Pharmacologic IKK/NF-κB inhibition causes antigen presenting cells to undergo TNFα dependent ROS-mediated programmed cell death

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Monocyte-derived antigen presenting cells (APC) are central mediators of the innate and adaptive immune response in inflammatory diseases. As such, APC are appropriate targets for therapeutic intervention to ameliorate certain diseases. APC differentiation, activation and functions are regulated by the NF-κB family of transcription factors. Herein, we examined the effect of NF-κB inhibition, via suppression of the IkB Kinase (IKK) complex, on APC function. Murine bone marrow-derived macrophages and dendritic cells (DC), as well as macrophage and DC lines, underwent rapid programmed cell death (PCD) after treatment with several IKK/NF-κB inhibitors through a TNFα-dependent mechanism. PCD was induced proximally by reactive oxygen species (ROS) formation, which causes a loss of mitochondrial membrane potential and activation of a caspase signaling cascade. NF-κB-inhibition-induced PCD of APC may be a key mechanism through which therapeutic targeting of NF-κB reduces inflammatory pathologies.
Moreover, our results indicate that APC death, in both macrophages and monocyte-derived DC, may contribute to the anti-inflammatory effects of NF-κB inhibitors observed in mammalian models of disease.

**Results**

**NF-κB suppression results in APC death.** Previously, studies by our group demonstrated that chronic treatment of a murine model of inflammatory bowel disease with the Nemo Binding Domain (NBD) peptide, a highly specific NF-κB/IKK inhibitor, fused to a protein transduction domain ameliorated disease. Moreover, levels of inflammatory cytokines derived from innate cells, including IL-12p40 and TNFα, were reduced in the NBD-treated animals compared with controls.

During the course of these experiments, it was observed that treatment with NBD resulted in macrophage cell death. For example, treatment of the RAW264.7 macrophage cell line with NBD conjugated to a protein transduction domain (TAT) resulted in rapid cell death (Figure 1). The majority of the macrophages exhibited characteristics of apoptosis, including membrane blebbing, nuclear condensation, cell shrinkage, and loss of symmetry (Figure 1A, Supplemental Figure 1). Further analysis showed that this cell death occurred rapidly with a majority of RAW cells treated with TAT-NBD, but not an inactivated form of the peptide TAT-mNBD, quantified by PI and Annexin V staining 4 hours after treatment (Figure 1B).

This cell death response in macrophages secondary to NF-κB inhibition was unexpected, as we hypothesized that the major effect would be to suppress cytokine signaling and inhibit phagocytosis. To determine whether our observation that rapid induction of cell death in a macrophage cell line was observed in other APC lines, we examined the induction of cell death by NBD using the MTT assay for both RAW264.7 macrophages and fetal skin dendritic cells (FSDC). To demonstrate that the death inducing doses of NBD were equivalent to the relative suppression of NF-κB by TAT-NBD, HEK293 cells stably transfected with a multimerized NF-κB DNA binding element-luciferase reporter (293NFκB cells; Figure 1C) were used to quantify NF-κB suppression. The survival curves for both FSDC and RAW cells and the NF-κB suppression profiles as determined in the 293NFκB cells were similar, suggesting that the observed APC death correlates with the level of NF-κB suppression.

To determine if the rapid induction of apoptosis in macrophages and DC was NF-κB/IKK inhibition-dependent and not an off-target effect of TAT-NBD, five additional NF-κB inhibitory compounds were tested: Compound A (CmpA) and TPCA-1, both of which inhibit IKKβ; MG-132, which inhibits IκBα proteasomal degradation; and IKKIVII and Wedelolactone, which inhibit both IκKα and IκKβ (see Supplemental Table 1). Each of these five inhibitors was evaluated for induction of APC death using FSDC. As with TAT-NBD experiments, the level of NF-κB suppression was quantified using the 293NFκB reporter cells (Figure 2). For each inhibitor used, the FSDC survival profile was similar to that of the NF-κB inhibition profile, demonstrating a direct correlation between the extent of NF-κB suppression and APC death.

**NF-κB inhibition induces apoptotic PCD in APC.** To determine whether NF-κB-inhibition induced PCD or necrosis, initially the
morphology of the treated FSDC was examined. The FSDC treated with each of the four NF-κB inhibitors exhibit several features of apoptosis including membrane blebbing, nuclear condensation, cell shrinkage, and loss of symmetry (Figure 1A, Supplemental Figure 1). To further elucidate whether these cells were undergoing necrotic or apoptotic cell death, caspase activation was measured in FSDC treated with two NF-κB inhibitors, Compound A and IKKIVII. Treatment with these inhibitors resulted in a significant increase in caspase-8, caspase-9 and caspase-3 cleavage over a 7-hour time course (Figure 3A). Caspase-3 activation was confirmed by immunofluorescence staining of CmpA treated FSDC (Figure 3B). Primary BMDM also underwent caspase-3 and -9 cleavage following NF-κB inhibition as determined by immunoblot (Supplemental Figure 2A). Pretreatment of FSDC and BMDM with the broad spectrum caspase inhibitor zVAD significantly increased APC survival nearly 4.5 fold with \( p < 0.0002 \) (Figure 3C and Supporting Figure 2B). These results suggest that the observed death mediated by NF-κB suppression is through a caspase-dependent apoptotic cell death pathway.

Apoptosis induced by NF-κB inhibition is specific to APC populations. To determine if apoptosis in response to NF-κB inhibition was APC-specific, we measured the ability of four different NF-κB inhibitors to induce cell death of HEK293 cells, immortalized D10 T cells, and primary mouse embryonic fibroblasts (MEFs) and compared their cell survival profiles with FSDC, using the MTT assay. Cell death occurred in FSDC at significantly lower concentrations of inhibitors compared to the three non-APC lines. CmpA and TPCA-1, both IKKβ selective inhibitors, showed very little toxicity in non-APC cell lines (Figure 4A and B). The two IKK complex inhibitors, IKKIVII and Wedelolactone, induced more cell death in FSDC compared to other cell types, but caused some toxicity in non-APC at high concentrations (Figure 4C and 4D). These high concentrations of inhibitors induced morphologic changes in non-APCs that were more consistent with necrosis than apoptosis (data not shown).

To determine if cell death secondary to NF-κB inhibition was unique to APC, other APC lines were tested, including primary bone marrow derived DC (BMDC) and macrophages (BMDM), as well as RAW264.7 and FSDC. The LD50 for CmpA was significantly higher with a minimum \( p < 0.003 \) in non-APC lines compared to all four APC types (Figure 4E). All non-APC lines tested had LD50 values 10-25-fold higher (Figure 4E), even when treated with CmpA for extended periods of time (supplemental Figure 3). In contrast, the APC lines had significantly lower LD50 values, ranging from 2.7±0.2 \( \mu M \) for FSDC to 8.9±0.4 \( \mu M \) for RAW264.7. These data demonstrate that the NF-κB-inhibition-induced cell death is APC-specific.

NF-κB-inhibition-induced cell death is TNFα dependent, but independent of the TNFα/JNK/Caspase-8 pathway. Activation of caspase-8 (Figure 3A) suggests involvement of the TNFα-dependent cell death pathway, which is the most commonly recognized NF-κB inhibition-induced PCD pathway. However, caspase-8 was activated hours after caspases-9 and -3 in both the CmpA and IKKIVII-treated FSDC (Figure 3A). This suggests that, in the APC death cascade initiated by NF-κB inhibition, caspase-8 may lie downstream of caspase-9, and is therefore not the initiating event. Furthermore, this brings into question whether NF-κB induced APC apoptosis is a TNFα-dependent event. To examine this, WT and TNFα-/- primary macrophages were treated with CmpA,
TCPA-1, and IKKiVII to evaluate the involvement of TNF/JNK/caspase-8 pathway activation in APC PCD. TNFα−/− BMDM were highly resistant to NF-κB/IKK inhibition-induced cell death compared to WT cells using three inhibitors (Figure 5A). The resistance of the TNFα−/− BMDM suggests that TNFα is necessary to induce APC death.

In further support of TNFα-dependence of this PCD pathway, TNFα−/− macrophages were treated with varying concentrations of TNFα, or left untreated in the presence of CmpA. Addition of exogenous TNFα at concentrations as low as 0.05 ng/ml caused TNFα−/− BMDM to become more sensitive to CmpA-induced cell death than WT BMDM treated with CmpA alone (Figure 5A, top panel). This same phenomenon was observed with additional NF-κB inhibitors, TCPA-1 and IKKiVII (Figure 5A). Thus, we hypothesized that endogenous TNFα produced by WT APC contributes to the minimal activation state required for APC apoptosis, and only minor activation of these cells is necessary for induction of NF-κB inhibition-mediated PCD. To test this, TNFα was depleted from WT macrophages 1 hour prior to CmpA treatment using 50 μg of etanercept (anti-TNF antibody), which resulted in inhibition of cell death (p<0.05) (Figure 5B).

To determine whether other NF-κB activators would also cause apoptosis in APC, RAW264.7 cells were pretreated with etanercept, followed by a number of varying NF-κB activators, including TNFα, IL-1β, CpG, and LPS, prior to the addition of CmpA (Figure 5B). Interestingly, in the presence of etanercept, only LPS allowed for CmpA-induced APC death to occur, while none of the other TLR and stimulatory ligands had any potentiating effect on cell death. LPS has previously been shown to induce apoptosis in IKKβ deficient macrophages, possibly in a PAI-2-dependent manner. These results illustrate that LPS is capable of inducing cell death in the absence of TNFα. In addition, these results further demonstrate that TNFα is necessary to induce apoptosis in NF-κB-suppressed APC.

We next measured the minimal amount of exogenous TNFα necessary to induce PCD. APC are typically stimulated in culture with 10 ng/ml of TNFα; however, amounts as low as 0.05 ng/ml of TNFα were sufficient to induce significant cell death in CmpA treated TNFα−/− macrophages, suggesting that only minor activation of these cells is necessary for induction of the NF-κB inhibition-induced PCD (Figure 5C).

To determine if the TNFα-dependent PCD is APC specific, the non-APC cell lines HEK293 cells and immortalized D10 T cells were pretreated with 10 ng/ml of TNFα, with RAW264.7 cells used as a positive control. As shown in supplemental Figure 4, NF-κB inhibition of RAW264.7 cells resulted in significant apoptosis. In contrast, there was no effect of TNFα and/or compound A on HEK293 cells. Interestingly, there was a slight increase in cell number following treatment of D10 cells with compound A. Taken together, these results suggest that the effect of the NF-κB inhibitory compounds is specific for APC cell lines, both in the absence and presence of TNFα.

The canonical mechanism of TNFα-mediated apoptosis involves downstream JNK signaling. Therefore, the effect of SP600125, a potent inhibitor of JNK1 and JNKII signaling, was evaluated in two APC lines. JNK inhibition had no effect on NF-κB-induced cell death in FSDC (Figure 5D) or in BMDC (data not shown), although it did reduce nitric oxide signaling, demonstrating that the inhibitor was indeed active (data not shown). Thus, APC apoptosis in response to TNFα suppression in APC leads to caspase-dependent apoptotic cell death. (A) Levels of caspase-8, -9, and -3 cleavage were determined in FSDC via immunoblot analysis at the indicated time-points following treatment with two different NF-κB inhibitors (Compound A and IKKiVII). β-actin was used as a positive control. The full-length gels are provided in the supplemental information. (B) Immunofluorescence shows an increase in the levels of cleaved caspase-3 (red) in FSDC after 5-hour treatment with 10 μM Compound A. Nuclei were counterstained with DAPI (blue). (C) MTT assays were used to measure survival of FSDC that were untreated or treated with the listed doses of CmpA, or IKKiVII in the presence of 75 μM zVAD.

* p<0.004; ** p<0.00002. Data are representative of 3 independent experiments in triplicate.
NF-κB inhibition is independent of JNK signaling. Collectively, these data suggest that NF-κB inhibition-induced PCD diverges from the well-documented TNFα/JNK/Caspase-8 cell death pathway, and suggests a novel TNFα-dependent PCD pathway.

APC NF-κB-inhibition-induced death is dependent on ROS production. The activation of caspase-9 during NF-κB inhibition-induced PCD (Figure 3A, Supplemental Figure 2A) suggested a mitochondrial dependent apoptotic response. Thus, mitochondrial ROS and membrane potential ($\Delta \Psi_m$) were measured using MitoSOX Red and DiOC6(3), respectively. FSDC were treated with 10 μM CmpA and fluorescence levels of MitoSOX Red and DiOC6(3) were visualized by microscopy, demonstrating a significant increase in ROS (red fluorescence) after incubation with CmpA, in particular in cells with apoptotic morphology (Figure 6A). Additionally, levels of MitoSOX Red and DiOC6(3) were measured by FACS at multiple time points (0, 3, 7, 15, and 24 hours) after treating cells with 10 μM CmpA. Following NF-κB inhibition, there was a progressive increase in ROS and a concomitant loss in $\Delta \Psi_m$, noted by decreased fluorescent levels of DiOC6 (Figure 6B).

Increased mitochondrial ROS is often considered a consequence of loss of $\Delta \Psi_m$. However, a few recent reports suggest that increased mitochondrial ROS can precede loss of $\Delta \Psi_m$. To elucidate the order of events, 200 μM BHA, an ROS scavenger that reduces $O_2^-$, was added to the cells for 1 hour prior to the treatment with CmpA (Figure 6C). ROS levels increased in FSDC at 3 hours post-treatment with BHA and CmpA, then stabilized and remained constant through the 15-hour time point, while ROS increased continually in samples treated with CmpA alone. Furthermore, pretreatment of FSDC with BHA protected against loss of $\Delta \Psi_m$ in response to CmpA (Figure 6B/C), suggesting that loss of $\Delta \Psi_m$ is ROS-dependent.

ROS production occurs upstream of caspase activation. To determine if ROS production was upstream or downstream of caspase activation, FSDC were pretreated with BHA or left untreated and analyzed for caspase cleavage at 4-7 hours after inhibition of NF-κB.
with CmpA. Caspase-8 and -3 cleavage was observed in cells treated with CmpA alone, but not in those treated with BHA plus CmpA (Figure 7A). Therefore, these results demonstrate that, like ΔΨm, caspase activation is ROS-dependent. These data also support the observation that NF-κB inhibition-induced PCD is independent of the TNF/ JNK/ Caspase-8 pathway. Furthermore, BHA treatment of FSDC results in suppression of cell death in response to both CmpA and IKKiVII (Figure 7B). This phenomenon was also observed in additional APC cell types, BMDM, BMDC and FSDC, treated with CmpA + BHA (Figure 7C). Another antioxidant deferoxamine (DFO)\textsuperscript{25}, also suppressed NF-κB inhibitor-induced PCD in primary WT macrophages (Figure 7D). However, neither BHA or DFO inhibited CmpA-induced cell death in APCs treated with LPS (Figure 7D), providing further evidence that LPS indeed acts by a separate mechanism to induce APC PCD in the presence of NF-κB inhibition. We also have demonstrated that treatment with the

Figure 5 | NF-κB-inhibition induced APC death is dependent on TNFα. Cellular survival was measured after 24 hours using MTT assays in the following experiments: (A) TNFα−/− (dark symbols) and WT (light symbols) primary macrophages were evaluated for survival in the presence of NF-κB inhibitors in unstimulated cells (blue) or following stimulation with 10 ng/ml of TNFα (red). (statistical variation was evaluated at max concentration using ANOVA analysis) (B) RAW264.7 cells were treated with etanercept (25 ng/ml) and compound A (10 μM) in the presence of several endogenous and exogenous known NF-κB activators, CpG (7 ng/ml), IL-1β (10 ng/ml), TNFα (10 ng/ml) and LPS (100 ng/ml). MTT assay was used to evaluate survival after overnight incubation. Controls included untreated RAW264.7 cells, etanercept treatment alone, and etanercept + LPS treatment. Representative results are from at least 3 independent experiments in triplicate (p-values determined by student T-test). (C) TNFα−/− macrophage survival was measured by MTT assay in the presence of 10 μM Compound A and varying concentrations of TNFα. Addition of 0.05 ng/ml TNFα led to significant (p<0.008) cell death compared with untreated cells using a student t-test. (D) Cell survival was determined by MTT assay for FSDC pretreated with either 10 or 25 μM of specific JNK inhibitor (SP600125) and in the presence of three NF-κB inhibitors. Data are representative of at least 3 independent experiments in triplicate.
Figure 6 | IKK suppression results in increased ROS production and secondary loss of ΔΨᵢ₁

(A) FSDC were treated with 10 μM Compound A and at the indicated time points, MitoSOX (red) and DiOC6(3) (green) were analyzed by fluorescent microscopy. Cells with apoptotic morphology are marked with yellow arrows. (B) FSDCs were treated with 10 μM Compound A. These FSDCs were either grown in serum alone or pretreated with 200 μM BHA. At 3, 7, 15, and 24 hours, flow cytometry was performed to analyze staining with MitoSOX Red [ROS production] and DiOC6(3) [mitochondrial membrane potential, ΔΨᵢ₁]. The results are representative of 3 independent experiments.
anti-oxidant nordihydroguaiaretic acid (NDGA), but not inhibitors of NADPH oxidase, diphenylene iodoine (DPI) or apocynin, was able to prevent CmpA-induced APC death (Supplemental Figure 5). Taken together, these data suggest that caspase activation occurs secondary to ROS formation and loss of ΔΨm, and that ROS is the initiating event in this process.

Discussion

The NBD peptide, when fused to a PTD, is therapeutic in animal models of inflammatory bowel disease (IBD), arthritis, type I diabetes, multiple sclerosis, Parkinson’s disease and muscular dystrophy. While examining the mechanism of action of NBD in a mouse model of IBD, we observed that treatment of APC with the NBD peptide results in rapid and extensive apoptosis. These results suggest a novel NF-κB-inhibition-induced cell death response in APC. The previously observed NF-κB-inhibition-induced PCD response was reported to occur via the TNFα/JNK/caspase-8 signaling pathway. However, in APC, while it appears that the process is TNFα dependent, secondarily there is increased ROS formation, which leads to a subsequent loss of ΔΨm (Figure 6) and activation of caspase-9/3 (Figures 3 & 7). Using TNFα−/− primary macrophages, we demonstrated that a minimal level of NF-κB activation is necessary for NF-κB inhibition-induced cell death, which is equivalent to basal levels of TNFα (0.05 ng/ml) produced by WT macrophages in cell culture36. Further, non-APC cell lines treated with exogenous TNFα in the presence of NF-κB inhibition did not undergo cell death, thus this process is likely specific for APCs. While LPS induces cell death to a similar extent as that induced by the addition of TNFα in TNFα−/− BMDM (data not shown), other NF-κB activators including IL-1β and CpG did not induce similar effects. Additionally, LPS-induced death in APC with suppressed NF-κB activity could not be reversed by the anti-oxidants BHA and DFO. Thus, the LPS and TNFα dependent death pathways in APCs are likely different.

JNK inhibition had no effect on APC PCD and caspase-8 activation occurred subsequent to ROS production (Figure 5) and likely downstream of activation of both caspases-9 and -3. Taken together, these data provide evidence that this PCD pathway is independent of TNFα/JNK/caspase-8 and death receptors, and suggests a novel NF-κB/NF-κB activation is necessary for NF-κB inhibition-induced, APC-specific apoptotic pathway.

Our results suggest that ROS formation is a proximal component of this NF-κB inhibition-induced PCD pathway as demonstrated by the early increase in superoxide formation in cells (Figure 6) and the fact that addition of the antioxidant BHA to cell culture caused a stabilization of mitochondrial membrane potential, and prevented caspase activation (Figures 6 & 7). NF-κB is known to transcriptionally regulate several anti-oxidant genes including SOD1, SOD2 and FHC37–39. Further, it is known that NADPH oxidase is up-regulated in activated APC30, and that macrophages up-regulate iNOS and COX-2 during activation as a part of their immune function31. This finding was expanded upon by Pagliari et al., who also used PTDC, but additionally examined NF-κB suppression using an adenovirus expressing IκBα-DN, which induced a clear collapse of ΔΨm. However, when using PTDC, it was observed that the cells were not rescued from apoptosis using caspase inhibitors, possibly due to zVAD toxicity in RAW264.7 cells. In addition, they did not observe caspase-3 cleavage, leading to the suggestion of a caspase-3-independent pathway, and did not address the role of ROS production in this apoptotic response32. Furthermore, since PTDC...
also has anti-oxidant properties, the role of ROS in NF-κB-inhibition-induced cell death pathway was not examined. Pagliari et al suggested a major role for A1/bfl-1, a bcl-2-like anti-apoptotic protein, in preventing NF-κB-inhibition-induced macrophage cell death. We also observed a slight downregulation of A1/bfl-1 at the mRNA level following treatment with CmpA with no effect on Bcl-2 and Bcl-XL mRNA levels. However, an increase in A1/bfl-1 protein levels was observed by immunofluorescence and immunoblot after NF-κB suppression (Supplemental Figure 6).

The findings presented here may help to explain physiologic and therapeutic responses to NF-κB inhibition. PCD secondary to NF-κB inhibition in APC may explain responses observed in specific pathogen infections. A few well-known pathogens, including *Yersinia* bacteria and Vaccinia virus, cause macrophage and DC apoptosis after infection, and are known to produce NF-κB inhibitory compounds. Studies indicate that APC death is induced secondary to one of two NF-κB inhibitors produced by *Yersinia*, YopP and YopJ, as a loss of these molecules leads to improved APC survival. Additionally, NF-κB-inhibition-induced PCD was enhanced by LPS signaling. Vaccinia virus inhibits NF-κB signaling via two proteins, N1L and B14. Furthermore, the N1L protein is deleted in attenuated Vaccinia virus, which does not induce APC death. Therefore, APC death induced by NF-κB inhibition may further elucidate the cause of macrophage cell death responses associated with other pathogen infections.

The apoptotic response following NF-κB inhibition is biologically important not only in regard to APC responses or protection against infection, but also in evaluating therapeutic strategies for cancer and inflammatory/autoimmune diseases. Recently, several groups have begun to examine the efficacy of NF-κB inhibition in treating myeloid leukemia, such as acute and chronic myeloid leukemia (AML and CML). AML and CML derive from myeloid progenitors, which give rise to non-lymphocyte white blood cells, including macrophages and DCs. In Imatinib-resistant CML cell lines, NF-κB/IKK was activated, and as expected these cells underwent caspase-dependent cell death in response to NF-κB/IKK suppression. Furthermore, mice injected with CML cell lines showed reduced tumor burdens when treated with an IKKβ inhibitor. The NF-κB initiated inflammatory response is an obvious pharmacologic target for the treatment of autoimmunity and inflammatory diseases. Not surprisingly, numerous NF-κB inhibitory compounds have been evaluated in the treatment of inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis, muscular dystrophy, Type-1 diabetes, as well as Parkinson’s and osteoporosis. In these diseases. Not surprisingly, numerous NF-κB inhibitory compounds have been evaluated in the treatment of inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis, muscular dystrophy, Type-1 diabetes, as well as Parkinson’s and osteoporosis. In these diseases, NF-κB inhibitors have been evaluated in the treatment of inflammatory diseases, such as chronic inflammatory bowel disease, rheumatoid arthritis, and muscular dystrophy. These compounds include: *Yersinia* infection, which does not induce APC death. Therefore, APC death induced by NF-κB inhibition may further elucidate the cause of macrophage cell death responses associated with other pathogen infections.

The nodal mechanism of NF-κB-inhibition-induced PCD in APC described here offers a potential explanation for many of the observed therapeutic effects following treatment with NF-κB/IKK inhibitors. Many of the inhibitors which suppress NF-κB signaling have extremely short half-lives in vivo, but can be injected only a few times per week and still maintain their immunosuppressive effects. Thus, we hypothesize that this APC death may result in a fundamental change in the immune system that prolongs the efficacy of NF-κB/IKK inhibition. In addition, our observations that minimal stimulation is required for cell death to occur would potentially mean that only activated APC would undergo apoptosis with this NF-κB inhibitor therapy, further reducing side effects, and improving the therapeutic potential of NF-κB inhibitors.

### Methods

**NF-κB inhibitors used include:** Compound A (gift from Bayer), IKK inhibitor VII (Calbiochem), IKBEB inhibitor IV (Calbiochem), Widelodactone (CellMetics), and MG-132 (Calbiochem). Caspase activation was inhibited by Z-VAD-fmk (Calbiochem). ROS production and apoptosis was inhibited by butylated hydroxyanisole (BHA) (Sigma), deferoxamine (DFO) (Calbiochem) and nordihydroguaiaeric acid (NDGA) (Aldrich). The NADPH oxidase inhibitors diphenylene iodonium (DPI) and apocynin were obtained from Sigma. All compounds were diluted as suggested by manufacturers in DMSO and then diluted in desired media. MTT (Sigma) was diluted to 5 mg/ml in Opti-mem media (Invitrogen). Etanercept was obtained from Wyeth Pharmaceuticals. The JNKII/II inhibitor, SP600125, was obtained from Sigma. NF-κB activators were obtained as TNFα (E Bioscience), LPS (Sigma), Cpg (Invitrogen), IL-1β (Peprotech). **Peptides.** Peptide NF-κB inhibitors were synthesized in association with the HIV TAT-protein transduction domain (PTD) or the 8-linase (8 K) PTD. The peptides TAT-NEMO Binding Domain (NBD; YGRKKRRQRRRGGTALDSWQLQTE-amide) and inactive (mutant) TAT-NBD (mNBD; YGRKKRRQRRRGGTALDAS) were synthesized as the peptide synthesized by the peptide synthesis unit of the University of Pittsburgh. Underlined amino acids represent tryptophan to alanine mutations. Peptides were purified and characterized by reversed-phase high performance liquid chromatography and mass spectrometry. For in vitro experiments, TAT-NBD and TAT-mNBD peptides were used, while in vivo 8K-NBD and 8K-mNBD peptides were used due to differing transduction rates.

**Murine macrophages, DC and other cell lines.** Wild-type bone marrow (BM)-derived macrophages (BMDM) were isolated from the femurs of C57BL/6 mice, both WT and TNFα−/−. BM was flushed with washing medium (RPMI 1640 supplemented with 10% FBS, 1% NEAA, and 10 μM 2-mercaptoethanol) into a 70 μm nylon cell strainer. Cells were spun down through a 50 ml conical tube, and spun down at 1500 rpm for 5 min. RBCs were lysed using ACK lysis buffer for 15 min, and resuspended in complete medium (washing medium with 10% FBS). BM cells were seeded in conditioned L-cell media (consisting of 20% precondition L-cell media, 60% DMEM, 20% FBS), supplemented with 1% L-glut, 1% sodium pyruvate, and 1% penicillin/streptomycin added to the media. Cells were seeded in 10 cm dishes, media was refreshed after 3 days and cells were collected on day 7. Cells were passed every 3-4 days and were discarded after one month.

Bone marrow–derived dendritic cells (BMDC) were isolated from mice as described for BMDM. BM cells were seeded at 36 cells per well in 6 well plates in complete RPMI supplemented with 10 ng/ml of GM-CSF (Cell Sciences) and 20 μg/ml IL-4 (Cell Sciences). Media was replaced on Day 3 (supplemented with GM-CSF and IL-4) and cells were collected on Day 7. Cells were then isolated using MACs columns (Miltenyi Biotech) with positive selection using CD11c beads. Isolated cells were then seeded in complete RPMI media and used for experiments.

Other cell lines used included L-cell, skin fibroblasts, immortalized murine DC cell line (ISDS), and immortalized murine DC cell line (ISDS). Cells were treated with either IFNγ or LPS, with or without peptides, after 24 hours of treatment. Medium was replaced with fresh media, and cells were incubated for an additional 24 hours. Cell viability was assessed by MTT assay.

**Peptide NF-κB luciferase assay.** HEK293 cells stably transfected with a multicloner NF-κB DNA binding element-luciferase reporter (293T-κBS), were pretreated for 1 hour with varying NF-κB inhibitory compounds (in Materials), and were activated for 3 hours with 10 ng/ml TNFα (R&D Systems). The cells were lysed in reporter lysis buffer and luciferase activity was measured with a luciferase assay system (Promega) using an Immune Fluorometer (Berthold Technologies). Due to the high binding affinity of the PTD fragments of the peptides, peptides were administrated in Opti-mem prior to addition of TNFα. All other NF-κB inhibitors were administered in the maintenance media of the specific cell type being treated.

**MTT Assay.** Cells were seeded in 96 well plates at a concentration of 40,000 cells per well for ISDC, 10,000 cells/well for MEFs, 30,000 cells/well for D10, 30,000 cells/well for BMDM and BMDC and 30,000 cells/well for 293T-κBS cells. Cells were treated with listed doses of the NF-κB inhibitors and allowed to grow for 24 hours or as described. 10 μl of MTT working solution (3 mg/ml) was added to each well and incubated for
Samples were then analyzed for A1/bfl-1 (Fwd: 5′-AATTCCAACAGCCCTCCAGA-3′ (Ambion). mRNA was then quantified using a NanoDrop (Thermo Scientific).

Confocal microscopy was completed using an Olympus FluoView 1000. Primary antibodies for 1 hour and then secondary antibodies conjugated to Alexafluor 488 were then added to cells for 30 seconds and coverslips were affixed to glass slides using gelvatol (Invitrogen), which was washed and blocked with 2% BSA for 45 minutes. Cells were then washed twice in complete IMDM media and were subsequently analyzed by flow cytometry and fluorescence microscopy. Flow cytometry was performed using a LSRII flow cytometer (BD Bioscience) and analyzed using FlowJo software (TreeStar, Inc.). Fluorescence imaging was performed using an Axiovert 200 Microscope and Axiosview software (Zeiss). To remove background fluorescence, all images had equally reduced green and channel levels for imaging but not quantification.

Immunofluorescence. FSCD were grown on poly-L-lysine-coated coverslips. Cells were fixed for 15 minutes using 2% paraformaldehyde (Sigma) and then washed with PBS. The cells were then permeabilized with 0.1% Triton X-100 (USB) in PBS and then washed and blocked with 2% BSA for 45 minutes. Cells were treated with indicated primary antibodies for 1 hour and then secondary antibodies conjugated to either Alexafluor 488 or 568 (1:500) (Jackson). A1/bfl-1 was then added to cells for 30 seconds and coverslips were affixed to glass slides using gelvatol solution. Confocal microscopy was completed using an Olympus FluoView 1000.

mRNA analysis. FSCD and BMDM were grown in 6 well plates and collected by trypsinization. mRNA was isolated from cell pellets using the RNAqueous Kit (Ambion). mRNA was then quantified using a NanoDrop (Thermo Scientific). Samples were then analyzed for A1/bfl-1 (Fwd: 5′-AATTCTAAGGACTGCTTGGATGAC-3′; Rev: 5′-GGAGAGATTGAGGACTGCTTGG-3′). RT-qPCR was then run for 3 cycles using a Taqman Gene expression Master Mix (Applied Biosystems).

Statistical analysis. Experiments shown are representative of 3–5 independent experiments as described in each experiment with p = values being determined in those experiments with at least three replicates per independent experiment. Error bars in all figures represent standard deviation. p-values were determined using the student t-test in cases of two variable analysis, and using ANOVA tukey analysis for multi-variable comparisons. Statistics were derived using SPSS (SPSS Inc., Chicago, IL).
40. Reuther, J. Y., Reuther, G. W., Cortez, D., Pendergast, A. M. & Baldwin, A. S., Jr. A requirement for NF-kappaB activation in Bcr-Abl-mediated transformation. Genes Dev 12, 968–981 (1998).
41. Lounnas, N. et al. NF-kappaB inhibition triggers death of imatinib-sensitive and imatinib-resistant chronic myeloid leukemia cells including T315I Bcr-Abl mutants. Int J Cancer 125, 308–317 (2009).
42. Kanters, E. et al. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 112, 1176–1185 (2003).
43. Niedernhofer, L. J. et al. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature 444, 1038–1043 (2006).

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Author contributions
J.S.T., L.J.N., S.E.P. and P.D.R. designed the experiments; J.S.T., D.F.G., J.Z. and S.H.D. performed the experiments; J.S.T., L.J.N. and P.D.R. analyzed the data, J.S.T. wrote the manuscript and D.F.G., J.Z., S.E.P., L.J.N. and P.D.R. edited the manuscript.

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