

# A Novel Role for I $\kappa$ B $\zeta$ in the Regulation of IFN $\gamma$ Production

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## Abstract

I $\kappa$ B $\zeta$  is a novel member of the I $\kappa$ B family of NF $\kappa$ B regulators, which modulates NF $\kappa$ B activity in the nucleus, rather than controlling its nuclear translocation. I $\kappa$ B $\zeta$  is specifically induced by IL-1 $\beta$  and several TLR ligands and positively regulates NF $\kappa$ B-mediated transcription of genes such as IL-6 and NGAL as an NF $\kappa$ B binding co-factor. We recently reported that the IL-1 family cytokines, IL-1 $\beta$  and IL-18, strongly synergize with TNF $\alpha$  for IFN $\gamma$  production in KG-1 cells, whereas the same cytokines alone have minimal effects on IFN $\gamma$  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 I $\kappa$ B $\zeta$  protein expression in KG-1 cells upon stimulation with IL-1 $\beta$ , IL-18 and TNF $\alpha$ . Our results demonstrated that IL-18, as well as IL-1 $\beta$ , induced moderate I $\kappa$ B $\zeta$  expression in KG-1 cells. However, TNF $\alpha$  synergized with IL-1 $\beta$  and IL-18, whereas by itself it had a minimal effect on I $\kappa$ B $\zeta$  expression. NF $\kappa$ B inhibition resulted in decreased IL-1 $\beta$ /IL-18/TNF $\alpha$ -stimulated IFN $\gamma$  release. Moreover, silencing of I $\kappa$ B $\zeta$  expression led to a specific decrease in IFN $\gamma$  production. Overall, our data suggests that I $\kappa$ B $\zeta$  positively regulates NF $\kappa$ B-mediated IFN $\gamma$  production in KG-1 cells.

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## Introduction

We previously showed that the Interleukin-1 (IL-1) family members, IL-1 $\beta$  and IL-18, synergize with tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) for interferon- $\gamma$  (IFN $\gamma$ ) production in the human acute myeloid leukemic KG-1 cell line [1]. IL-1 $\beta$  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 NF $\kappa$ B and MAPKs.

Although the IL-1 $\beta$  and TNF $\alpha$  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 NF $\kappa$ B and MAPKs [6–8]. Therefore, many of the genes induced by IL-1 $\beta$  and TNF $\alpha$  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  $\beta$ -defensin 2 (hBD2) [10–14], extracellular matrix metalloprotease 3 (MMP-3) [15] and IL-6 [16–18], is specific for IL-1 $\beta$ . In the same way, expression of other genes, such as complement factor H, is specific for the TNF $\alpha$  signaling pathway [17]. Moreover, expression of the novel member of the I $\kappa$ B family of NF $\kappa$ B regulators, I $\kappa$ B $\zeta$ , has been shown to be specific to the IL-

1R/TLR pathway (e.g. upon IL-1 $\alpha/\beta$ , LPS stimulation), not the TNF $\alpha$  pathway [2–5,19–24].

I $\kappa$ B $\zeta$  expression is immediately induced upon stimulation with TLR ligands and IL-1 $\beta$  [19–31]. Moreover, I $\kappa$ B $\zeta$  is essential for NF $\kappa$ B-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]. I $\kappa$ B $\zeta$  has been shown to positively and negatively regulate NF $\kappa$ B-mediated transcription by binding to the p50 subunit of NF $\kappa$ B dimers [19,21,24,26,29,30,32–34,37]. This is in contrast to other I $\kappa$ B members, such as I $\kappa$ B $\alpha/\beta$ , which are mainly found in the cytosol and modulate NF $\kappa$ B nuclear translocation. I $\kappa$ B $\zeta$  is most homologous to the nuclear I $\kappa$ B protein, Bcl-3 [19,23,26,29], which also regulates NF $\kappa$ B-mediated transcription as a binding co-factor [38–50].

We recently showed that both, IL-1 $\beta$  and IL-18, synergize with TNF $\alpha$  for IFN $\gamma$  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-1 $\beta$ /IL-18 and TNF $\alpha$  for IFN $\gamma$  production. Even though both, the IL-1R and IL-18R, belong to the IL-1R/TLR superfamily, and I $\kappa$ B $\zeta$  is specifically induced upon stimulation with several IL-1R/TLR ligands, I $\kappa$ B $\zeta$  expression has not been investigated in response to IL-18 stimulation. Therefore, we analyzed I $\kappa$ B $\zeta$  expression in KG-1 cells upon IL-18 and IL-1 $\beta$  stimulation, and the role of I $\kappa$ B $\zeta$  in

IFN $\gamma$  production in response to combined IL-1 $\beta$ /IL-18 and TNF $\alpha$  stimulation. Our results indicate that stimulation with IL-1 $\beta$  and/or IL-18 results in moderate levels of I $\kappa$ B $\zeta$  production, while TNF $\alpha$  has no effect. However, when combined with IL-1 $\beta$  or IL-18, TNF $\alpha$  strongly enhances I $\kappa$ B $\zeta$  protein expression. Moreover, NF $\kappa$ B inhibition, as well as silencing of I $\kappa$ B $\zeta$  expression, resulted in decreased IL-1 $\beta$ /IL-18/TNF $\alpha$ -induced IFN $\gamma$  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 TNF $\alpha$ , but not IL-1 $\beta$  and TNF $\alpha$  combined stimulation. In summary, our findings indicate that stimulation with the IL-1 cytokines, IL-1 $\beta$  and IL-18, in combination with TNF $\alpha$  results in synergistic KG-1 IFN $\gamma$  production in an I $\kappa$ B $\zeta$ /NF $\kappa$ B 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-1 $\beta$ , rIL-18 and rIL-8 were purchased from R&D Systems (Minneapolis, MN). An I $\kappa$ B $\alpha$  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), TNF $\alpha$  capture monoclonal Ab (clone 2C8) from Advanced Immunochemical (Long Beach, CA), IL-8 ELISA detection polyclonal Ab from Endogen (Rockford, IL), rTNF $\alpha$  from Knoll Pharmaceuticals (Whippany, NJ), Interleukin-1 receptor antagonist (IL-1ra) from Amgen (Thousand Oaks, CA), and IFN $\gamma$  and IL-6 ELISA kits from eBioscience (San Diego, CA). Rabbit anti-serum against I $\kappa$ B $\zeta$  and IL-1 $\beta$  and pre-immune serum were developed in our laboratory. The NF $\kappa$ B inhibitor, JSH23, was purchased from Calbiochem (San Diego, CA). A mixture of three different small interfering RNA (siRNA) oligonucleotides against I $\kappa$ B $\zeta$  and three different scrambled siRNA oligonucleotides were purchased from Dharmacon RNA Technologies (Lafayette, CO). The cell line nucleofector kit R was purchased from Amaxa (Gaithersburg, MD).

### 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% CO<sub>2</sub>. Peripheral blood monocytes were isolated from human blood by density gradient centrifugation using lymphocyte separation media (Cell Grow, Media Tech, Herndon, VA) followed by CD14 positive selection (Miltenyi Biotec, Auburn, CA), which yields >95% purity as determined by FACS. Isolated monocytes were cultured at 10<sup>6</sup>/ml or at 12.5×10<sup>6</sup>/ml in RPMI 1640 (Cambrex, East Rutherford, New Jersey), supplemented with 5% FBS (<0.0005 EU/ml) (HyClone, Logan, UT) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY), in a 37°C humidified incubator with 5% CO<sub>2</sub>. Monocytes were stimulated, or not, with E. coli LPS (10 ng/ml) for 4 h and ATP (5 mM) for the last 15 min of LPS stimulation, or with LPS or ATP alone. The supernatants (conditioned media) from monocytes cultured at 10<sup>6</sup>/ml were harvested by centrifugation (5 min; 3 600 rpm) and used to stimulate KG-1 cells.

KG-1 cells were plated at a final cell density of 10<sup>6</sup>/ml and incubated with test samples (monocyte conditioned media, 1/3 of the final volume) or recombinant proteins (10 ng/ml each). In selected experiments, neutralizing agents for IL-1 $\beta$  (IL-1 $\beta$  Ab – clone 2805, IL-1ra), IL-18 (IL-18 Ab – clone 125-2H, or IL-18R Ab – clone 70625), and/or TNF $\alpha$  (TNF $\alpha$  Ab – clone 2C8) were used at the indicated concentrations to neutralize the activities of rIL-18, rIL-1 $\beta$  and/or rTNF $\alpha$ , or the same endogenous cytokines present in the monocyte conditioned media, prior to incubation with KG-1 cells. Alternatively, the neutralizing agents, IL-1ra and IL-18R Ab, were used to neutralize IL-18 or IL-1 receptors prior to addition of rIL-18, rIL-1 $\beta$ , or monocyte conditioned media, to the KG-1 cells. KG-1 supernatants and cells were harvested at various time points for subsequent ELISA and Western blot analysis. In selected experiments, cells were treated with the NF $\kappa$ B inhibitor, JSH-23 (30  $\mu$ M) (an NF $\kappa$ B nuclear translocation inhibitor), in combination with rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$  for 24 h. KG-1 cells were harvested at 12 h for subsequent nuclear extraction and Western blot analysis. KG-1 supernatants were harvested at 24 h for IL-6 and IFN $\gamma$  ELISA.

### Western blotting

KG-1 cells were lysed in a 60 mM Tris-HCl (pH 6.8), 2% SDS buffer. Total cell extracts were sonicated and spun (5 min; 10 000 rpm, R.T.) to remove cell debris. KG-1 nuclear/cytosolic extracts were also analyzed by Western blotting. Protein concentration 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 min or heated at 70°C for 10 min in NuPAGE Sample Reducing Agent (Invitrogen). 10–40  $\mu$ g of total protein were loaded per well on pre-cast 10% Tris-Glycine or 7% 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 h at R.T. 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 (10<sup>6</sup>/ml) were stimulated, or not, with the indicated combinations of rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$  (10 ng/ml each) for 24 h. Cells were Fc-blocked by treating with 1  $\mu$ g of human IgG/10<sup>5</sup> for 15 min at R.T. Cells (10<sup>5</sup>/25  $\mu$ l\*reaction) were transferred to a 5 ml tube. Phycoerythrin (PE)-conjugated anti-IL-18R $\alpha$  or fluorescein (FITC)-conjugated anti-IL-1R1 reagent (10  $\mu$ l 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 (10<sup>5</sup>/200  $\mu$ l) for flow cytometric analysis. As controls, cells were also treated with phycoerythrin-labeled murine IgG<sub>1</sub> and fluorescein-labeled goat IgG.

### Nuclear/Cytosolic extraction

KG-1 cells (10<sup>6</sup>/ml) were stimulated, or not, with rIL-1 $\beta$ , rIL-18, rTNF $\alpha$  (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 KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol [DTT]; 1×Complete Mini protease inhibitor cocktail, Roche) at 400  $\mu$ l/0.5–1×10<sup>6</sup> cells. Cells were allowed to swell for 15 min. 10%

Nonidet NP-40 was added to the solution (25  $\mu$ l per 400  $\mu$ l). 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 $\times$ Complete Mini protease inhibitor cocktail, Roche) at 50  $\mu$ l/0.5–1 $\times$ 10<sup>6</sup> 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 $\times$ 10<sup>6</sup>/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 I $\kappa$ B $\zeta$  or 3 different scrambled siRNA oligonucleotides (3  $\mu$ g per 2 $\times$ 10<sup>6</sup> cells). After 2 h, cells were stimulated with a combination of rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$  (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<sup>6</sup>/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 IFN $\gamma$ , IL-6 and IL-8. Values were normalized to two house-keeping genes, CAP-1 and GAPDH.

### Statistical analysis

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

## Results

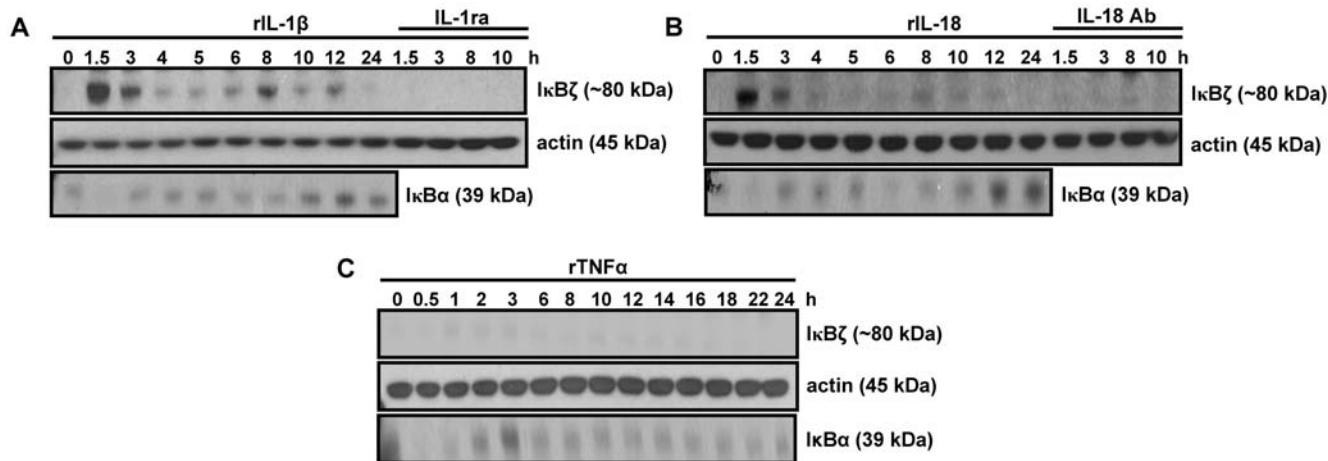
### IL-1 $\beta$ and IL-18, but not TNF $\alpha$ , induce I $\kappa$ B $\zeta$ protein expression in KG-1 cells

We have recently demonstrated that IL-1R and IL-18R agonists synergize with TNF $\alpha$  for IFN $\gamma$  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 I $\kappa$ B protein family member, I $\kappa$ B $\zeta$ , is known to be induced downstream of the IL-1R and TLRs [19–31], we chose to evaluate its potential role in IFN $\gamma$  production in response to IL-1 $\beta$ /IL-18 and TNF $\alpha$  combined stimulation. We analyzed induction of I $\kappa$ B $\zeta$  protein expression in response to IL-1 $\beta$  and IL-18, with the idea that I $\kappa$ B $\zeta$  may be the common factor downstream of the IL-1R and IL-18R, responsible for the observed synergy between IL-1 $\beta$ /IL-18 and TNF $\alpha$  for IFN $\gamma$  production [1].

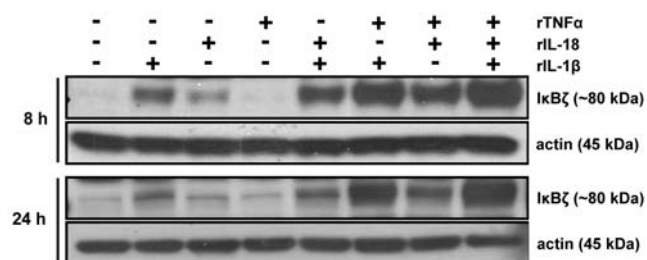
In this context, KG-1 cells were stimulated with rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$  for various time points, with and without co-addition of IL-1ra or IL-18 Ab. Total cell extracts were analyzed for I $\kappa$ B $\zeta$  protein expression by Western blotting. Results indicated that both rIL-1 $\beta$  and rIL-18, but not rTNF $\alpha$ , induced I $\kappa$ B $\zeta$  expression in KG-1 cells (Fig. 1A–C). The lack of rTNF $\alpha$ -mediated I $\kappa$ B $\zeta$  expression was not due to lack of biological activity of rTNF $\alpha$ , as judged by the modulation (degradation and *de novo* protein synthesis) of I $\kappa$ B $\alpha$  expression upon rTNF $\alpha$  stimulation (Fig. 1C). The finding that IL-1 $\beta$ , as well as IL-18, both induce I $\kappa$ B $\zeta$  protein expression supports the hypothesis that I $\kappa$ B $\zeta$  may be the common factor downstream of the IL-1R and IL-18R that allows for synergy between IL-1 cytokines (IL-1 $\beta$  and IL-18) and TNF $\alpha$  for IFN $\gamma$  production in KG-1 cells.

### TNF $\alpha$ enhances IL-1 $\beta$ /IL-18-mediated I $\kappa$ B $\zeta$ expression.

Stimulation with TNF $\alpha$  alone does not lead to I $\kappa$ B $\zeta$  expression (Fig. 1C) [19–21,24]. However, TNF $\alpha$  has been shown to induce I $\kappa$ B $\zeta$  transcription to a greater extent than IL-1 $\beta$  and LPS [20]. In contrast, upon actinomycin D treatment, the half-life of ectopically expressed I $\kappa$ B $\zeta$  mRNA was prolonged with IL-1 $\beta$  and LPS, but unaffected by TNF $\alpha$ . Therefore, even though TNF $\alpha$  has strong I $\kappa$ B $\zeta$  transcriptional activity, IL-1/LPS may provide additional mRNA stabilization (absent with solo TNF $\alpha$  stimulation) leading to subsequent protein expression. Based on this information, we



**Figure 1. I $\kappa$ B $\zeta$  protein expression in KG-1 cells in response to IL-1 $\beta$ , IL-18 and TNF $\alpha$  stimulation.** KG-1 cells (10<sup>6</sup>/ml) were stimulated with rIL-1 $\beta$  (A), rIL-18 (B), or rTNF $\alpha$  (C) (10 ng/ml each) for the indicated time points. At selected time points, the cells were incubated with rIL-1 $\beta$  and rIL-18 in the presence of IL-1ra (100  $\mu$ g/ml) or IL-18 Ab (2  $\mu$ g/ml), respectively. Protein-matched total cell extracts were analyzed by Western blotting using anti-serum against I $\kappa$ B $\zeta$ , I $\kappa$ B $\alpha$  Ab and actin Ab. Results are representative of at least 3 separate experiments. doi:10.1371/journal.pone.0006776.g001



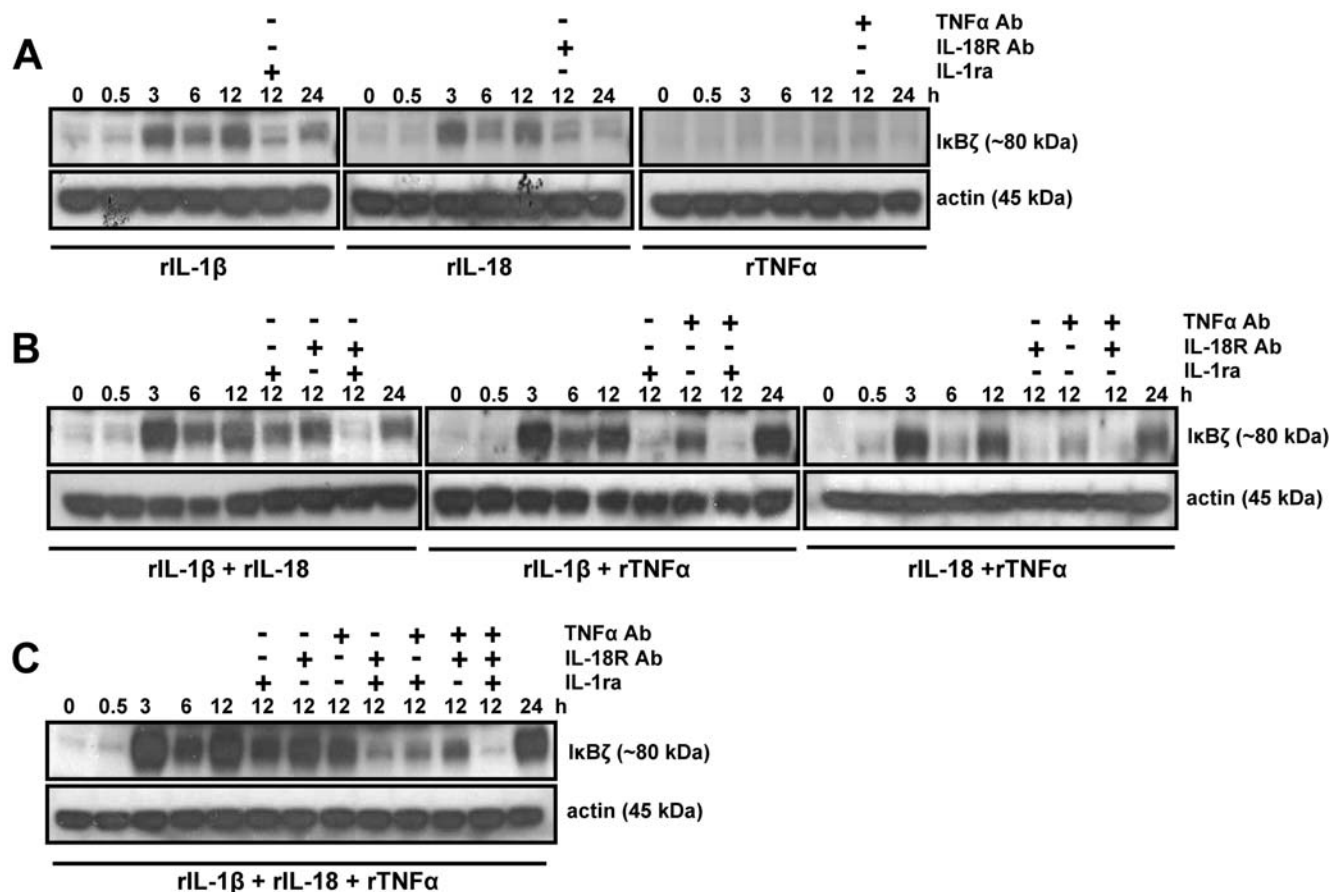
**Figure 2. TNF $\alpha$  enhances IL-1 $\beta$ /IL-18-induced I $\kappa$ B $\zeta$  protein expression in KG-1 cells.** KG-1 cells ( $10^6$ /ml) were stimulated with rIL-1 $\beta$ , rIL-18, rTNF $\alpha$  (10 ng/ml each), or different combinations of these cytokines for 8 and 24 h. Protein-matched total cell extracts were analyzed by Western blotting using anti-serum against I $\kappa$ B $\zeta$  and actin Ab.

doi:10.1371/journal.pone.0006776.g002

hypothesized that TNF $\alpha$  may enhance IL-1 $\beta$ /IL-18-mediated I $\kappa$ B $\zeta$  protein expression by providing strong transcriptional activation, even though by itself it does not lead to I $\kappa$ B $\zeta$  protein expression.

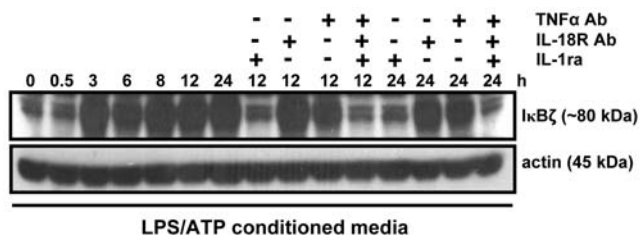
To test this hypothesis, KG-1 cells were stimulated with rTNF $\alpha$  alone and in combination with rIL-1 $\beta$ , rIL-18, or both, for 8 and 24 h. Total cell extracts were analyzed for I $\kappa$ B $\zeta$  protein expression

by Western blotting. Recombinant TNF $\alpha$  enhanced rIL-1 $\beta$ - and rIL-18-mediated I $\kappa$ B $\zeta$  protein expression at both time points (Fig. 2). We then analyzed the kinetics of KG-1 I $\kappa$ B $\zeta$  protein expression in response to different combinations of rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$ , with and without co-addition of IL-1ra, IL-18R Ab, TNF $\alpha$  Ab, or different combinations of these neutralizing agents. Interestingly, I $\kappa$ B $\zeta$  protein expression followed an oscillating pattern (Fig. 3), which is typical of I $\kappa$ B proteins, such as I $\kappa$ B $\alpha$ . Moreover, it was evident that the observed induction of I $\kappa$ B $\zeta$  protein upon stimulation with rTNF $\alpha$  combined with rIL-1 $\beta$ , rIL-18, or both, was in part due to rTNF $\alpha$ , since the induction was only partially suppressed with a TNF $\alpha$  neutralizing Ab (Fig. 3B and C). The remaining I $\kappa$ B $\zeta$  protein expression after TNF $\alpha$  neutralization was likely due to IL-1 $\beta$  and/or IL-18. As expected, neutralization with IL-1ra or IL-18R Ab, resulted in complete inhibition of I $\kappa$ B $\zeta$  expression, indicating that TNF $\alpha$  by itself has no I $\kappa$ B $\zeta$  inducing activity. Interestingly, rIL-1 $\beta$ , rIL-18 or the combination of these cytokines (at a dose of 10 ng/ml) results in minimal amounts of IFN $\gamma$  production by KG-1 cells, despite their ability to induce I $\kappa$ B $\zeta$  protein expression [1]. Therefore, the levels of IL-1 $\beta$ /IL-18-induced I $\kappa$ B $\zeta$  protein may either not be sufficient for significant IFN $\gamma$  production (in the absence of TNF $\alpha$  stimulation), or may require an additional TNF $\alpha$ -induced factor for activation of the IFN $\gamma$  promoter.



**Figure 3. The synergistic effect of TNF $\alpha$  and IL-1 $\beta$ /IL-18 on I $\kappa$ B $\zeta$  protein expression is partially suppressed with a TNF $\alpha$ -specific Ab, but completely blocked with IL-1 $\beta$  and/or IL-18 neutralization.** KG-1 cells ( $10^6$ /ml) were stimulated with rIL-1 $\beta$ , rIL-18, rTNF $\alpha$  (10 ng/ml each) (A), or the indicated combinations of these cytokines (B and C) for the indicated time points. At selected time points, the cells were incubated with the recombinant proteins in the presence of IL-1ra (100  $\mu$ g/ml), IL-18R Ab (10  $\mu$ g/ml), TNF $\alpha$  Ab (10  $\mu$ g/ml), or different combinations of these neutralizing agents. Protein-matched total cell extracts were analyzed by Western blotting using anti-serum against I $\kappa$ B $\zeta$  and actin Ab. Results are representative of at least 3 separate experiments.

doi:10.1371/journal.pone.0006776.g003



**Figure 4. The conditioned media from LPS/ATP-stimulated monocytes induces I $\kappa$ B $\zeta$  expression in an IL-1 $\beta$ /TNF $\alpha$ -dependent, but IL-18-independent manner.** KG-1 cells ( $10^6$ /ml) were incubated with conditioned media from monocytes ( $10^9$ /ml) that were stimulated with LPS (10 ng/ml, 4 h) and ATP (5 mM, last 15 min) for the indicated time points. For selected time points, the cells were incubated with the conditioned media in the presence of IL-1ra (100  $\mu$ g/ml), IL-18R Ab (10  $\mu$ g/ml), TNF $\alpha$  Ab (10  $\mu$ g/ml), or different combinations of these neutralizing agents. Protein-matched total cell extracts were analyzed by Western blotting using anti-serum against I $\kappa$ B $\zeta$  and actin Ab. Results are representative of 3 separate experiments. doi:10.1371/journal.pone.0006776.g004

#### The conditioned media from LPS/ATP-stimulated monocytes induces I $\kappa$ B $\zeta$ expression in an IL-1 $\beta$ -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-1ra, IL-18R Ab, TNF $\alpha$  Ab, or different combinations of these neutralizing agents, and analyzed I $\kappa$ B $\zeta$  protein expression. The monocyte conditioned media induced I $\kappa$ B $\zeta$  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 I $\kappa$ B $\zeta$  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(s) 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 I $\kappa$ B $\zeta$  and IFN $\gamma$  production. Moreover, the greater IFN $\gamma$  production in response to rIL-18 in combination with rTNF $\alpha$ , compared to rIL-1 $\beta$  in combination with rTNF $\alpha$  [1], may be explained by additional TNF $\alpha$ -mediated upregulation of the IL-18R.

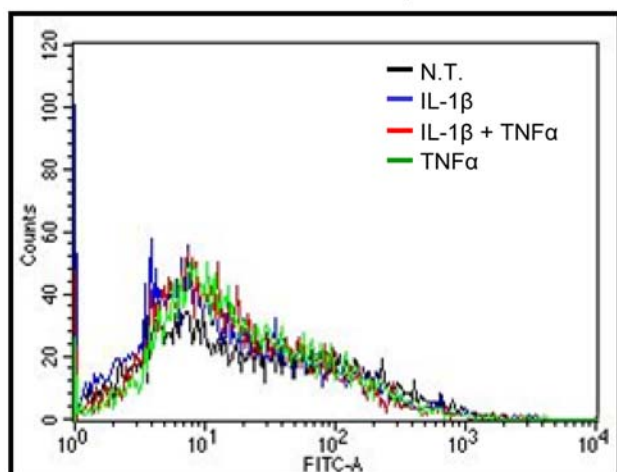
#### I $\kappa$ B $\zeta$ protein localizes to the nucleus

I $\kappa$ B $\zeta$  protein has been shown to localize to the nucleus in most cell types [19,23,26,28,34]. However, I $\kappa$ B $\zeta$  has also been shown to localize to the cytoplasm in B cell rich regions of immune organs, such as lymphoid follicles in the spleen [28]. In order to confirm the cellular localization of I $\kappa$ B $\zeta$  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 I $\kappa$ B $\zeta$  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.

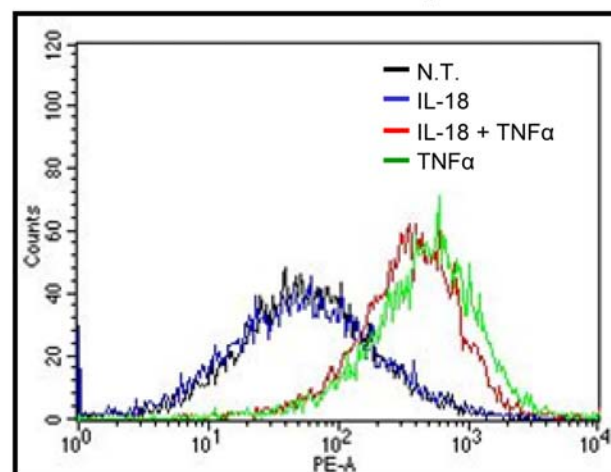
#### NF $\kappa$ B inhibition leads to decreased IFN $\gamma$ and IL-6 release

I $\kappa$ B $\zeta$  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

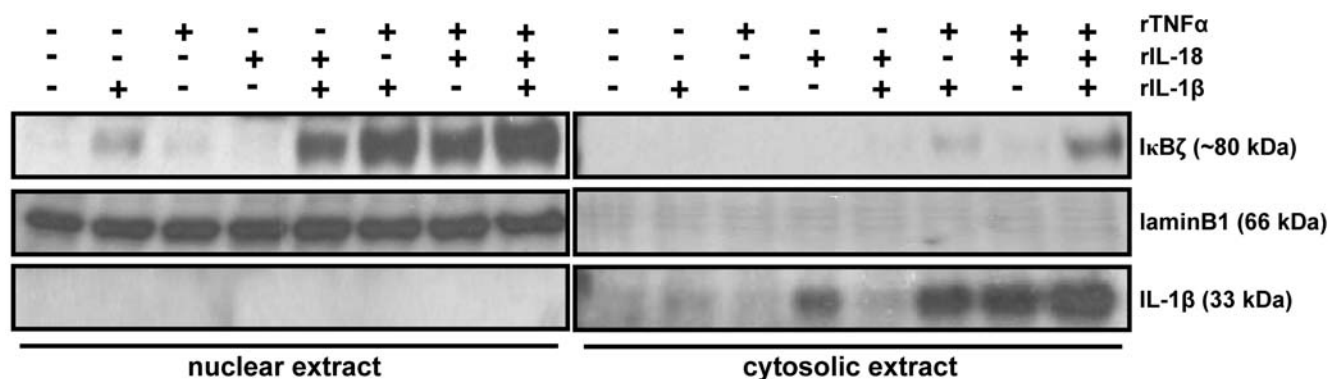
#### IL-1R cell surface expression



#### IL-18R cell surface expression



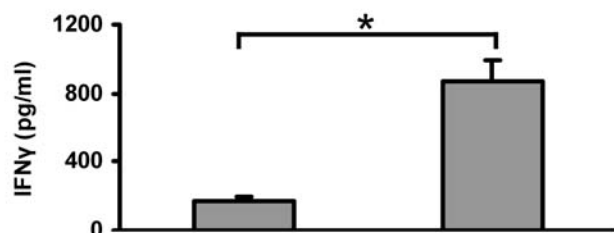
**Figure 5. Effect of TNF $\alpha$  treatment on KG-1 IL-18R and IL-1R surface expression.** KG-1 cells ( $10^6$ /ml) were incubated with the indicated combinations of rIL-1 $\beta$ , rIL-18, rTNF $\alpha$  (10 ng/ml each) for 24 h. Cells were stained with IL-18R-PE and IL-1R-FITC followed by flow cytometry analysis. Results are representative of 3 separate experiments. doi:10.1371/journal.pone.0006776.g005



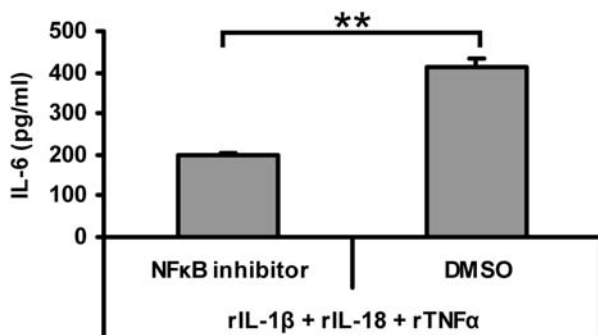
**Figure 6. I $\kappa$ B $\zeta$  mainly localizes to the nucleus.** KG-1 cells ( $10^6$ /ml) were stimulated with rIL-1 $\beta$ , rIL-18, rTNF $\alpha$  (10 ng/ml each), or different combinations of these cytokines for 8 h. Cells were harvested for subsequent cytosol and nuclear extraction. Protein-matched nuclear and cytosolic extracts were then analyzed by Western blotting with anti-serum against I $\kappa$ B $\zeta$ , laminB1 Ab (nuclear marker) and IL-1 $\beta$  Ab (cytosolic marker). Results are representative of 3 separate experiments.  
doi:10.1371/journal.pone.0006776.g006

translocation followed by IL-1 $\beta$ , IL-18 and TNF $\alpha$  combined stimulation. Western blot analysis with nuclear extracts indicated a reduction in p50 and p65 nuclear localization, indicative of a decrease in NF $\kappa$ B activity (data not shown). Moreover, IL-6 and IFN $\gamma$  release were significantly reduced with NF $\kappa$ B inhibition (Fig. 7). Therefore, I $\kappa$ B $\zeta$  may regulate IFN $\gamma$  release in KG-1 cells in response to IL-1 $\beta$ , IL-18 and TNF $\alpha$  combined stimulation by acting as a co-factor for NF $\kappa$ B-mediated transcription.

#### IFN $\gamma$



#### IL-6

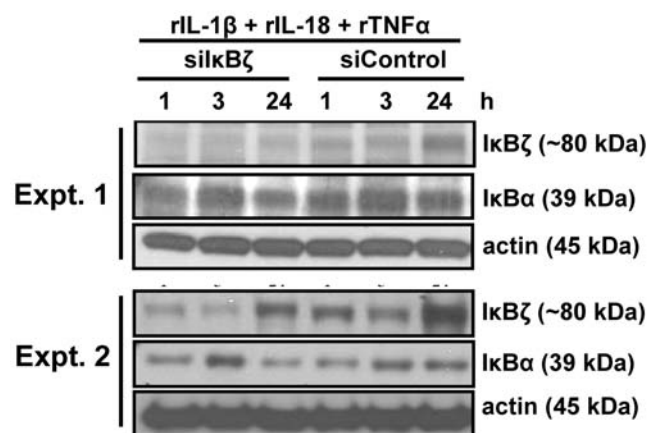


**Figure 7. NF $\kappa$ B inhibition results in decreased IFN $\gamma$  and IL-6 release.** KG-1 cells ( $10^6$ /ml) were incubated with the NF $\kappa$ B inhibitor, JSH23 (30  $\mu$ M), and subsequently stimulated with a combination of rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$  (10 ng/ml each). Supernatants were harvested after 24 h and analyzed for cytokine release by IL-6 and IFN $\gamma$  ELISAs. Results are shown as mean  $\pm$  S.E.M. \*,  $p < 0.05$ ; \*\*,  $p < 0.005$  ( $n = 3$ ).  
doi:10.1371/journal.pone.0006776.g007

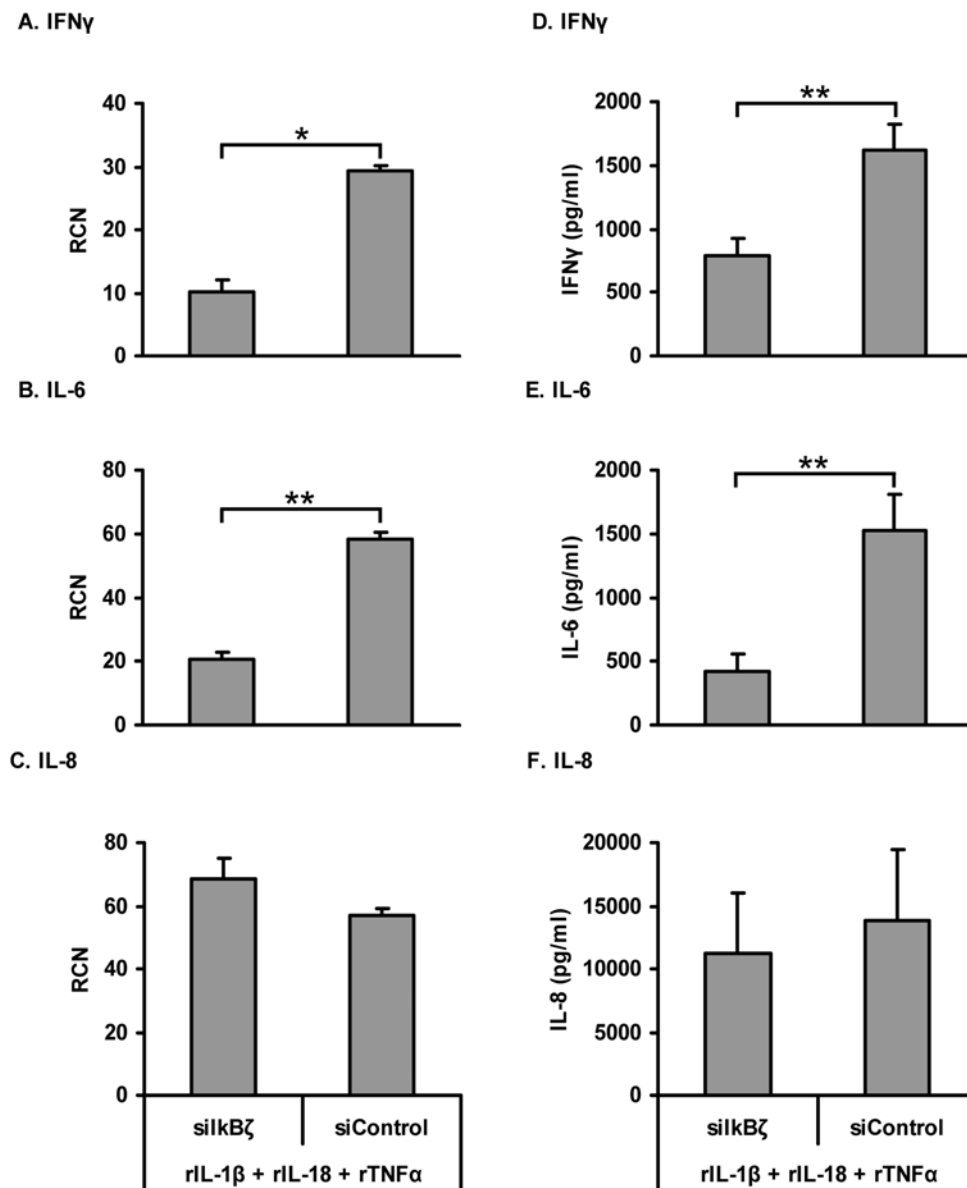
#### Silencing of I $\kappa$ B $\zeta$ suppresses IFN $\gamma$ and IL-6, not IL-8 production

I $\kappa$ B $\zeta$  has been shown to either negatively or positively regulate NF $\kappa$ B 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-1 $\beta$ /IL-18 or TNF $\alpha$  blockade inhibits both I $\kappa$ B $\zeta$  protein expression and IFN $\gamma$  production in KG-1 cells, we hypothesized that I $\kappa$ B $\zeta$  positively regulates IFN $\gamma$  production.

In order to test this hypothesis, KG-1 cells were nucleofected with a mixture of 3 different small interfering RNA (siRNA) oligonucleotides against I $\kappa$ B $\zeta$  or 3 different scrambled siRNA oligonucleotides. Cells were then stimulated with a combination of rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$ . Western blot analysis indicated a reduction in I $\kappa$ B $\zeta$  protein expression with anti-I $\kappa$ B $\zeta$  siRNA delivery (Fig. 8). In order to determine the effect of I $\kappa$ B $\zeta$  silencing on IFN $\gamma$  protein production, we measured IFN $\gamma$  mRNA levels and protein release in KG-1 cells upon rIL-1 $\beta$ , rIL-18 and rTNF $\alpha$  combined stimulation (Fig. 9). As a positive



**Figure 8. Silencing of I $\kappa$ B $\zeta$  expression.** 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 $\zeta$  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 the indicated time points. Protein-matched total cell extracts were analyzed by Western blotting using anti-serum against I $\kappa$ B $\zeta$  and actin Ab. Results are representative of 5 separate experiments.  
doi:10.1371/journal.pone.0006776.g008



**Figure 9. Silencing of I $\kappa$ B $\zeta$  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 $\zeta$  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  $\pm$  S.E.M. \*,  $p < .05$ ; \*\*,  $p < 0.005$  (A, B and C,  $n = 3$ ) (D, E and F,  $n = 5$ ). doi:10.1371/journal.pone.0006776.g009

control, we measured IL-6 mRNA and protein release since this cytokine has been shown to be positively regulated by I $\kappa$ B $\zeta$  [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 $\zeta$  [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 $\zeta$  siRNA delivery (Fig. 9). These results implicate a role for I $\kappa$ B $\zeta$  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, NF $\kappa$ B, 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



stimulation. As an example, the combination of LY49 activating receptor crosslinking and IL-12 or IL-18 synergistically enhance IFN $\gamma$  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 IFN $\gamma$  gene expression. However, at high doses (50 ng/ml), IL-18 alone can induce IFN $\gamma$  production in the human acute myeloid leukemic KG-1 cell line [1,24]. KG-1 cells have been widely used to study IL-18-mediated signaling events leading to IFN $\gamma$  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].

NF $\kappa$ B has been shown to regulate the expression of many pro-inflammatory genes, IFN $\gamma$  being no exception. Two putative NF $\kappa$ B binding sites have been identified in the IFN $\gamma$  promoter region ( $\kappa$ BB site and CD28RE) and one in the first intron (C3) [78]. The requirement for NF $\kappa$ B in IFN $\gamma$  gene expression appears to be contingent on the cell type and the specific stimulus. IL-18 signaling via the IL-18R leads to NF $\kappa$ B activation [78–82]. Moreover, stimulation of KG-1 cells with high doses (50 ng/ml) of IL-18 leads to IFN $\gamma$  production in an NF $\kappa$ B-dependent manner [78].

We have previously described a novel synergistic role for the members of the IL-1 family, IL-1 $\beta$  and IL-18, in combination with TNF $\alpha$  in IFN $\gamma$  production in KG-1 cells [1]. Importantly, at the dose of 10 ng/ml, the individual cytokines induced only minimal amounts of IFN $\gamma$  release. Given the striking similarities between the IL-1 $\beta$  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-1 $\beta$ /IL-18 and TNF $\alpha$ . The latter is supported by the fact that induction of the IFN $\gamma$  promoter is generally mediated by multiple signals leading to activation of multiple transcription factors that synergistically induce IFN $\gamma$  gene expression [58–61].

The novel member of the I $\kappa$ B family of NF $\kappa$ B regulators, I $\kappa$ B $\zeta$ , 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 I $\kappa$ B $\zeta$  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 I $\kappa$ B $\zeta$  expression. We have shown for the first time that IL-18 stimulation also leads to I $\kappa$ B $\zeta$  protein expression in KG-1 cells.

I $\kappa$ B $\zeta$  has been shown to positively regulate NF $\kappa$ B-mediated transcription of secondary response genes such as IL-6 and NGAL, as a co-factor binding to the p50 NF $\kappa$ B subunit [21,24,26,32,33,35]. Moreover, NF $\kappa$ B has been shown to play an important role as a positive regulator of IFN $\gamma$  gene expression in thymocytes, peripheral blood T lymphocytes and KG-1 cells [66,78,83]. Therefore, we hypothesized that I $\kappa$ B $\zeta$  may be the common factor downstream of the IL-1R and IL-18R pathways, which allows for synergy between IL-1 cytokines and TNF $\alpha$  for IFN $\gamma$  production in KG-1 cells.

Interestingly, we observed that TNF $\alpha$  enhanced IL-1 $\beta$ /IL-18-mediated I $\kappa$ B $\zeta$  protein expression, even though by itself it had no effect on I $\kappa$ B $\zeta$  protein expression. However, TNF $\alpha$ , IL-1 $\beta$  and LPS have all been shown to induce I $\kappa$ B $\zeta$  mRNA transcription in NIH3T3 and A549 cells [20]. Importantly, TNF $\alpha$  stimulation alone results in strong activation of the I $\kappa$ B $\zeta$  promoter without subsequent protein expression. Moreover, nuclear run-on analysis in NIH3T3 cells also indicates that TNF $\alpha$  is a stronger transcriptional activator of the I $\kappa$ B $\zeta$  gene,

compared to IL-1 $\beta$  or LPS. Furthermore, decay analysis of ectopically expressed I $\kappa$ B $\zeta$  mRNA upon actinomycin D treatment, indicates that degradation of I $\kappa$ B $\zeta$  mRNA is delayed by IL-1 $\beta$  and LPS stimulation, but not by TNF $\alpha$  stimulation. Moreover, the N-terminal half, not the C-terminal half, of the I $\kappa$ B $\zeta$  ORF confers IL-1/LPS-mediated I $\kappa$ B $\zeta$  mRNA stabilization. Therefore, the specificity of IL-1/LPS stimulus for I $\kappa$ B $\zeta$  mRNA and protein induction is most likely at the post-transcriptional level and due to stabilization of I $\kappa$ B $\zeta$  mRNA. A *cis*-element in the N-terminal half of the I $\kappa$ B $\zeta$  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-1 $\beta$ , not by TNF $\alpha$  [9,21,84,85]. NGAL promoter activity requires NF $\kappa$ B activation and an intact NF $\kappa$ B binding site [24]. Even though IL-1 $\beta$  and TNF $\alpha$ , both induce NF $\kappa$ B nuclear translocation and recruitment to the NGAL promoter, only IL-1 $\beta$  is able to induce NGAL expression. I $\kappa$ B $\zeta$  has been shown to be the co-factor which allows NF $\kappa$ B to mediate NGAL gene expression downstream of the IL-1R/TLR signaling pathway [24]. The latter was shown by I $\kappa$ B $\zeta$  over expression in A549 cells, which rescued TNF $\alpha$ -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-1 $\beta$ , not TNF $\alpha$  stimulation, and to other genes specifically induced downstream of the IL-1R/TLR pathway. As an additional example, hBD2 is also stimulated by IL-1 $\beta$ , but not TNF $\alpha$  stimulation in human keratinocytes [9,84] and A549 cells [24]. Moreover, siRNA experiments have shown that I $\kappa$ B $\zeta$  is critical for IL-1 $\beta$  mediated hBD2 mRNA expression in A549 cells [24].

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

In summary, we have shown a positive role for I $\kappa$ B $\zeta$  on IFN $\gamma$  production in response to IL-1 $\beta$ , IL-18 and TNF $\alpha$  combined stimulation in KG-1 cells. This regulation is most likely dependent on the ability of I $\kappa$ B $\zeta$  to regulate NF $\kappa$ B mediated transcription of the IFN $\gamma$  gene. This finding represents a new addition to the complex and continuously growing literature on the regulation of IFN $\gamma$  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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