the TNFα pathway [2–5,19–24].

IκBζ expression is immediately induced upon stimulation with TLR ligands and IL-1β [19–31]. Moreover, IκBζ is essential for NFκB-mediated induction of genes encoding for proteins such as IL-6, NGAL, hBD2, IL-12 p40, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [21,24,26,30,32–36], and for the suppression of E-selectin expression [33]. IκBζ has been shown to positively and negatively regulate NFκB-mediated transcription by binding to the p50 subunit of NFκB dimers [19,21,24,26,29,30,32–34,37]. This is in contrast to other IκB members, such as IκBα/β, which are mainly found in the cytosol and modulate NFκB nuclear translocation. IκBζ is most homologous to the nuclear IκB protein, Bcl-3 [19,23,26,29], which also regulates NFκB-mediated transcription as a binding co-factor [38–50].

We recently showed that both, IL-1β and IL-18, synergize with TNFα for IFNγ production in KG-1 cells [1]. Given the similarities between the IL-1R and IL-18R signaling pathways, we hypothesized that a common event downstream of these two receptors is crucial for the observed synergy between IL-1β/IL-18 and TNFα for IFNγ production. Even though both, the IL-1R and IL-18R, belong to the IL-1R/TLR superfamily, and IκBζ expression is specifically induced upon stimulation with several IL-1R/TLR ligands, IκBζ expression has not been investigated in response to IL-18 stimulation. Therefore, we analyzed IκBζ expression in KG-1 cells upon IL-18 and IL-1β stimulation, and the role of IκBζ in...
IFNγ production in response to combined IL-1β/IL-18 and TNFα stimulation. Our results indicate that stimulation with IL-1β and/or IL-18 results in moderate levels of IkBα production, while TNFα has no effect. However, when combined with IL-1β or IL-18, TNFα strongly enhances IkBα protein expression. Moreover, NFκB inhibition, as well as silencing of IkBα expression, resulted in decreased IL-1β/IL-18/TNFα-induced IFNγ production. Furthermore, IL-1R and IL-18R expression analysis indicated that the observed synergy may take place at the receptor level in the case of IL-18 and TNFα, but not IL-1β and TNFα combined stimulation. In summary, our findings indicate that stimulation with the IL-1 cytokines, IL-1β and IL-18, in combination with TNFα results in synergistic KG-1 IFNγ production in an IkBα/NFκB dependent manner.

Methods

Reagents

Purified Escherichia coli (E. coli) LPS (serotype 0111:B4) was obtained from Axxora (San Diego, CA) and cell culture tested. ATP disodium salt from Sigma-Aldrich (St. Louis, MO). Anti-human IL-8 ELISA capture monoclonal Ab (clone 6217), IL-18 monoclonal Ab (clone 159-12B), IL-18 receptor (IL-18R) monoclonal Ab (clone 70625), and human rIL-1β, rIL-18 and rIL-8 were purchased from R&D Systems (Minneapolis, MN). An IkBα polyclonal Ab was obtained from Upstate (Billerica, Massachusetts), actin monoclonal Ab (clone C4) from MP Biomedicals (Solon, OH), p50 monoclonal (clone E-10) Ab and p65 polyclonal Ab from Santa Cruz (Santa Cruz, CA), lamin B1 polyclonal Ab from Abcam (Cambridge, MA), TNFα capture monoclonal Ab (clone 2C8) from Advanced Immunohu scmunochemical (Long Beach, CA), IL-8 ELISA detection polyclonal Ab from Endogen (Rockford, IL), rTNFα from Knoll Pharmaceuticals (Whippenny, NJ), Interleukin-1 receptor antagonist (IL-1ra) from Amgen (Thousand Oaks, CA), and IFNγ and IL-6 ELISA kits from eBioscience (San Diego, CA). Rabbit anti-serum against IkBα and IL-1β and primary immune serum were developed in our laboratory. The NFκB inhibitor, JSH23, was purchased from Calbiochem (San Diego, CA). Rabbit anti-serum against IκBβ was obtained from Upstate (Billerica, Massachusetts).

Cell Culture

KG-1 cells (American Type Culture Collection, ATCC; Manassas, VA) were maintained in RPMI 1640 (Mediatech Inc., Herndon, VA), supplemented with 20% FBS (Atlas Biologicals, Fort Collins, CO) and 1% penicillin/streptomycin in a 37°C humidified incubator with 5% CO2. Peripheral blood monocytes were isolated from human blood by density gradient centrifugation in total, nuclear or cytosolic extracts was estimated using the Bio-Rad Dc protein Lowry assay (Bio-Rad). Samples were boiled in Laemmli’s buffer for 5 minutes or heated at 70°C for 10 minutes in NuPAGE Sample Reducing Agent (Invitrogen). 10–40 μg of total protein were loaded per well on pre-cast 10% Tris-Glycine or 7.5% Tris-Acetate SDS-PAGE gels and transferred to a PVDF or nitrocellulose membrane. Membranes were blocked with 10% nonfat milk (Carnation, Nestle) in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween for 1 hour at RT. The membranes were probed with the indicated primary Abs, followed by peroxidase-conjugated secondary antibodies. Protein bands were visualized by chemiluminescence (GE Healthcare).

ELISA

Sandwich ELISAs were used to measure cytokine release in the supernatants of KG-1 cells.

Flow Cytometry

KG-1 cells (106/ml) were stimulated, or not, with the indicated combinations of rIL-1β, rIL-18 and rTNFα (10 ng/ml each) for 24 h. Cells were Fc-blocked by treating with 1 μg of human IgG/105 for 15 min at RT. Cells (105/25 μl reaction) were transferred to a 5 ml tube. Phycocythrithin (PE)-conjugated anti-IL-18Rα or fluorescein (FITC)-conjugated anti-IL-1R1 reagent (10 μl of each per reaction) were added to the cells. Cells were incubated for 30 min at 4°C, washed twice with 1×PBS and re-suspended in 1×PBS (107/200 μl) for flow cytometric analysis. As controls, cells were also treated with phycocythrithin-labeled murine IgG1, and fluorescein-labeled goat IgG.

Nuclear/Cytosolic extraction

KG-1 cells (106/ml) were stimulated, or not, with rIL-1β, rIL-18, rTNFα (10 ng/ml each), or a combination of this cytokines for the indicated time points. Cells were washed twice in 1×PBS and gently re-suspended in cold Buffer A (10 mM HEPES, pH 7.9; 10 mM KC1; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM diithiothreitol [DTT]; 1×Complete Mini protease inhibitor cocktail, Roche) at 400 μl/0.5–1×105 cells. Cells were allowed to swell for 15 min. 10%
Nonidet NP-40 was added to the solution (25 µl per 400 µl). Samples were vortexed for 10 sec and centrifuged for 30 sec (4°C, 13,200 rpm). Supernatants containing cytosolic contents were transferred to fresh tubes containing an equal volume of Buffer B (10 mM Tris-HCl, pH 7.5; 7 M urea; 1% SDS; 0.3 M NaAc; 20 mM EDTA) and stored immediately at −20°C. The pellets containing the nuclear contents were re-suspended in cold Buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol (DTT); 1 mM Complete Mini protease inhibitor cocktail, Roche) at 50 µl/0.5–1×10^6 cells. The samples were vigorously shook for 15 min at 4°C on a shaking platform and then centrifuged for 5 min (4°C, 13,200 rpm). The supernatants with the nuclear contents were stored at −20°C. Nuclear and cytosolic extracts were subsequently analyzed for protein concentration using the Bio-Rad Dc protein Lowry assay (Bio-Rad). Nuclear and cytosolic extracts were then prepared for Western blot analysis.

Small interfering RNA

KG-1 cells (2×10^6/ml) were nucleofected following the protocol for KG-1 cell nucleofection provided with the cell line nucleofector kit R from Amaxa (Gaithersburg, MD) with a mixture of 3 different small interfering RNA (siRNA) oligonucleotides against IκBα or 3 different scrambled siRNA oligonucleotides (3 µg per 2×10^6 cells). After 2 h, cells were stimulated with a combination of rIL-1β, rIL-18 and rTNFα (10 ng/ml each). Cells and supernatants were harvested at 24 h for subsequent RNA and protein analysis (qPCR and Western blot, respectively).

Quantitative PCR (qPCR)

KG-1 cells (10^6/ml) were lysed in TRIzol reagent (Invitrogen Life Technologies) and mRNA was extracted and converted to cDNA using the Thermoscript RT-PCR system (Invitrogen Life Technologies). qPCR was performed using specific primers for IFNγ, IL-6 and IL-8. Values were normalized to two housekeeping genes, CAP-1 and GAPDH.

Statistical analysis

Data are presented mean±S.E.M. from≥3 independent experiments. Comparisons were done by paired t-test with p<0.05 defined as statistically significant.

Results

IL-1β and IL-18, but not TNFα, induce IκBα protein expression in KG-1 cells

We have recently demonstrated that IL-1R and IL-18R agonists synergize with TNFα for IFNγ production in KG-1 cells [1]. The IL-1R, IL-18R and TLRs all belong to the IL-1R/TLR superfamily [2]. Because expression of the IκB protein family member, IκBα, is known to be induced downstream of the IL-1R and TLRs [19–31], we chose to evaluate its potential role in IFNγ production in response to IL-1β/IL-18 and TNFα combined stimulation. We analyzed induction of IκBα protein expression in response to IL-1β and IL-18, with the idea that IκBα may be the common factor downstream of the IL-1R and IL-18R, responsible for the observed synergy between IL-1β/IL-18 and TNFα for IFNγ production [1].

In this context, KG-1 cells were stimulated with rIL-1β, rIL-18 and rTNFα for various time points, with and without co-addition of IL-1α or IL-18 Ab. Total cell extracts were analyzed for IκBα protein expression by Western blotting. Results indicated that both rIL-1β and rIL-18, but not rTNFα, induced IκBα expression in KG-1 cells (Fig. 1A-C). The lack of rTNFα-mediated IκBα expression was not due to lack of biological activity of rTNFα, as judged by the modulation (degradation and de novo synthesis) of IκBβ expression upon rTNFα stimulation (Fig. 1C).

The finding that IL-1β, as well as IL-18, both induce IκBα protein expression supports the hypothesis that IκBα may be the common factor downstream of the IL-1R and IL-18R that allows for synergy between IL-1 cytokines (IL-1β and IL-18) and TNFα for IFNγ production in KG-1 cells.

TNFα enhances IL-1β/IL-18-mediated IκBα expression.

Stimulation with TNFα alone does not lead to IκBα expression (Fig. 1C) [19,21,24]. However, TNFα has been shown to induce IκBα transcription to a greater extent than IL-1β and LPS [20]. In contrast, upon actinomycin D treatment, the half-life of ectopically expressed IκBα mRNA was prolonged with IL-1β and LPS, but unaffected by TNFα. Therefore, even though TNFα has strong IκBα transcriptional activity, IL-1/LPS may provide additional mRNA stabilization (absent with solo TNFα stimulation) leading to subsequent protein expression. Based on this information, we...
hypothesized that TNFα may enhance IL-1β/IL-18-mediated IκBα protein expression by providing strong transcriptional activation, even though by itself it does not lead to IκBα protein expression.

To test this hypothesis, KG-1 cells were stimulated with rTNFα alone and in combination with rIL-1β, rIL-18, or both, for 8 and 24 h. Total cell extracts were analyzed for IκBα protein expression by Western blotting. Recombinant TNFα enhanced rIL-1β- and rIL-18-mediated IκBα protein expression at both time points (Fig. 2). We then analyzed the kinetics of KG-1 IκBα protein expression in response to different combinations of rIL-1β, rIL-18 and rTNFα, with and without co-addition of IL-1ra, IL-18R Ab, TNFα Ab, or different combinations of these neutralizing agents. Interestingly, IκBα protein expression followed an oscillating pattern (Fig. 3), which is typical of IκB proteins, such as IκBα. Moreover, it was evident that the observed induction of IκBα protein upon stimulation with rTNFα combined with rIL-1β, rIL-18, or both, was in part due to rTNFα, since the induction was only partially suppressed with a TNFα neutralizing Ab (Fig. 3B and C). The remaining IκBα protein expression after TNFα neutralization was likely due to IL-1β and/or IL-18. As expected, neutralization with IL-1ra or IL-18R Ab, resulted in complete inhibition of IκBα expression, indicating that TNFα by itself has no IκBα inducing activity. Interestingly, rIL-1β, rIL-18 or the combination of these cytokines (at a dose of 10 ng/ml) results in minimal amounts of IFNγ production by KG-1 cells, despite their ability to induce IκBα protein expression [1]. Therefore, the levels of IL-1β/IL-18-induced IκBα protein may either not be sufficient for significant IFNγ production (in the absence of TNFα stimulation), or may require an additional TNFα-induced factor for activation of the IFNγ promoter.
The conditioned media from LPS/ATP-stimulated monocytes induces \( \text{I} \kappa \text{B} \gamma \) expression in an IL-1\( \beta \)/TNF\( \alpha \)-dependent, but IL-18-independent manner.

We have recently shown that the conditioned media from LPS/ATP-treated monocytes induces IFN\( \gamma \) release by KG-1 cells and that this induction is due to the synergistic effect of IL-1\( \beta \) and TNF\( \alpha \), and independent of IL-18 [1]. Herein, we incubated KG-1 cells with conditioned media from LPS/ATP-stimulated monocytes for various time points, with and without co-addition of IL-1\( \alpha \), IL-18R Ab, TNF\( \alpha \) Ab, or different combinations of these neutralizing agents, and analyzed \( \text{I} \kappa \text{B} \gamma \) protein expression. The monocyte conditioned media induced \( \text{I} \kappa \text{B} \gamma \) protein expression in an IL-1\( \beta \)-dependent, but IL-18-independent manner (Fig. 4). This finding correlates with our previous observation that endogenous IL-18 present in the conditioned media from LPS/ATP-stimulated monocytes does not induce IFN\( \gamma \) production by KG-1 cells [1]. The lack of IL-18 IFN\( \gamma \) inducing activity in the supernatants of LPS/ATP-stimulated monocytes may be due to low levels of IL-18 being released or to IL-18 being bound to its biological inhibitor, IL-18BP [1].

**TNF\( \alpha \) upregulates IL-18R, not IL-1R expression**

TNF\( \alpha \) has been shown to upregulate expression of the IL-18R in KG-1 cells [51–57]. Therefore, TNF\( \alpha \) may synergize with IL-1\( \beta \) in a similar manner for \( \text{I} \kappa \text{B} \gamma \) and IFN\( \gamma \) production — by upregulating surface expression of the IL-1R. In order to explore this possibility, KG-1 cells were treated with the indicated combinations of rIL-1\( \beta \), rIL-18 and rTNF\( \alpha \) for 24 h. TNF\( \alpha \) treatment resulted in upregulation of IL-18R, not IL-1R expression, as determined by flow cytometry (Fig. 5). Therefore, a signaling event downstream of the IL-1R and IL-18R, rather than TNF\( \alpha \)-mediated receptor upregulation, is likely to be crucial for the observed synergy between IL-1 cytokines and TNF\( \alpha \) for \( \text{I} \kappa \text{B} \gamma \) and IFN\( \gamma \) production. Moreover, the greater IFN\( \gamma \) production in response to rIL-1\( \beta \) in combination with rTNF\( \alpha \), compared to rIL-1\( \beta \) in combination with rTNF\( \alpha \), may be explained by additional TNF\( \alpha \)-mediated upregulation of the IL-18R.

**\( \text{I} \kappa \text{B} \gamma \) protein localizes to the nucleus**

\( \text{I} \kappa \text{B} \gamma \) protein has been shown to localize to the nucleus in most cell types [19,23,26,28,34]. However, \( \text{I} \kappa \text{B} \gamma \) has also been shown to localize to the cytoplasm in B cell rich regions of immune organs, such as lymphoid follicles in the spleen [20]. In order to confirm the cellular localization of \( \text{I} \kappa \text{B} \gamma \) protein in the KG-1 cell line, cells were stimulated with rIL-1\( \beta \), rIL-18, rTNF\( \alpha \) and different combinations of these cytokines for 8 h and harvested for cytosol and nuclear extraction. Results demonstrate that \( \text{I} \kappa \text{B} \gamma \) protein localizes predominantly to the nucleus of KG-1 cells (Fig. 6), consistent with its role as a co-factor for NF\( \kappa \)B-mediated transcription.

**NF\( \kappa \)B inhibition leads to decreased IFN\( \gamma \) and IL-6 release**

\( \text{I} \kappa \text{B} \gamma \) has been shown to act as an NF\( \kappa \)B binding co-factor by associating with the p50 NF\( \kappa \)B subunit. Therefore, we decided to test whether IFN\( \gamma \) release in KG-1 cells in response to IL-1\( \beta \), IL-18 and TNF\( \alpha \) combined stimulation is NF\( \kappa \)B dependent. KG-1 cells were incubated with an inhibitor of NF\( \kappa \)B nuclear...
translocation followed by IL-1β, IL-18 and TNFα combined stimulation. Western blot analysis with nuclear extracts indicated a reduction in p50 and p65 nuclear localization, indicative of a decrease in NFκB activity (data not shown). Moreover, IL-6 and IFNγ release were significantly reduced with NFκB inhibition (Fig. 7). Therefore, IkBζ may regulate IFNγ release in KG-1 cells in response to IL-1β, IL-18 and TNFα combined stimulation by acting as a co-factor for NFκB-mediated transcription.

Silencing of IkBζ suppresses IFNγ and IL-6, not IL-8 production

IkBζ has been shown to either negatively or positively regulate NFκB activity depending upon the context. Genes, such as E-selectin, are negatively regulated [33], whereas genes such as IL-6 and NGAL, are positively regulated [21,24,26,32–35]. Because IL-1β/IL-18 or TNFα blockade inhibits both IkBζ protein expression and IFNγ production in KG-1 cells, we hypothesized that IkBζ positively regulates IFNγ production.

In order to test this hypothesis, KG-1 cells were nucleofected with a mixture of 3 different small interfering RNA (siRNA) oligonucleotides against IkBζ or 3 different scrambled siRNA oligonucleotides. Cells were then stimulated with a combination of rIL-1β, rIL-18 and rTNFα. Western blot analysis indicated a reduction in IkBζ protein expression with anti-IkBζ siRNA delivery (Fig. 8). In order to determine the effect of IkBζ silencing on IFNγ protein production, we measured IFNγ mRNA levels and protein release in KG-1 cells upon rIL-1β, rIL-18 and rTNFα combined stimulation (Fig. 9). As a positive
control, we measured IL-6 mRNA and protein release since this cytokine has been shown to be positively regulated by I\(\kappa\)B\(\alpha\) [21,26,32,34,35]. As a negative control, we measured IL-8 mRNA and protein levels, which have been shown not to be regulated by I\(\kappa\)B\(\alpha\) [24,33]. Results indicated that the mRNA and protein levels of IFN\(\gamma\) and IL-6, but not IL-8, were significantly reduced with anti-I\(\kappa\)B\(\alpha\) siRNA delivery (Fig. 9). These results implicate a role for I\(\kappa\)B\(\alpha\) as a positive regulator of IFN\(\gamma\) production.

Discussion

Regulation of IFN\(\gamma\) gene transcription involves the action of many different transcription factors including STATs, AP-1, GATA-3, NFAT, T-bet, Eomesodermin, NFKB, NFAT, T-bet, YY-1, DREAM, ERM and SMADs. In most cases, multiple signals synergize for IFN\(\gamma\) production via induction of different transcription factors that act in concert to induce gene expression [58–61]. The combination of IL-12 and IL-18 is the most well-known example of synergy between two cytokines for IFN\(\gamma\) production in T cells, NK/NKT cells, B cells, macrophages and dendritic cells [62–70]. Synergy between IL-12 and IL-18 occurs not only at the transcription factor level via STAT4 and AP-1 activation, respectively, but also at the receptor level, with both cytokines upregulating cell surface expression of each other’s receptors. Synergy for IFN\(\gamma\) production has also been observed with the combination of receptor crosslinking and cytokine

Figure 9. Silencing of I\(\kappa\)B\(\alpha\) expression suppresses IFN\(\gamma\) and IL-6, not IL-8, mRNA and protein production. KG-1 cells (2\(\times\)10^6/ml) were nucleofected with a mixture of 3 different small interfering RNA (siRNA) oligonucleotides against I\(\kappa\)B\(\alpha\) or 3 different scrambled siRNA oligonucleotides. After 2 h, cells were stimulated with a combination of rIL-1\(\beta\), rIL-18 and rTNF\(\alpha\) (10 ng/ml each) for 24 h. Cells were lysed for mRNA extraction. Messenger RNA (mRNA) was converted to cDNA, followed by quantitative PCR (qPCR) using primers specific for IFN\(\gamma\) (A), IL-6 (B) and IL-8 (C). Supernatants were harvested and analyzed for cytokine release by IFN\(\gamma\) (D), IL-6 (E) and IL-8 (F) ELISA. Results are shown as mean±S.E.M. *, \(p<0.05\); **, \(p<0.005\) (A, B and C, \(n=3\)) (D, E and F, \(n=5\)).
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stimulation. As an example, the combination of LY49 activating receptor crosslinking and IL-12 or IL-10 synergistically enhance IFNγ production in NK cells via the p38 MAP kinase and the ERK-dependent signal transduction pathways [71].

In general, IL-12 and IL-18 require each other for IFNγ gene expression. However, at high doses (50 ng/ml), IL-18 alone can induce IFNγ production in the human acute myeloid leukemia KG-1 cell line [1,24]. KG-1 cells have been widely used to study IL-18-mediated signaling events leading to IFNγ expression. The responsiveness of KG-1 cells to IL-18 (in absence of IL-12) is partly due to constitutive expression of both chains of the IL-18R [72,73], whereas primary NK and T cells require IL-12 stimulation for expression of the binding chain of the IL-18R [70,74–77].

NFκB has been shown to regulate the expression of many proinflammatory genes, IFNγ being no exception. Two putative NFκB binding sites have been identified in the IFNγ promoter region (κBB site and CD23RE) and one in the first intron (C3) [78]. The requirement for NFκB in IFNγ gene expression appears to be contingent on the cell type and the specific stimulus. IL-18 signaling via the IL-18R leads to NFκB activation [78–82]. Moreover, stimulation of KG-1 cells with high doses (50 ng/ml) of IL-18 leads to IFNγ production in an NFκB-dependent manner [78].

We have previously described a novel synergistic role for the members of the IL-1 family, IL-1β and IL-18, in combination with TNFα in IFNγ production in KG-1 cells [1]. Importantly, at the dose of 10 ng/ml, the individual cytokines induced only minimal amounts of IFNγ release. Given the striking similarities between the IL-1β and IL-18 signaling pathways, we proposed that a common factor downstream of the IL-1R and IL-18R is responsible for the observed synergy between IL-1β/IL-18 and TNFα. The latter is supported by the fact that induction of the IFNγ promoter is generally mediated by multiple signals leading to activation of multiple transcription factors that synergistically induce IFNγ gene expression [58–61].

The novel member of the κB family of NFκB regulators, IkBκ, is known to be induced by IL-1R/TLR ligands. Moreover, even though the TNFR signaling pathway shares some similarities with the IL-1R/TLR pathway, such as the use of TRAF adaptor molecules, TNF signaling alone does not result in IkB protein expression [2-5,19–24]. Although IL-18 signals via a member of the IL-1R family, it has never been tested as an inducer of IkBκ expression. We have shown for the first time that IL-18 stimulation also leads to IkBκ protein expression in KG-1 cells.

IkBκ has been shown to positively regulate NFκB-mediated transcription of secondary response genes such as IL-6 and NGAL, as a co-factor binding to the p50 NFκB subunit [21,24,26,32,33,35]. Moreover, NFκB has been shown to play an important role as a positive regulator of IFNγ gene expression in thymocytes, peripheral blood T lymphocytes and KG-1 cells [66,70,93]. Therefore, we hypothesized that IkBκ may be the common factor downstream of the IL-1R and IL-18R pathways, which allows for synergy between IL-1 cytokines and TNFα for IFNγ production in KG-1 cells.

Interestingly, we observed that TNFα enhanced IL-1β/IL-18-mediated IkBκ protein expression, even though by itself it had not effect on IkBκ protein expression. However, TNFα, IL-1β and LPS have all been shown to induce IkBκ mRNA transcription in NIH3T3 and A549 cells [20]. Importantly, TNFα stimulation alone results in strong activation of the IkBκ promoter without subsequent protein expression. Moreover, nuclear run-on analysis in NIH3T3 cells also indicates that TNFα is a stronger transcriptional activator of the IkBκ gene, compared to IL-1β or LPS. Furthermore, decay analysis of ectopically expressed IkBκ mRNA upon actinomycin D treatment, indicates that degradation of IkBκ mRNA is delayed by IL-1β and LPS stimulation, but not by TNFα stimulation. Moreover, the N-terminal half, not the C-terminal half, of the IkBκ ORF confers IL-1/LPS-mediated IkBκ mRNA stabilization. Therefore, the specificity of IL-1/LPS stimulus for IkBκ mRNA and protein induction is most likely at the post-transcriptional level and due to stabilization of IkBκ mRNA. A cis-element in the N-terminal half of the IkBκ gene appears to be crucial for this stabilization [20].

In support of the latter findings, the NGAL promoter has been shown to be specifically induced by IL-1β, not by TNFα [9,21,84,85]. NGAL promoter activity requires NFκB activation and an intact NFκB binding site [24]. Even though IL-1β and TNFα, both induce NFκB nuclear translocation and recruitment to the NGAL promoter, only IL-1β is able to induce NGAL expression. IkBκ has been shown to be the co-factor which allows NFκB to mediate NGAL gene expression downstream of the IL-1R/TLR signaling pathway [24]. The latter was shown by IkBκ over expression in A549 cells, which rescued TNFα-induced NGAL expression. The same may apply to expression of the IL-6 gene, which at least in the KG-1 cell line (data not shown) and other cell types [16–18] is specific to IL-1β, not TNFα stimulation, and to other genes specifically induced downstream of the IL-1R/TLR pathway. As an additional example, hBD2 is also stimulated by IL-1β, but not TNFα stimulation in human keratinocytes [9,84] and A549 cells [24]. Moreover, siRNA experiments have shown that IkBκ is critical for IL-1β mediated hBD2 mRNA expression in A549 cells [24].

Based on this information, we conclude that while IL-1β and IL-18 may provide the signal(s) required for IkBκ transcriptional activation, mRNA stabilization and subsequent IkBκ protein expression, TNFα may enhance IL-1β/IL-18-mediated IkBκ expression by providing strong transcriptional activation of the IkBκ gene. Moreover, TNFα stimulation of KG-1 cells provides robust binding of the p50 and p65 NFκB subunits to EMSA probes containing the NFκB binding sites present in the IFNγ promoter and first intron (data not shown), compared to weaker binding provided by IL-1β and IL-18. Therefore, robust NFκB activation provided by TNFα may also result in increased IFNγ gene expression since IkBκ regulates transcription as a co-factor for NFκB and KG-1 IFNγ production in response to IL-1/TLR combination stimulation is NFκB dependent (Fig. 7). Thus, the synergy between IL-1β/IL-18 and TNFα may be due to their combined effects on IkBκ expression, as well as on NFκB activation. Alternatively, other transcription factors induced by TNFα may synergize with IkBκ/NFκB for IFNγ gene expression. Receptor expression analysis indicated that TNFα-mediated upregulation of IL-1/IL-18R expression did not account for the synergy between these cytokines for IkBκ production.

In summary, we have shown a positive role for IkBκ on IFNγ production in response to IL-1β, IL-18 and TNFα combined stimulation in KG-1 cells. This regulation is most likely dependent on the ability of IkBκ to regulate NFκB mediated transcription of the IFNγ gene. This finding represents a new addition to the complex and continuously growing literature on the regulation of IFNγ expression.

Author Contributions
Conceived and designed the experiments: RMR SS. Performed the experiments: RMR YK VBA. Analyzed the data: RMR YK SS MDW. Contributed reagents/materials/analysis tools: HW DCG MDW. Wrote the paper: RMR MDW.
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