Published in final edited form as:

Immunol Lett. 2015 December ; 168(2): 319–324. doi:10.1016/j.imlet.2015.10.011.

A quinoxaline urea analog uncouples inflammatory and pro-survival functions of IKK β

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Abstract

Activation of the NF- κ B pathway is causally linked to initiation and progression of diverse cancers. Therefore, IKK β , the key regulatory kinase of the canonical NF- κ B pathway, should be a logical target for cancer treatment. However, existing IKK β inhibitors are known to induce paradoxical immune activation, which limits their clinical usefulness. Recently, we identified a quinoxaline urea analog 13–197 as a novel IKK β inhibitor that delays tumor growth without significant adverse effects in xenograft tumor models. In the present study, we found that 13–197 had little effect on LPS-induced NF- κ B target gene induction by primary mouse macrophages while maintaining considerable anti-proliferative activities. These characteristics may explain absence of inflammatory side effects in animals treated with 13–197. Our data also demonstrate that the inflammation and proliferation-related functions of IKK β can be uncoupled, and highlight the utility of 13–197 to dissect these downstream pathways.

Keywords

NF- κ B; IKK β inhibitor; bone marrow-derived macrophages; LPS; inflammatory cytokines

1. Introduction

The NF- κ B pathway was originally identified as essential transcriptional regulators of the immune responses, but ensuing studies revealed its critical involvement in diverse cellular processes including cell growth, differentiation and survival.

Numerous studies linked the NF- κ B pathway activation to cancer. While mutations in the actual NF- κ B pathway genes are mostly limited to lymphoid malignancies [1], both cancer

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cell-intrinsic as well as –extrinsic mechanisms have been proposed to contribute to NF- κ B activation in many solid tumors. For example, the crosstalk between signal transduction pathways enables oncogenic mutant RAS, present in a large fraction of lung, pancreas and colon cancers, to activate the NF- κ B pathway through the adaptor protein p62 [2], GSK3 [3], IL-1 [4] and PI3K-AKT [5]. Pro-inflammatory cytokines produced by activated fibroblasts and immune/inflammatory cells in the tumor microenvironment stimulate cancer cells and activate the NF- κ B pathway in a cancer cell-extrinsic fashion [6].

Essential roles of the NF- κ B pathway activation in tumor initiation and progression are clearly demonstrated by multiple independent studies that genetic inactivation of this pathway protected mice from various solid tumors including pancreas [4], colon [7], breast [8–11] and lung [12,13]. Collectively, these data support that the NF- κ B pathway is a rational target for therapeutic intervention for cancer.

In the absence of stimulation, NF- κ B transcription factors are sequestered in the cytoplasm by binding to I κ B. Upstream signals lead to activation of a tripartite I κ B kinase (IKK, comprises α , β , and NEMO subunits) and phosphorylation of I κ B, which are ubiquitinated and degraded in the proteasome. This liberates NF- κ B proteins, which then translocate to the nucleus and initiate transcription of genes including pro-inflammatory and anti-apoptotic genes.

Given the central roles of IKK complexes in the NF- κ B pathway activation, considerable effort has been made towards developing selective IKK inhibitors [14]. Of the two major catalytic subunits of IKK, early studies established that IKK β was the primary regulator of the NF- κ B activity in a wide range of cell types [15–17]. Consequently, much effort was made on developing specific IKK β inhibitors. ATP-competitive IKK β inhibitors such as ML120B [18] and TPCA-1 [19] acutely inhibited pro-inflammatory cytokine production *in vitro* and successfully reduced the severity of arthritis in mouse models.

However, chronic *in vivo* administration of ML120B induced significant granulocytosis [18] and sensitized mice to LPS-induced septic shock [20]. This was found to be due to paradoxical overproduction of a pro-inflammatory cytokine IL-1 β in the absence of IKK β activity. IL-1 β production is regulated at multiple levels including transcription, translation, post-translational processing and secretion [21]. Chronic IKK β inhibition reduced transcription of IL-1 β (as anticipated from inhibition of NF- κ B), but it also reduced the transcription of serine protease inhibitors in granulocytes and caspase-1 inhibitors in macrophages. Downregulation of these post-translational regulators resulted in elevated plasma IL-1 β levels and predisposed mice to septic shock [20].

Recently, a quinoxaline urea analog 13–197 was identified by its ability to promote cancer cell apoptosis [22,23], and was shown to inhibit the kinase activity of IKK β [24]. Treatment with 13–197 significantly reduced the tumor load, proliferation index and microvessel density in xenograft models of pancreatic cancer [24] and treatment-resistant lymphoma [25]. Importantly, unlike existing IKK β inhibitors, chronic administration of 13–197 did not cause overt side effects in mice; neutrophil count remained normal, and mice treated chronically with 13–197 were protected from LPS-induced septic shock [24]. These results

demonstrate that anti-tumor/anti-survival and pro-inflammatory effects of IKK β inhibitors can be uncoupled, restoring IKK β as a viable and promising therapeutic target.

In the present study, in order to further characterize this novel IKK β inhibitor and gain insights into its disparate effects on downstream signaling pathways, we examined the effect of 13–197 on activation and cytokine production by bone marrow-derived primary mouse macrophages.

2. Materials and Methods

2.1. Mice and bone marrow-derived macrophages (BMDM)

All animal studies were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. BMDM were prepared as previously described [26]. Briefly, bone marrow was flushed from the femurs and tibiae of 8–10 week old C57BL/6 mice. The cells were plated in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine, penicillin/streptomycin, and 10% L929 cell conditioned medium (CM). Cells were fed with fresh media on days 3 and 5. On day 7, non-adherent cells were removed and remaining cells were subjected to experiments in DMEM-10% FBS medium.

2.2. Characterization of BMDM by flow cytometry

Freshly isolated mouse bone marrow cells were cultured in the presence of M-CSF (20 ng/ml) or L929 cell CM for 7 days. Adherent cells were released with trypsin and stained with anti-F4/80-PE (BioLegend, San Diego, CA), anti-CD11b-FITC, anti-CD11c-APC and anti-Ly-6G-PE/Cy7 (BD Biosciences, San Jose, CA). Flow cytometry was performed on a BD LSRII at the UNMC Flow Cytometry Research Facility. Data were analyzed using FlowJo software (FlowJo, Ashland, OR).

2.3. Western blot analysis

In vitro-differentiated BMDMs were pre-treated for 1 hour with 20 μ M 13–197 or TPCA-1 in DMEM-10% FBS. Cells were then stimulated with TNF α (20 ng/ml) or LPS (100 ng/ml) at 37 °C in the presence of inhibitors for indicated period of time. At the end of stimulation, cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, and protease and phosphatase inhibitor cocktails). Equal amount of protein was separated on 8% SDS-polyacrylamide gels and transferred to PVDF membrane. Membranes were incubated with primary antibodies overnight at 4°C, washed, and incubated with secondary antibody conjugated with HRP for 1 hour. Membranes were developed by using Pierce ECL Western Blotting Substrate (Life Technologies, Grand Island, NY).

Antibodies against I κ B α , phospho-I κ B α , 4E-BP1, phospho-4E-BP1, S6K and phospho-S6K were from Cell Signaling Technology (Danvers, MA). Antibody against HSC70 was from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4. Thymidine incorporation assay

Freshly isolated mouse bone marrow cells were seeded in triplicate in 48-well plates at 1×10^5 /well in DMEM-10% FCS containing 20 ng/ml M-CSF and various concentrations of IKK β inhibitors (13–197 or TPCA-1). [3 H] thymidine (4 μ Ci/ml) was added for the last 6 hours of the 48 hour incubation period. To terminate reaction, cells were washed once with ice-cold PBS and incubated in 10% trichloroacetic acid at 4°C. After 30 minutes, wells were washed with ice-cold PBS and cells were solubilized with 0.2 M NaOH at room temperature. Incorporated [3 H] thymidine level was determined by liquid scintillation counting.

2.5. MTT assay

In vitro-differentiated BMDMs were treated for 48 hours with various concentrations of 13–197 or TPCA-1 in DMEM-10% FBS in 48-well plates. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) was added at the final concentration of 0.5 mg/ml for the last 4 hours of incubation. Next, the media was removed and DMSO was added to each well. Optical density was read with a spectrophotometer (SPECTRAMax PLUS, Molecular devices, Union City, CA) at the wavelength of 570 nm.

2.6. RNA isolation and quantitative real-time (qRT) PCR

In vitro-differentiated BMDMs were pre-treated for 1 hour with 20 μ M 13–197 or TPCA-1 in DMEM-10% FBS in 6-well plates. Cells were then stimulated with LPS (100 ng/ml) for various period of time and RNA was isolated with QIAGEN RNeasy kit (Qiagen, Valencia, CA). Maxima cDNA Synthesis kit (Life Technologies) was used to generate cDNA from RNA. Quantitative real-time PCR was performed using Maxima SYBR Green kit on an ABI Prism 7700 Sequence Detection System. Relative transcript quantity was calculated with the values of unstimulated control samples set as 1. The primer sequences are as follows: *Csf1* (M-CSF), TAGAAAGGATTCTATGCTGGG and CTCTTTGTTGAGAGTCTAAG; *Csf2* (GM-CSF), CTACTACCAGACATACTGCC and GCATTCAAAGGGGATATCAG; *Tnf* (TNF α), CTATGTCTCAGCCTCTTCTC and CATTTGGGAACCTTCTCATCC; *Ccl5* (Rantes), AGGAGTATTTCTACACCAGC and CAGGGTCAGAATCAAGAAAC; *Cxcl2* (MIP-2), GGGTTGACTTCAAGAACATC and CCTTGCCTTTGTTTCAGTATC; *Ccl2* (MCP-1), CAAGATGATCCCAATGAGTAG and TTGGTGACAAAACTACAGC; *Il1b* (IL-1 β), GGATGATGATGATAACCTGC and CATGGAGAATATCACTTGTGG; *Il6* (IL-6), AAGAAATGATGGATGCTACC and GAGTTTCTGTATCTCTCTGAAG; *Sod2* (MnSOD), CCATTTTCTGGACAAACCTG and GACCTTGCTCCTTATTGAAG; *Nfkbia* (I κ B α), CAGAATTCACAGAGGATGAG and CATTCTTTTGGCCACTTTCC; *Gapdh* (GAPDH), CCTGGAGAAACCTGCCAAGTATG and AGAGTGGGAGTTGCTGTTGAAGT.

2.7. Cytokine measurement

In vitro-differentiated BMDMs were pre-treated for 1 hour with 20 μ M 13–197 or TPCA-1 in DMEM-10% FBS in 12-well plates. Cells were then stimulated with LPS (100 ng/ml). Supernatants were harvested at various time points and immediately frozen at -80°C until analysis. Cytokine concentrations were measured using a Magnetic Luminex Screening

Assay (R&D Systems, Minneapolis, MN) on a Luminex LX200 Analyzer (Luminex, Austin, TX).

2.8. Data processing

All graphs represent mean $\pm$ SEM. Error bars were often smaller than data point symbols. All experiments were repeated 2–4 times and a data set from a representative experiment is shown.

3. Results and Discussion

3.1. 13–197 has little effect on I κ B α phosphorylation in response to TNF α in BMDMs

Previous studies demonstrated that systemic administration of 13–197 inhibited tumor cell growth without overt toxicity in mice [24,25]. Specifically, in contrast to existing IKK β inhibitors such as ML120B, which enhanced susceptibility to LPS-induced septic shock in mice [20], 13–197 actually protected mice from harmful LPS effects [24]. Because LPS-induced septic shock is primarily mediated by inflammatory cytokines and chemokines released by myeloid lineage cells, we decided to examine the effects of 13–197 on myeloid lineage cells.

To this end, we isolated bone marrow cells from mice and induced them to differentiate into macrophages (bone marrow-derived macrophages, BMDMs) in culture. Successful differentiation into macrophages, as defined by the expression of F4/80, CD11b (Mac-1) and Ly6G and lack of the dendritic cell marker CD11c was confirmed by flow cytometry (Supplementary Figure 1).

Typically, stimulation-induced I κ B α phosphorylation shows a biphasic pattern, the early peak (within 10 minutes) reflecting phosphorylation of the pre-existing I κ B α and the late one (30–180 minutes) that of the newly-synthesized protein. Because phosphorylation of I κ B α triggers its ubiquitination and degradation, the total I κ B α level declines after the early peak of I κ B α phosphorylation. Simultaneously, I κ B α degradation liberates the NF- κ B dimers in the cytoplasm and enables nuclear translocation, where they initiate transcription of NF- κ B target genes, which includes I κ B α itself. This restores I κ B α levels and terminates signal transduction by binding to NF- κ B and re-sequestering it in the cytoplasm.

Prior studies by us showed that 13–197 inhibited TNF α -induced phosphorylation of I κ B α in HEK293 cells with unique kinetics. Namely, 13–197 had little effects on the early phase phosphorylation of I κ B α but reduced the late phase phosphorylation of I κ B α considerably [24]. Based on these data, we first examined the effects of 13–197 on TNF α -stimulated BMDMs. Cells were pre-treated with 13–197 or TPCA-1 (an ATP-competitive IKK β inhibitor [19]) for 1 hour followed by stimulation with exogenous TNF α .

As shown in Figure 1, the effect of 13–197 on I κ B α phosphorylation was very modest, both at the early and the late phases, while TPCA-1 completely inhibited I κ B α phosphorylation. Of note, a significant portion of TPCA-1 treated cells lost viability within 120 minutes of TNF α stimulation, which likely contributed to the reduced total I κ B α signal at later time points.

3.2. 13–197 reduces proliferation and survival of BMDMs

It was previously demonstrated that 13–197 reduced proliferation of cancer cells both in vitro and in vivo [24,25]. In order to assess whether 13–197 exerts similar effects on non-cancer cells, we examined BMDM cell growth and survival.

It has been known that bone marrow cells proliferate in response to exogenous M-CSF immediately after isolation, but the proliferative response declines gradually and almost completely ceases when they are fully differentiated into macrophages in vitro [27]. Therefore, in order to evaluate the effects of IKK β inhibitors on proliferation, we treated freshly-isolated bone marrow cells with the inhibitors prior to terminal differentiation. As shown in Figure 2A, 13–197 inhibited DNA synthesis of bone marrow cells as measured by [3 H]thymidine incorporation in a dose dependent manner. This effect is accompanied by reduced phosphorylation of 4E-BP1 and p70 S6K (Figure 2B), similar to what was previously reported with cancer cells [24,25].

We also tested the effects of 13–197 on fully-differentiated BMDMs. Bone marrow cells were cultured in the presence of L929 cell CM for 7 days to promote differentiation into macrophages before they were treated with 13–197. As shown in Figure 2C, 13–197 reduced conversion of MTT into formazan, reflecting reduced cell viability in a dose-dependent manner. Collectively, we concluded that 13–197 showed marginal effects on I κ B α phosphorylation upon TNF α stimulation but inhibited growth/survival of BMDMs.

3.3. 13–197 does not affect LPS-triggered I κ B α phosphorylation in BMDMs

LPS, a key mediator of septic shock, binds to the cell surface TLR4 and triggers pro-inflammatory cytokine transcription by activating the canonical NF- κ B pathway [28,29]. Previous studies on 13–197 as an IKK β inhibitor focused primarily on its effects upon TNF α stimulation but it has not been fully clarified how 13–197 works when the NF- κ B pathway is activated with other stimuli. Because pre-treatment with 13–197 protected mice from LPS-induced septic shock [24], we examined how 13–197 affects IKK β activation upon LPS stimulation. As expected, when BMDMs were stimulated with LPS, pre-treatment with TPCA-1 almost completely blocked I κ B α phosphorylation (Figure 3). In contrast, 13–197 treatment showed only marginal reduction on the levels of I κ B α phosphorylation compared to vehicle treatment up to 180 minutes after LPS stimulation.

3.4. 13–197 does not affect LPS-triggered cytokine transcription in BMDMs

Numerous studies clearly demonstrate that the NF- κ B pathway activation is required for transcription of pro-inflammatory cytokines and chemokines. In light of marginal effects of 13–197 on I κ B α phosphorylation, we next examined the transcript levels of classical NF- κ B target genes including inflammatory cytokines in response to LPS stimulation by BMDMs treated with 13–197 and TPCA-1. BMDMs were pre-treated with respective inhibitors and transcript levels were evaluated by qRT-PCR. While TPCA-1 almost completely blocked transcription of most of the NF- κ B target genes, 13–197 showed very little effect on the levels of all the transcripts examined (Figure 4A). Combined with the marginal effects on I κ B α phosphorylation, we concluded that 13–197 had little effects on the NF- κ B pathway transcription program initiated by LPS stimulation.

3.5. 13–197 does not affect LPS-triggered cytokine secretion by BMDM

While the levels of transcript are informative of cellular responses, the actual biological effects are mediated by secreted protein products. Importantly, because secretion of some cytokines, most notably IL-1 β , is known to depend heavily on post-translational processing [21], transcript levels may not reflect that of biologically active protein accurately. To address this, we measured the levels of inflammatory cytokines/chemokines in the culture supernatant of BMDMs stimulated with LPS in the presence or absence of IKK β inhibitors. As anticipated from the previous report that an IKK β inhibitor paradoxically increases IL-1 β secretion [20], TPCA-1 treatment inhibited the secretion of most cytokines and chemokines except for IL-1 β and M-CSF, which were actually detected at higher concentration than in control cells (Figure 4B). In contrast, 13–197 treatment showed little effects on the levels of most cytokines examined (Figure 4B).

The elevated IL-1 β secretion upon IKK β inhibition has been attributed to the changes in post-translational processing, but similar mechanisms for M-CSF have not been described. Importantly, whereas control cells produced abundant M-CSF transcripts upon LPS stimulation (Figure 4A), no significant amount of M-CSF protein was detected in the corresponding culture supernatant (Figure 4B). These discrepancies may indicate that, in the control culture, secreted M-CSF protein was immediately consumed by BMDMs in an autocrine/paracrine fashion. Likewise, the increased M-CSF in IKK β inhibitor-treated culture supernatants may reflect release of receptor-bound and/or intracellular M-CSF pools as the cell viability declined and the plasma membrane integrity was compromised (Figure 2C). This pool may also include a carryover from the *in vitro* differentiation process because BMDMs were derived in the presence of L929 cell CM which contains abundant murine M-CSF.

In the present study, we demonstrated that the quinoxaline urea analog 13–197 showed only marginal effects on I κ B α phosphorylation in BMDM upon stimulation with TNF α or LPS. We also confirmed that 13–197 inhibited pro-survival/anti-apoptotic functions of IKK β in BMDMs. This unique mode of action is likely one of the basis for its apparent lack of immunological side effects while maintaining anti-tumor activity. The present study demonstrates that 13–197 is a unique reagent to uncouple multiple downstream effectors of IKK β .

Although IKK β was originally identified as the essential kinase in the NF- κ B pathway, it is now well documented that IKK β is involved in a variety of NF- κ B-independent functions [30]. Among them, IKK β -mediated phosphorylation of TSC1, a member of the TSC1/2 tumor suppressor complex, has been shown to suppress the repressor function of the TSC complex towards mTOR [31]. Thus, IKK β activation results in mTOR activation and subsequent phosphorylation of S6K and 4E-BP1. On the basis of these reports and our present findings, we propose a model that 13–197 inhibits IKK β activity not by binding to the ATP binding pocket of the kinase, but by interacting with IKK β in the substrate recognition interface. According to this model, we expect that the binding affinity of 13–197 to IKK β is higher than TSC1 but lower than I κ B α in BMDMs, inhibiting only the NF- κ B-independent pathway without affecting the NF- κ B-dependent signals. We further posit that

the relative abundance, post-translational modifications, interaction with other molecules in higher order complexes and diverse other factors may influence effective binding affinity between IKK β and its substrates inside the cells. Thus, although 13–197 inhibited IKK β kinase activity towards a peptide substrate derived from the I κ B α sequence at the IC₅₀ of a low μ M concentration in an *in vitro* kinase assay [24], it is conceivable that 13–197 is less effective in displacing I κ B α protein from the substrate recognition interface of IKK β in select cell types.

The exact molecular/biochemical mechanisms how 13–197 inhibits IKK β remain to be clarified. The proposed model is experimentally testable. Future studies should decipher exact mechanisms of IKK β inhibition by 13–197.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from UNMC-Buffett Cancer Center to M.N. and the National Institutes of Health to A.N. (CA182820). Authors would like to thank the UNMC Flow Cytometry Core for flow cytometry and Eppley High Throughput Screening Core for Luminex assays. Support for the core facilities are provided, in part, by the Nebraska Research Initiative and the Fred and Pamela Buffett Cancer Center Support Grant (P30CA036727).

Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E-binding protein
BMDM	bone marrow-derived macrophage
CM	conditioned medium
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
FBS	fetal bovine serum
GSK3	glycogen synthase kinase 3
HRP	horseradish peroxidase
IκB	inhibitor of NF- κ B
IKKβ	I κ B kinase β
IL	interleukin
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor

ML120B	<i>N</i> -(6-Chloro-7-methoxy-9 <i>H</i> -pyrido[3,4- <i>b</i>]indol-8-yl)-2-methyl-3-pyridinecarboxamide dihydrochloride
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor of kappa chain enhancer of activated B cells
PI3K	phosphoinositide 3-kinase
S6K	ribosomal protein S6 kinase
SEM	standard error of the mean
TLR	Toll-like receptor
TSC	tuberous sclerosis
TNFα	tumor necrosis factor α
TPCA-1	2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide

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Highlights

- Unlike ATP competitive IKK β inhibitor TPCA1, 13–197 lacks inflammatory side effects.
- We examined the effects of 13–197 on primary mouse macrophages.
- 13–197 inhibited growth and survival of primary mouse macrophages.
- 13–197 did not affect LPS-induced NF- κ B target gene regulation in macrophages.

Figure 1.

13–197 shows marginal effects on IKK β activation in bone marrow-derived macrophages (BMDMs) stimulated with TNF α . Mouse bone marrow cells were cultured in the presence of L929 cell CM to promote differentiation into the macrophage lineage. After 7 days, media was replaced with DMEM-10% FBS, BMDMs were pre-treated for 1 hour with 13–197 or TPCA-1 at 20 μ M and stimulated with 20 ng/ml TNF α in the presence of inhibitors.

Figure 2.

13-197 inhibits proliferation and survival of primary bone marrow cells. **(A)** Freshly-isolated mouse bone marrow cells were seeded in DMEM-10% FBS containing 20 ng/ml M-CSF and treated with indicated amount of 13-197 or TPCA-1. Cell proliferation was determined by the incorporation of 3 H-thymidine. **(B)** Freshly-isolated mouse bone marrow cells were seeded in DMEM-10% FBS containing 10% L929 cell CM in the presence (+) or absence (-) of 20 μ M 13-197. Cell lysates were collected after 48 hours and probed with indicated antibodies. **(C)** Freshly-isolated mouse bone marrow cells were cultured in the

presence of L929 cell CM for 7 days to generate BMDMs. BMDMs were treated with indicated amount of 13–197 or TPCA-1 in DMEM-10% FBS. Cell viability was measured by MTT assay.

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Figure 3.

13-197 shows marginal effects on IKK β activation in BMDMs stimulated with LPS. Mouse bone marrow cells were cultured in the presence of L929 cell CM to promote differentiation into the macrophage lineage. After 7 days, media was replaced with DMEM-10% FBS, cells were pre-treated with 20 μ M 13-197 or 20 μ M TPCA-1 for 1 hour and BMDMs were stimulated with 100 ng/ml LPS.

Figure 4.

13–197 shows marginal effects on the NF- κ B-dependent transcriptional program in LPS-stimulated BMDMs. **(A)** Mouse bone marrow cells were cultured in the presence of L929 cell CM to promote differentiation into the macrophage lineage. After 7 days, media was replaced with DMEM-10% FBS, BMDMs were pre-treated with μ M 13–197 or 20 μ M TPCA-1 for 1 hour before stimulation with 100 ng/ml LPS. Cells were harvested at indicated time after LPS stimulation, RNA was prepared and transcript levels were measured by qRT-PCR. **(B)** Mouse bone marrow cells were prepared and stimulated as

above. Supernatant was harvested at indicated time points and cytokine/chemokine levels were measured by Luminex-based ELISA.

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