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The Tyrosine Kinase BMX Is an Essential Mediator of Inflammatory Arthritis in a Kinase-Independent Manner

Marie Gottar-Guillier,* Francis Dodier,†‡ Francis Huesken,* Vadim Iourgenko,† Craig Mickenan,‡ Mark Labow,‡ Samuel Gaveraux,* Bernd Kinzel,* Matthias Mueller,* Kari Alitalo,‡ Amanda Littlewood-Evans,* and Bruno Cenni*†

Inflammatory cytokines like TNF play a central role in autoimmune disorders such as rheumatoid arthritis. We identified the tyrosine kinase bone marrow kinase on chromosome X (BMX) as an essential component of a shared inflammatory signaling pathway. Transient depletion of BMX strongly reduced secretion of IL-8 in cell lines and primary human cells stimulated by TNF, IL-1β, or TLR agonists. BMX was required for phosphorylation of p38 MAPK and JNK, as well as activation of NF-κB. The following epistasis analysis indicated that BMX acts downstream of or at the same level as the complex TGF-β activated kinase 1 (TAK1)–TAK1 binding protein. At the cellular level, regulation of the IL-8 promoter required the pleckstrin homology domain of BMX, which could be replaced by an ectopic myristylation signal, indicating a requirement for BMX membrane association. In addition, activation of the IL-8 promoter by in vitro BMX overexpression required its catalytic activity. Genetic ablation of BMX conferred protection in the mouse arthritis model of passive K/BxN serum transfer, confirming that BMX is an essential mediator of inflammation in vivo. However, genetic replacement with a catalytically inactive BMX allele was not protective in the same arthritis animal model. We conclude that BMX is an essential component of inflammatory cytokine signaling and that catalytic, as well as noncatalytic functions of BMX are involved. The Journal of Immunology, 2011, 186: 6014–6023.

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or VEGF-B (28). Furthermore, a role of BMX in TLR signaling and cytokine secretion has been suggested based on its activation by TLR ligands (29–31). Finally, skin-specific overexpression of BMX has been shown to generate skin hyperplasia and inflammatory angiogenesis (32).

In the current study, we show that BMX plays a central role in the secretion of proinflammatory cytokines induced by TNF and TLR ligands. Dissection of the signaling pathways indicates that BMX acts downstream of or at the same level as the TAK1–TAB1 complex. We further show that the kinase activity and the membrane localization of BMX are required for TNF signaling. We finally show that whereas BMX deficiency protects from arthritis in an animal model, the BMX kinase activity is not required for its role in arthritis. We conclude that BMX has a nonredundant role in inflammation and that some functions are kinase-independent.

Materials and Methods

Materials

The plasmids with the human coding sequences pCMV6-XL5-BMX and pCMV6-XL5-RIPK6 were purchased from OriGene, and the plasmids pcDNA3.1-TAB1 and pcDNA3.1-TAK1 were constructed at Novartis. The pRL–TK reporter plasmid was from Promega, and the pGL3-IL8 promoter reporter plasmid was constructed with the mouse IL-8 promoter (GenBank accession no. AF385628). The BMX constructs were cloned in pcDNA3.1, and point mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene) with the primers listed in Supplementary Table 1. All small interfering RNAs (siRNAs) were synthesized at Novartis with the target sequences listed in Supplementary Table 2. The siRNA libraries have been described earlier and comprise siRNA targeting 24,373 predicted human genes (33), as well as 5000 “druggable” genes, predominately enzymes and receptors (34, 35). Synthesis of siRNA was performed with standard phosphoramidite chemistry. All siRNAs are of 21 oligonucleotides length with a 19-base-pair ribo-nucleotide duplex region and two deoxynucleotides overhangs on the 3′ terminus of each strand. The 5′ ends of both strands are unphosphorylated. Recombinant human TNF and IL-1β were produced at Novartis. LPS was purchased from Sigma, human EGF from R&D Systems, Pam3Cys-SKHK from EMC Microcollections, and polyinosinic:polycytidylic acid (polyI:C) from GE Healthcare. Reporter gene assays were analyzed with the Dual Luciferase Reporter Assay from Promega. The following Abs were used: anti-p65, anti-BMX (H220), and HRP-conjugated secondary Abs from Cell Signaling Technology. The following Abs were used: anti–anti-p65, anti–BMX (H220), and HRP-conjugated secondary Abs from Cell Signaling Technology.

Animals

The BMX knockout (KO) mice have been described previously (24). The BMX KO mice were used after being back-crossed 10 times into a BALB/c background. The kinase-deficient BMX K421R (equivalent to the human K454R mutation) knock-in mice were generated in a pure BALB/c background as follows. Murine BMX genomic sequences from intron 12 to intron 14 and containing the codon for K421 on exon 14 were amplified from murine BMX mouse genomic DNA and subcloned into a modified pRay2 vector (GenBank accession no. U63120). Site-directed mutagenesis was performed to introduce the K421R point mutation. The targeting plasmid was transfected into embryonic stem cells from BALB/c mice. Transfected embryonic stem cells were selected for neomycin resistance. Homologous recombination was identified by PCR and confirmed by Southern hybridization. The loxP-flanked neomycin cassette was deleted after homologous recombination in BMX KO mice. Chimeric offspring were further crossed with BALB/c, and F1 heterozygotes were interbred to obtain homozygous mutant mice C-BMXKO/−/−.

All mice were housed in filter-top cages under specified pathogen-free conditions, and a standard diet and water were provided ad libitum. All mice were used for experiments between 10 and 14 wk of age and for all experiments littermates were used as controls. All animal procedures were approved by the institutional ethics committee.

Arthritis model and histology

The model of arthritis induced by transfer of serum from K/BxN mice has been described (36, 37). Arthritis was induced by i.p. injection of 250 μl serum from K/BxN mice. Arthritis was scored visually in each paw using a scale of 0 (no signs of inflammation) to 3 (maximal inflammation and systolic pressure with separate scores for the proximal and distal paws in a ratio of maximum score 24 per animal). These assessments were performed by two trained individuals who were blinded to the study groups.

For histological analysis, animals were sacrificed and hind paws were isolated at day 13 after serum transfer. Samples were fixed for 4 h in 4% paraformaldehyde at 4˚C. After fixation, the samples were dehydrated, infiltrated in methyl-methacrylate (Fluka), dibutylphthalate (Merck), Per- klux (Merck), Dr. Grogg Chemie, and Novocane (Lonza) for a maximum of 10 passages. HUVECs were purchased with culture medium from Promocell and cultured for a maximum of 10 passages. All cell cultures were maintained at 37˚C in a humidified incubator with 5% CO2. For siRNA transfection, HeLa cells or HDFs were seeded in 150 μl culture medium in 96-well plates, and 50 μl OptiMEM (Invitrogen) containing 2 nM (final concentration) siRNA and 0.75 μl HiPerfect (Qiagen) were added. Cells were incubated 48 h before further analysis. For siRNA transfection, HeLa cells or HDFs were seeded in 6-well plates and transfected in 100 μl OptiMEM containing 2 nM siRNA and 12 μl HiPerfect. After 48 h, cells were trypsinized, seeded in 96-well plates, and 4 h later transfected with plasmid DNA as described above.

Cell culture and transfections

HeLa cells (from American Type Culture Collection) and HEK293-EBNA cells (from Invitrogen) were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS (Amimed). Human dermal fibroblasts (HDFs) from neonatal skin (Lonza) were cultured in FGM-2 medium (Lonza) for a maximum of 10 passages. HUVECs were purchased with culture medium from Promocell and cultured for a maximum of 10 passages. All cell cultures were maintained at 37˚C in a humidified incubator with 5% CO2. For siRNA transfection, HeLa cells or HDFs were seeded in 150 μl culture medium in 96-well plates, and 50 μl OptiMEM (Invitrogen) containing 2 nM (final concentration) siRNA and 0.75 μl HiPerfect (Qiagen) were added. Cells were incubated 48 h before further analysis. For plasmid transfections, HeLa cells were plated and transfected the next day with 100 μl transfection mix containing 100 μl OptiMEM, 200 ng DNA, and 1 μl GenePORTER (Gene Therapy Systems) during 4 h. For sequential transfection of siRNA and plasmid DNA, HeLa cells were seeded in 6-well plates and transfected in 100 μl OptiMEM containing 2 nM siRNA and 12 μl HiPerfect. After 48 h, cells were trypsinized, seeded in 96-well plates, and 4 h later transfected with plasmid DNA as described above. For reporter gene assays, plasmids were transfected in HEK293-EBNA cells after seeding cells in 100 μl culture medium in 96-well plates and adding 10 μl OptiMEM containing 200 ng DNA and 0.8 μl Fugene6 (Roche). HUVECs were transfected after seeding the cells in 24-well plates in 500 μl medium and adding 200 μl OptiMEM containing 100 nM (final concentration) siRNA and 2 μl Lipofectin (Invitrogen).

Immunoblotting and subcellular fractionation

Cells were lysed in 50 mM Tris pH 7.4, 1% SDS, 5 mM EDTA, 10 mM glycerophosphate, 10 mM NaF, PhosSTOP (Roche), and protease inhibitor cocktail (Roche). Lysates were ultracentrifuged, and the protein concentration was determined by the BCA test (Sigma). Equal amounts of protein were re-solved on ∼4–12% Bis-Tris polyacrylamide NuPAGE gels (Invitrogen). After transfer onto nitrocellulose membranes, proteins were detected with appropriate Abs and Immobilon Western Reagent (Millipore). Chemiluminescence intensity of immunoblot membranes was quantified with a ChemiDoc system (Bio-Rad). Nuclear proteins were isolated using NE-PER reagent as recommended by the manufacturer (Pierce).

IL-8 cytokine secretion and mRNA detection

Cell culture supernatants were collected, and the IL-8 concentration was determined in 10 μl supernatant by homogenous time-resolved fluorescence assays as recommended by the manufacturer (Cisbio). The mRNA levels were determined by quantitative real-time PCR after isolating total RNA (RNeasy microkit; Qiagen) and transcribing cDNA (Superscript III; Invitrogen). Duplex real-time PCR was performed with TaqMan Fast Universal PCR mix (Applied Biosystems) as recommended by the manufacturer using probes for IL-8 and for ribosomal S18 RNA in the same reaction (Applied Biosystems).

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Results

Depletion of BMX protein results in decreased secretion of IL-8

Genome-wide siRNA libraries were screened for regulation of TNF-induced IL-8 secretion in HeLa cells (33–35), and several siRNAs targeting BMX were found to be potent inhibitors (data not shown). A selection of siRNAs designed to target different sequences in the human BMX mRNA were transfected in HeLa cells according to the HiPerfect protocol, and TNF-induced IL-8 was measured (Fig. 1A). Cells transfected with siRNA directed against TNFR1 (siTNFR1) or TAK1 (siTAK1) showed approximately a 70% decrease in IL-8 secretion in comparison with cells transfected with an siRNA targeting luciferase (siControl). The transfection of siRNAs against BMX (siBMX 56, 57, and 88) led to a strong reduction of IL-8 secretion similar to the positive controls, whereas siBMX87 showed intermediate effects. A pool of these four siRNA against BMX showed maximum reduction of IL-8 secretion. All BMX siRNAs, except siBMX88, did not show cytotoxic effects for the duration of our experiments. The cell lines used in our cytokine secretion studies did not express sufficiently high levels of BMX to assess accurately the knockdown of endogenous BMX protein by the siRNA with the available reagents. We therefore confirmed the efficacy of BMX depletion in primary HUVECs, which express high levels of endogenous BMX. All four siRNAs led to a strong reduction of endogenous BMX protein (Fig. 1B). The reduction in IL-8 protein secretion was correlated with reduced levels of IL-8 mRNA in HeLa cells (Fig. 1C). Knockdown of BMX prevented TNF-induced IL-8 mRNA accumulation to almost the same degree as the siRNA against TNFR1. This suggests that BMX is required for TNF-induced IL-8 production at a stage earlier than mRNA transcription.

We expanded the validation of BMX to primary cells and chose HDFs to test the role of BMX in several inflammatory signaling pathways (Fig. 1D). The siRNAs against BMX strongly reduced IL-8 secretion after stimulation with the TLR4 agonist LPS, the synthetic TLR1/2 agonist Pam3Cys, and the synthetic TLR3 agonist poly(I:C). In addition, the closely related signaling pathway of IL-1β was similarly inhibited. Altogether, these results indicate that BMX plays an important role in a pathway shared by several inflammatory stimuli.

FIGURE 1. Depletion of BMX inhibits inflammatory cytokine response. A, HeLa cells were transfected with 2 nM siRNA against luciferase (siControl), TNFR1 (siTNFR1), BMX (siBMX 56, 57, 87, and 88), and a pool of these four siRNAs (each at 0.5 nM final concentration). After 48 h, the cells were stimulated with 30 ng/ml TNF for 8 h, and IL-8 was measured in the supernatants. The levels of IL-8 in unstimulated cells ranged from 50 to 300 pg/ml and in stimulated cells transfected with siControl from 1540 to 2970 pg/ml. Bars indicate the means ± SEM of three independent experiments. B, The levels of endogenous BMX protein were measured in HUVECs 48 h after transfection with several different siRNAs against BMX. Equal amounts of cell lysates were loaded and analyzed with Abs against BMX and actin. C, Knockdown of BMX inhibits TNF-induced IL-8 mRNA accumulation. HeLa cells were transfected with siRNA and 48 h later stimulated with 30 ng/ml TNF for the indicated time period. Total RNA was extracted, and IL-8 mRNA levels were measured by real-time PCR. Data points represent averages of IL-8 mRNA normalized to 18S rRNA from three independent experiments. D, BMX is required for IL-8 secretion induced by multiple inflammatory signals. Primary HDFs were transfected with siRNA and 48 h later stimulated for 4 h with 0.5 ng/ml IL-1β, for 8 h with 30 ng/ml TNF or 10 μg/ml poly(I:C), or for 16 h with 10 ng/ml LPS or with 1 μg/ml Pam3Cys. The levels of IL-8 in stimulated cells transfected with siControl were in the range 680–4330 pg/ml, depending on stimulus and duration. Bars indicate means ± SEM of three independent experiments.
**BMX regulates TNF-mediated MAPK and NF-κB signaling**

The known pleiotropic effects of TNF prompted us to examine the impact of BMX depletion on MAPK and NF-κB pathways. We found that depletion of BMX in HeLa cells completely blocked TNF-induced phosphorylation of JNK (p46 and p54 isoforms), p38, and p65 NF-κB (Fig. 2A). In addition, the TNF-induced degradation of IκB was completely prevented. The inhibition by the siRNA against BMX was comparable with the TNFR1 siRNA reference, as shown by direct quantification of the chemiluminescence signals of several experiments (Fig. 2B). Similarly, the levels of nuclear p65 NF-κB were reduced by ∼50% (Fig. 2C).

These data suggest that BMX is required for TNF-induced activation of the JNK and p38 MAPK as well as NF-κB pathways.

**BMX acts downstream or at the TAK1–TAB1 complex**

The protein kinase TAK1 and its subunit TAB1 (7, 38, 39) as well as RIPK1 (40–43) are equally critical for the activation of NF-κB.

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**FIGURE 2.** Essential role of BMX in MAPK and NF-κB signaling pathways. **A**, Knockdown of BMX inhibits TNF-induced phosphorylation of p38, of the JNK p54 and p46, and of p65 NF-κB. Similarly, TNF-induced degradation of IκB is blocked. HeLa cells were transfected with siRNA according to the jetPRIME protocol and 48 h later stimulated with 30 ng/ml TNF. Equal amounts of lysate proteins were loaded and immunoblotted with the indicated Abs. One representative immunoblot of at least three separate experiments is shown. **B**, Knockdown of BMX leads to similar inhibition of TNF signaling as the TNFR1 reference siRNA. The chemiluminescence intensity of immunoblots from three independent experiments was quantified, and the ratios to the actin bands in the same experiment were calculated. Protein/actin ratios normalized to the unstimulated siControl are shown as averages ± SEM. **C**, Depletion of BMX reduces nuclear accumulation of p65 NF-κB. HeLa cells were transfected with siRNA according to the HiPerfect protocol and 48 h later stimulated with 30 ng/ml TNF for 5 min. Nuclear extracts were prepared, and equal amounts of proteins were loaded and immunoblotted with an anti-p65 Ab. Transfection of an siRNA against BMX led to an approximate reduction of 50% in nuclear p65 compared with the reference siRNA against TNFR1. **D**, BMX depletion does affect EGF-induced phosphorylation of STAT3 S727 or Y705. HeLa cells were transfected with siRNA according to the jetPRIME protocol and 48 h later stimulated with 10 ng/ml EGF. Equal amounts of lysate proteins were loaded and immunoblotted with the indicated Abs. One representative immunoblot of at least three separate experiments is shown.
and MAPKs by proinflammatory cytokines and microbial pathogens. Overexpression of RIPK1 is known to activate NF-κB (40). Similarly, coexpression of TAK1 and TAB1 leads to activation of TAK1, and in turn NF-κB (44). As shown above, TAK1 knockdown strongly inhibited TNF-induced IL-8 secretion (Fig. 1A). We used this approach to dissect at which level BMX regulates the TNF signaling cascade.

The overexpression in HeLa cells of RIPK1 (Fig. 3A) or TAK1 plus TAB1 (Fig. 3B) induced dose-dependent IL-8 secretion. As expected, this effect was independent of TNFR1 depletion, and, conversely, potently inhibited by depletion of the downstream effectors TAK1 or NF-κB p65. Depletion of BMX reduced IL-8 production in both systems, indicating that BMX acts downstream of RIPK1 and downstream or at the same level as the TAK1–TAB1 complex.

We attempted to elucidate the molecular interaction of BMX with the TAK1 complex by immunoprecipitation from BMX transfected cells. Endogenous TAK1 could be copurified after immunoprecipitation of endogenous TAB2, but not with a TAB3 Ab. Similarly, transfected BMX was found to copurify both with endogenous TAB2 and TAB3 (Supplemental Fig. 1). Whereas the TAB3 Ab allowed copurification of BMX and TAB2, it did not permit the detection of endogenous TAB3 (data not shown). The reverse copurification of the TABs or TAK1 with BMX could not be detected with any of the available Abs against BMX or tagged versions of BMX (data not shown). It is therefore not possible to conclude whether BMX physically interacts with the TAK1–TAB complex.

**BMX PH domain and kinase activity are required for in vitro IL-8 promoter activation**

To elucidate further the molecular mechanism by which BMX regulates inflammatory signaling, we generated several BMX mutants (Fig. 4). Transient overexpression of wild-type (WT) full-length human BMX in HEK293-EBNA cells resulted in a robust activation of the IL-8 promoter reporter gene in a dose-dependent manner (Fig. 5A, 5D). Similar levels of activation by BMX overexpression were obtained with a synthetic NF-κB reporter gene construct (data not shown).

We first assessed whether BMX kinase activity was required for IL-8 activation by testing the mutations of the ATP coordination site K445R (Fig. 5A) and E460D (data not shown) or of the activation loop Y566F (data not shown). IL-8 promoter activation was completely abolished for all these kinase-dead mutants. In addition, the three deletion mutants lacking the kinase domain (PH-TH, PH-TH-SH3, and PH-TH-SH3-SH2) did not activate the IL-8 promoter. This indicates that the BMX kinase activity is required to activate the IL-8 promoter after BMX overexpression.

We then addressed the question whether the PH domain was required for BMX function by testing the N-terminal deletion mutant TH-SH3-SH2-KD and found that it failed to activate the IL-8 reporter (Fig. 5A). The proposed function of the PH domain is to confer localization of BMX in membrane subdomains enriched in phosphatidylinositol phosphates (18). This hypothesis was substantiated by the fact that replacing the PH domain by the ectopic myristylation signal from the N terminus of c-Src (45) fully restored IL-8 reporter activation by the Myr-dPH construct (Fig. 5A). We conclude that the PH domain is required for in vitro IL-8 promoter activation by BMX overexpression.

The expression levels of all BMX constructs were assessed with an Ab recognizing an epitope present in all constructs, and all mutants were found to be expressed at similar levels as the WT BMX (Fig. 5B). Mutants consisting of the isolated PH domain or the SH2-KD domains are probably not stable (data not shown), as they could not be detected, even as tagged proteins.

We noticed that upon overexpression, the WT BMX and E42K mutants showed a shift to higher apparent m.w. compared with that of the kinase-deficient K445R point mutant (Supplemental Fig. 2). This suggested that posttranslational modifications might occur when BMX is overexpressed. Indeed, additional studies using an anti-phosphotyrosine Ab to detect the tyrosine phosphorylation status of immunoprecipitated BMX showed that overexpression of BMX in HeLa cells led to an increase in tyrosine phosphorylated BMX (Fig. 5C). This is also the case for the E42K mutant, which is thought to represent a gain-of-function of the PH domain resulting in increased membrane association (46) in analogy to the IL-8 expression.

**FIGURE 4.** Schematic representation of the mutant BMX constructs. All constructs were based on the human BMX coding sequence, and the start and end amino acids of the selected domain constructs are indicated with dotted lines. Point mutations are indicated by stars and the respective amino acid code changed at the given position.
well-described E41K mutation in BTK (47, 48). This is, however, not the case for the PH loss-of-function mutation R29N, further underscoring the requirement for membrane localization. As the double E42K+K445R mutant did not activate the IL-8 promoter, we conclude that BMX activation triggered by overexpression and constitutive membrane association is dependent on BMX kinase activity.

BMX-deficient mice are protected from arthritis

To assess the relevance of our in vitro findings, we selected a well-characterized mouse model of arthritis based on the passive transfer of arthritogenic serum of the K/BxN mice (36, 37). This model was chosen because it is dependent on macrophages and IL-1β. The arthritis induced by the K/BxN serum was strongly reduced in the BMX-deficient mice (BMX KO) compared with that of WT littermates (Fig. 6A). The protection from clinical signs of arthritis was consistently reproduced in three independent experiments (Fig. 6B). In line with the observed reduction of clinical signs for arthritis, the histological analysis of BMX-deficient mice clearly showed reduced histopathology compared with that of WT littermates. In contrast to BMX WT mice, sections from the BMX KO mice (Fig. 6C, lower row) showed a marked absence of cartilage damage and bone erosion, while the joint space was well preserved. These histological findings were similar to those of non-arthritic mice (data not shown). The infiltration of inflammatory cells into the tarsal and metatarsal regions and in the hyperplastic synovium was quantified (Fig. 6D). BMX-deficient mice showed a statistically significant reduction of cell infiltration and fibrosis formation. These results underscore that the regulation of inflammatory cytokines by BMX is relevant in vivo.

Protection from arthritis is independent of BMX kinase activity

We sought to determine whether the kinase-dependent regulation of the IL-8 promoter was confirmed in vivo. Mice in which the BMX gene was replaced by a catalytically inactive allele expressing the K421R mutation (equivalent to the human K445R) were tested in the passive K/BxN serum transfer model. The K421R knock-in mice did not show any significant protection from arthritis compared with WT littermates (Fig. 7A). Although a first experiment showed a slight trend for reduction of arthritis in the K421R animals, this was not substantiated in a second experiment (Fig. 7B). This indicates that although in vitro BMX overexpression...
FIGURE 6. BMX-deficient mice are protected from arthritis induced by K/BxN serum. A, Age-matched BMX WT and BMX KO mice were injected with K/BxN serum, and the development of arthritis was monitored. The BMX KO mice (open circles) showed strongly diminished disease scoring compared with the BMX WT mice (closed circles). Paws were scored individually using a score of 0 (no signs of inflammation) to 3 (maximal inflammation and swelling). The data are shown as averages ± SEM (n = 7 in each group). B, Data from three independent experiments comparing the cumulative total of paw scores for each mouse on day 12 are shown relative to the BMX WT groups (n = 5 to 7 per group). The centerline of the bars indicates the median, and the upper and lower borders of the bars represent the 25th percentile. Each circle shows the cumulative score of a single mouse. C, A histopathological analysis of representative sections of the tarsal regions from BMX WT mice (upper panels) and BMX KO mice (lower panels) taken 12 d after injection of K/BxN serum is shown. The Giemsa-stained section in the top left panel shows inflammatory infiltrates and destruction of the synovial lining in the BMX...
activates the IL-8 promoter in a kinase-dependent manner, this is not sufficient to inhibit inflammation in vivo.

Discussion

In this study, we identify an essential role for the tyrosine kinase BMX in cytokine signaling and inflammation. We first show that BMX is an essential component of inflammatory cytokine secretion induced via a signaling pathway shared by TNF, as well as by IL-1β and several TLR ligands. In addition, BMX regulates TNF-dependent signaling at a central node, which equally affects the three efferent signaling branches of JNK, p38 MAPK, and NF-κB. Epistasis studies reveal that BMX functionally regulates inflammatory signaling at or below the level of the shared TAK1–TAB complex. In vitro, the IL-8 promoter is shown to be regulated by overexpression of BMX in a manner requiring kinase activity and membrane localization. These findings are then extended to an in vivo model of inflammatory arthritis where genetic ablation of BMX is shown to prevent disease. However, in contrast to the in vitro regulation of the IL-8 promoter, genetic replacement of the WT BMX with a catalytically inactive allele did not confer protection from arthritis.

Previous reports indicated a role of BMX in vitro for endotoxin-induced cytokine secretion in macrophages and synovial fibroblasts from RA patients. Two studies (29, 30) found that overexpression of BMX mediated an increase in LPS-induced stabilization of the IL-6 mRNA, whereas overexpression of BMX in the absence of LPS did not induce IL-6 mRNA. The effects of BMX overexpression on LPS-induced IL-6 were found to be independent of p38, based on the use of the p38 inhibitor SB203580. As this compound has been shown to inhibit also RIPK2 (49–51), it is difficult to draw firm conclusions on the requirement of p38 for mRNA stabilization in this context. Although these studies did not include experiments based on BMX depletion, they showed that BMX overexpression affected LPS-mediated mRNA stabilization via the 3’ untranslated regions of the cytokines. Notably, these studies indicated that the effects of BMX overexpression were specific for the IL-6 and TNF mRNA, but not for the IL-8 mRNA. In our studies, we could not find any differential effects on IL-8 and IL-6.

A similar study described in synovial fibroblasts a functional and physical interaction of BMX with MyD88 and Mal, components of the LPS signaling pathway (31). In this case, siRNA against BMX showed inhibition of LPS-induced IL-6 secretion, very similar to our results. As neither MyD88 nor Mal have been reported to be involved in TNF signaling, it is unlikely that these two mediators of TLR signaling are the targets of BMX in the context of our TNF signaling experiments. These studies also suggested that BMX could be replaced by BTK or other TEC family kinases, as functional redundancy within the TEC family kinases is well established (52, 53).

BMX had previously been shown to interact with TNFR2 (46), and this interaction is thought to mediate arteriogenesis and angiogenesis after ischemia (25). Our data expand the current knowledge about the role of BMX in TNFR1 and other inflammatory signaling pathways. The cellular systems used in our studies specifically reflect TNF signaling mediated by TNFR1. First, non-immune cells such as HeLa, HEK293, and HDF are known to express only TNFR1, but not TNFR2, and TNFR1 is thought to mediate most inflammatory TNF signals (3). Second, siRNAs against TNFR1 were sufficient to block completely TNF-induced responses, and third, soluble TNF as used in our in vitro studies is known to primarily activate TNFR1 and not TNFR2 (54). Because we found that BMX regulates TNF signaling at the level of the TAK1–TAB complex, it was not surprising that several inflammatory signaling pathways sharing this complex were all regulated by BMX. These effects did, however, not reflect a general inhibition of cellular signaling, as EGF-induced STAT3 phosphorylation was not affected by the siRNA against BMX. Whereas a role for tyrosine phosphorylation in TNF signaling has often been reported (55–57), direct evidence for a specific kinase is missing. Our report suggests that BMX may be the relevant tyrosine kinase in the tissues that express BMX.

Our efforts to map the interaction at the functional level indicate that BMX regulates the TAK1–TAB complex. As the analysis of the physical interaction of BMX with TAK1–TAB was not conclusive, it is likely that the interaction is not direct. The complexity of the assembly and regulation of the proximal TNF signalosome has recently been expanded (58), and it is conceivable that BMX is one component of this multiprotein complex even though we could not yet define the direct interaction partners of BMX.

Our data support a molecular mechanism for the regulation of TNF signaling by BMX in which the PH domain and membrane-tethering of BMX are required. This is reminiscent of the well-established role of the PH domain for BTK function, as underlined by mutations in the PH domain of BTK leading to a loss of function and X-linked agammaglobulinemia (59). Furthermore, our in vitro studies on the regulation of the IL-8 promoter indicate

WT mice, whereas the Giemsa-stained section in the lower left panel indicates a strong reduction of joint inflammation in the BMX KO mice (original magnification ×50). The center panels (Giemsa stain, original magnification ×200) show a higher magnification of the solid square area in the left panels. The right panels (safranin O, original magnification ×100) show a higher magnification of the dotted square area in the left panels. The top right panel shows cartilage proteoglycan depletion in the BMX WT mice as revealed by safranin O staining, which was strongly reduced in the BMX KO mice (lower right panel). The histopathology of the BMX KO mice was similar to that of naïve nonarthritic animals (not shown). D, Quantitative analysis of the histological findings. The BMX KO mice show significantly less inflammatory cell infiltrates and joint damage, as well as a trend to reduced cartilage damage. Shown are means ± SEM, n = 5. *p < 0.05 (Mann–Whitney U test).
a requirement for the BMX kinase activity. In contrast, the results of the in vivo arthritis model suggest that if BMX expression is essential, its kinase activity is dispensable. Although our BMX kinase-deficient mouse model is, to our knowledge, the first full genetic replacement reported for the kinases in this family, previous studies with BTK involved a B cell-specific transgenic expression of a kinase-deficient BTK allele on the background of the BTK KO mouse (60). These studies indicated only a partial kinase requirement with regard to many BTK-dependent B cell signaling events. It is therefore likely that BMX, like BTK, may function as a kinase and as an adapter protein, depending on cellular context. A possible explanation for the differential requirements for BMX kinase activity is that whereas transcription of cytokine mRNA is kinase-dependent, as detected in our IL-8 promoter reporter assay, a second kinase-independent process could compensate the former. This additional process could involve mRNA stabilization, as described by Palmer et al. (30). Alternatively, the kinase-dependent effects on IL-8 transcription might be evident in our in vitro system where the stimulus is limited to BMX overexpression, whereas the complex and strong inflammatory stimuli in the in vivo arthritis model might bypass or override the kinase requirement. This could imply that to block all cellular functions, a BMX inhibitor may need a mode of action more complex than competition with ATP binding. It is noteworthy that in vivo active BTK inhibitors have been reported (61), and therefore it is possible that selective and active BMX inhibitors could be designed. The arthritis model of passive K/BxN serum transfer mainly reflects the terminal inflammatory part of RA and is dependent on macrophages, neutrophils, and mast cells, as well as on complement, Fc receptors, and IL-1β (37, 62). Because BMX is expressed in monocytes, macrophages, and other myeloid cells (12) and as we show that BMX is required for IL-1β signaling, the reduction of arthritis in the BMX-deficient mice confirms and validates the relevance of our in vitro findings. It is noteworthy that the BMX-deficient mice were not protected from arthritis induced by local injection of murine Bmx tyrosine kinase in the granulo-monozygotic lineage. Blood 90: 4323–4340.


The tyrosine kinase BMX regulates inflammatory cytokine signaling

**Supplementary tables**

Supplemental Table 1 – Primers

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward (5’ to 3’)</th>
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<tbody>
<tr>
<td>BMX K445R</td>
<td>GGGGCCAATATGATGTGGCTGTAAGAATGATCAAGGAGGG</td>
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<tr>
<td>BMX R29N</td>
<td>GTCACCAAATAATTACAAAGAAAACCTTTTTGTTTTGACCAAAC</td>
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<tr>
<td>BMX E42K</td>
<td>CTTTTTCTACTATAAAATATGACAAAATGAAAAGGGGCAGC</td>
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Supplemental Table 2 – siRNA

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Target sequence (5’ to 3’)</th>
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<tr>
<td>siTAK1 (in HeLa)</td>
<td>CAGAGTGAATCTGGACGTT</td>
</tr>
<tr>
<td>siTAK1 (in HDF)</td>
<td>CCGCTGGTACAGGAACATA</td>
</tr>
<tr>
<td>siNFkB p65</td>
<td>GGCAGAGGCCAGACAGA</td>
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<td>siControl (luciferase)</td>
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<tr>
<td>siTNFR1</td>
<td>CGGCATTATGGAGTGAAA</td>
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</table>
Supplementary figure legends

Supplemental Figure 1: Potential interaction of BMX with the TAB/TAK1 complex.
HeLa cells were transfected with BMX or a control plasmid and 48 hours later lysates were prepared. Immunoprecipitation with an anti-TAB3 antibody resulted in co-enrichment of BMX and low levels of TAB2. While the TAB3 antibody allowed to co-purify BMX, it did not allow the detection of endogenous TAB3 (not shown). Immunoprecipitation with an anti-TAB2 antibody efficiently enriched for BMX, TAK1 and TAB2.

Supplemental Figure 2: Overexpression of BMX leads to post-translational modifications. HEK293-EBNA cells were transfected with plasmids for BMX and 48 hours later assayed for BMX expression in absence of stimulation by immunoblotting for total BMX protein. Over-expression of BMX led to a slight shift towards higher apparent molecular weight, which was also detected for the E42K gain of function mutant. This was not the case for the kinase-dead K445R mutant alone or in the context of the E42K/K445R double mutant, as well as in the PH-domain loss of function R29N mutation.