Published in final edited form as:

Ann Rheum Dis. 2009 February ; 68(2): 257–263. doi:10.1136/ard.2008.095356.

Synergistic benefit in inflammatory arthritis by targeting IκB kinase ε and interferon β

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Abstract

Objectives—The IκB kinase (IKK)-related kinase IKKε regulates type I interferon expression and responses as well as proinflammatory mediator production. We examined the role of IKKε in arthritis and its ability to enhance the therapeutic response to systemic interferon (IFN) β therapy in passive murine K/BxN arthritis.

Methods—IKKε^{−/−}, IFNα~βR^{−/−} and wild type mice were given K/BxN serum and treated with polyinosinic polycytidylic acid (poly(I:C)), IFNβ, or normal saline. Clinical response and histological scores were assessed. Gene expression in the paws was measured by quantitative PCR. Serum interleukin 1α receptor agonist (IL1Ra) and IL10 were measured by ELISA and multiplex bead array.

Results—Arthritis was almost completely blocked in wild type mice if arthritogenic K/BxN serum and the Toll-like receptor (TLR)3 ligand, poly(I:C), were coadministered at the onset of the model, but not in established disease. Mice deficient in IFNα~βR had an accelerated course of arthritis, and did not respond to poly(I:C). IKKε null mice had a modest decrease in clinical arthritis compared with heterozygous mice. Low doses of IFNβ that were ineffective in wild type mice significantly decreased clinical arthritis in IKKε null mice. Articular chemokine gene expression was reduced in the IKKε^{−/−} mice with arthritis and secreted IL1Ra (sIL1Ra) mRNA was significantly increased. Serum levels of IL1Ra were increased in low dose IFNβ-treated IKKε^{−/−} mice.

Conclusions—Subtherapeutic doses of IFNβ enhance the anti-inflammatory effects of IKKε deficiency, possibly by increasing production of IL1Ra and unmasking the antichemokine effects. Combination therapy with low dose IFNβ and an IKKε inhibitor might improve efficacy of either agent alone and offers a novel approach to RA.

Type I interferons (IFNs) stimulate antiviral responses, but they paradoxically exhibit anti-inflammatory properties. For instance, IFNβ reduces tumour necrosis factor (TNF)α, interleukin (IL)1β and IL6 production and enhances the release of anti-inflammatory mediators such as IL1 receptor antagonist (IL1Ra) and IL10.^{1–3} Preclinical studies emphasising the anti-inflammatory functions of IFNβ strongly supported its therapeutic potential in rheumatoid arthritis (RA).¹⁴⁵ Local gene therapy or systemic treatment of arthritic rodents with IFNβ in rodent models of arthritis results in clinical improvement.¹⁶⁷ However, IFNβ did not demonstrate clinical efficacy in RA or evidence of improved synovial histology.⁸⁹ While the explanation for negative results in RA is not certain, side effects from IFNβ therapy, preventing the use of high doses, could contribute.⁸

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Competing interests: None declared.

The potential utility of modulating IFN production and the difficulty developing IFN β as a therapeutic agent led us to explore other mechanisms of IFN β regulation in arthritis. This cytokine is tightly regulated by Toll-like receptors (TLR), interferon regulatory factor 3 (IRF3), and two I κ B kinase (IKK)-related kinases, IKK ϵ and TBK1 (TRAF family member-associated nuclear factor (NF) κ B activator (TANK)-binding kinase 1).^{10–13} IKK ϵ phosphorylates IRF3, which in turn induces the transcription of many genes, including chemokines and type I IFNs.^{14,15} IKK ϵ , like IFN β , is expressed in the rheumatoid synovial intimal lining and by cultured fibroblast-like synoviocytes (FLS).^{16–19}

Like IFN β , IKK ϵ blockade has potential for beneficial and deleterious effect on synovitis by virtue of its role in IFN β , chemokine and matrix metalloproteinase (MMP) expression. We explored whether dichotomous effects could be leveraged in a novel therapeutic paradigm. Our studies demonstrated that IKK ϵ deficiency and low dose IFN β therapy separately have modest effects on murine passive K/BxN arthritis. However, combining the two approaches was synergistic. Therefore, IKK ϵ blockade in combination with a low dose IFN β might have minimal side effects and could be clinically useful in RA. Using this approach, innate host defences that rely on type I IFN might be spared while still gaining the beneficial effects of modulating interferon-regulated genes.

METHODS

Mice

KRN T cell receptor (TCR) transgenic mice were a gift from Drs D Mathis and C Benoist (Harvard Medical School, Boston, Massachusetts, USA) and Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France),²⁰ and were maintained on a C57BL/6 background (K/B). Arthritic mice were obtained by crossing K/B with NOD/Lt (N) animals (K/BxN). C57BL/6 and NOD/Lt mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). *Ikk ϵ ^{-/-}* (IKK ϵ ^{-/-}) mice were a generous gift of Dr T Maniatis (Harvard University, Cambridge, Massachusetts, USA).²¹ IFN α / β R^{-/-} (where R = receptor) and background strain 129SvEv were originally obtained from B&K Universal Limited (Hull, UK). The mice were bred and maintained under standard conditions in the University of California, San Diego Animal Facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols received prior approval by the institutional review board.

Serum transfer and arthritis scoring

Arthritic adult K/BxN mice were bled and the sera were pooled. Groups of three to eight recipient mice were injected with 150 μ l intraperitoneally on day 0. Some groups of mice also received IFN β (Chemicon (3.6×10^7 IU/mg; or 1000 IU=28 ng)), normal saline (NS) or polyinosinic polycytidylic acid (poly(I:C); Sigma, St Louis, Missouri, USA) at the indicated doses intraperitoneally. Clinical arthritis scores were evaluated using a scale of 0–4 for each paw for a total score of 16. Ankle thickness was measured with a calliper (Manostat, Herisau, Switzerland) in mm.²²

Histology

Whole knee joint sections were stained with haematoxylin and eosin and safranin O (HistoTox, Boulder, Colorado, USA). Histopathological scoring was performed as previously described on a scale of 0–4 for inflammation and erosion.²²

Gene expression

The wrists of mice were snap frozen and pulverised as a pool. Total RNA is isolated using Qiagen RNeasy kit and cDNA was prepared with the qscript cDNA SuperMix kit (Quanta, Gaithersburg, Maryland, USA). The mRNA levels for chemokine (C–C motif) ligand (CCL) 2/monocyte chemoattractant protein (MCP)1, chemokine (C–X–C motif) ligand (CXCL)1/keratinocyte-derived chemokine (KC), CXCL2/macrophage inflammatory protein (MIP)2a, CXCL5/granulocyte chemotactic protein (GCP)2, CXCL10/IFN-inducible protein (IP)10, IL1 β , IL10, IFN β , TNF α , IL1Ra were quantified by real time reverse transcriptase (RT) PCR using commercially designed and prepared primer and probe sets (Applied Biosystems, Foster City, California, USA) and normalised to actin as previously described.¹⁸ Fold induction was calculated by $2^{-(\Delta\Delta Ct)}$, normalising to actin and using wrist RNA from uninjected mice of the same genotype as the baseline comparator.

Cytokine measurement

The sera were assayed for IL1Ra using a commercial capture ELISA (R&D Systems, Minneapolis, Minnesota, USA) and IL10 using Bioplex beads (Bio-Rad, Hercules, California, USA) on a Luminex analyser (Austin, Texas, USA).

Statistics

Significance was assessed by analysis of variance (ANOVA) using the area under the curve for arthritis studies. The Mann–Whitney U test was used for pairwise comparisons using Prism software V. 4.0 (GraphPad Software, San Diego, California, USA).

RESULTS

Passive K/BxN arthritis is attenuated by the TLR3 ligand, poly(I:C) and by IFN β treatment

To explore the complex role of innate immunity in arthritis, we first confirmed that pretreatment with poly(I:C) decreases clinical signs of joint inflammation in passive K/BxN arthritis (fig 1A).²³ Poly(I:C)-treated mice had markedly reduced joint swelling. Surprisingly, poly(I:C) had no effect when it was administered at the peak of disease. The role of type I IFN was further explored in type I IFN α/β R^{-/-} mice. As shown in fig 1, IFN α/β R^{-/-} mice had an accelerated course and more severe swelling (fig 1B). More importantly, poly(I:C) had no beneficial effect in the IFN α/β R^{-/-} mice (fig 1C). Finally, we showed that mice treated with recombinant mouse IFN β (1000 IU/day) had significantly less clinical arthritis (fig 1D). A lower dose of IFN β (500 IU/day) had no beneficial effect (see below). Unlike a murine bowel inflammation model,²⁴ every other day dosing also did not alter the clinical course of arthritis (data not shown).

IKK ϵ -deficient mice are less sensitive to arthritis induction and respond to poly(I:C)

The IFN response is regulated, in part, through the IKK-related kinases, including IKK ϵ .²⁵ We previously showed that IKK ϵ regulates transcription of several matrix metalloproteinases (MMP) and proinflammatory chemokines (eg, monocyte chemoattractant protein 1 (MCP1) and CCL5 (formerly known as RANTES)).^{18 19} Therefore, the relative benefit of IKK ϵ inhibition would reflect a balance between the benefit of chemokine/MMP inhibition and potentially deleterious effects of either decreased IFN production or decreased IFN sensitivity. To evaluate the net contribution of IKK ϵ to synovial inflammation, we used the passive K/BxN arthritis in heterozygous and *Ikk ϵ* ^{-/-} mice. As shown in fig 2A, *Ikk ϵ* ^{-/-} mice demonstrated decreased inflammatory arthritis. Although the clinical benefit was modest, the effect on histological damage was more striking with significantly reduced inflammatory cell infiltration, little bone erosion and a trend toward reduced cartilage damage in the IKK ϵ -deficient mice (fig 3). The IKK ϵ null mice had minimal bone erosions on haematoxylin and eosin staining of the knees,

but there were areas of proteoglycan loss in the cartilage as evidenced by reduced intensity in safranin O staining.

To evaluate the contribution of IKK ϵ to endogenous type I IFN production, IKK ϵ null mice were pretreated with poly(I:C) followed by K/BxN serum injection. Poly(I:C) provided limited protection compared with wild type mice (fig 1A and fig 2C). These data suggest that IKK ϵ blockade might provide some benefit in arthritis, and that the effect is potentially limited by impaired IFN responsiveness.

IFN β and IKK ϵ are synergistic in passive K/BxN arthritis

Based on the impaired inflammatory antiviral response seen in IKK ϵ -deficient mice,²¹ we reasoned that the mice would also have a muted response to exogenous interferon. To assess this response, we treated mice with subtherapeutic doses of IFN β (500 IU/day). Surprisingly, the low dose of IFN β significantly decreased clinical arthritis in the IKK ϵ -deficient mice even though it had no effect in the wild type animals (fig 4). These data suggest that low dose therapy could be used as an adjunct to IKK ϵ blockade to enhance efficacy and potentially decrease the suppressive effects on host defence.

Mechanism of IKK ϵ deficiency and IFN β in arthritis: potential role of IL1Ra

The mechanism by which IKK ϵ deficiency modulates arthritis is complex due to its impact on IFN responses and proinflammatory gene expression. To evaluate the role of IKK ϵ , the relative levels of synovial mRNA transcripts were evaluated on day 4 after K/BxN serum injection. As shown in fig 5A, synovial expression of several chemokines was lower in *Ikk ϵ ^{-/-}* mice, which is consistent with the observations that this pathway regulates the IFN response. Synovial IFN β mRNA levels were similar in wild type and *Ikk ϵ ^{-/-}* joints, suggesting that the effect was more likely related to the response to IFN β rather than its production. Expression of several chemokines, most notably MIP2a, IP10 and MCP1, was also lower in the IKK ϵ null mice.

One striking finding was an increase in the secreted IL1Ra isoform mRNA expression in the joints of IKK ϵ -deficient mice (fig 5B), which could contribute to the anti-inflammatory effect in the model due to its IL1 dependence.^{22,26} We also examined expression of the same genes in the mice treated with IFN β , which had little effect other than an increase in IP10 in the IKK ϵ -deficient mice. Finally, serum IL1Ra levels were assayed in wild type and IKK ϵ null mice on day 9 (fig 5C). The levels of IL1Ra were increased in IKK ϵ -deficient mice that received daily low dose IFN β .

DISCUSSION

RA is a destructive immune-mediated disease that involves elements of adaptive and innate immunity. Like other autoimmune diseases, type I interferon production has been implicated as a potential contributory factor. An interferon signature, for example, has been detected in the peripheral blood and synovial tissue of patients with RA^{27,28} and IFN β is expressed in rheumatoid synovium.¹⁷ Although type I interferons can potentially instigate autoimmune responses,^{29,30} IFN β treatment is paradoxically beneficial in multiple sclerosis and decreases severity in several animal models of arthritis.^{14–7} IFN β has potential salutary effects in RA, such as inhibiting production of inflammatory mediators and MMPs by cultured fibroblast-like synoviocytes and limiting osteoclast differentiation.^{19,31–33} However, IFN β therapy has little effect on synovial histology or clinical manifestations of RA.⁸ One possible explanation is that the doses required for efficacy are not tolerated in RA. In fact 45% of patients withdrew in one RA study evaluating high dose treatment due to side effects.⁸ Therefore, we explored the signalling pathways involved in interferon production and response as an alternative approach to rheumatoid synovitis.

Recent data have shed light on the TLRs and intracellular mechanisms that regulate interferon production. TLR3 and TLR4 activate two IKK-related kinases known as IKK ϵ and TBK1 through a mechanism that requires the adaptor protein Toll/IL1 receptor domain-containing adapter-inducing IFN β (TRIF).^{10 12} These two kinases have limited effect on the NF κ B pathway, but instead phosphorylate IRF3,²⁵ leading to interferon gene transcription. In addition to regulating type I interferon production, IKK ϵ is also required for some elements of IFN-mediated signalling and IFN-induced gene expression.²¹ IKK ϵ is activated in RA synovium and regulates expression of MMPs, chemokines and IFN β in cultured fibroblast-like synoviocytes.^{18 19} Based on these observations, we considered whether IKK ϵ is a potential therapeutic target in inflammatory arthritis. Its utility in disease would depend on the relative effects on proinflammatory products such as chemokines and anti-inflammatory proteins such as IFN β .

Initial studies were performed to evaluate the role of IFN β , TLR3 and type I IFN receptors in the passive K/BxN model of arthritis. These experiments confirmed that pretreatment with the TLR3 ligand poly(I:C) suppresses clinical arthritis and that this effect requires type I interferon signalling.²³ Exogenous IFN β therapy also decreased disease severity, as previously described in activate and passive murine arthritis.^{1 23} The role of TLR3, poly(I:C) and IFNs are paradoxical in that direct injection of poly(I:C) into rodent joints can induce arthritis.^{34 35} In addition, TLR stimulation in passive K/BxN arthritis was only beneficial when mice were pretreated; delayed therapy had little or no effect. The reasons for escape from the anti-inflammatory effects are not yet defined, but could potentially involve decreased IFN β production, decreased type I interferon responses, downregulation of TLRs, or other signalling defects induced by established inflammation.

We then evaluated the consequences of IKK ϵ deficiency in the same model. The *Ikk ϵ ^{-/-}* mice had a modest reduction in clinical swelling and improved histological evidence of disease. Decreased chemokine expression was noted in the joints, which is consistent with in vitro data in cultured synoviocytes.¹⁸ Lower synovial IFN β production or responses could counterbalance some of the beneficial effect of IKK ϵ deficiency and limit efficacy. The gene expression profile showed normal IFN β mRNA levels in the joint; however, IFN β expression might have been defective at extra-articular sites. More likely, defective responses to interferon, which was observed after virus infection in mice lacking IKK ϵ ,²¹ could account for the observations. To test the response to exogenous IFN β , we treated mice with a subtherapeutic dose of IFN β . This low dose of interferon had no effect on clinical arthritis in wild type mice, but markedly decreased disease severity in IKK ϵ deficiency. The synergistic response raises the possibility that low dose IFN β offsets the negative impact of IKK ϵ deficiency and unmask the anti-inflammatory action.

Previous mechanistic studies suggested that beneficial effects of type I interferon treatment were associated with the induction of IL10 and IL1Ra.^{13 36–39} However, the benefit in our experiments was not likely due to IL10 as transcripts of this cytokine were minimally induced in disease or with IFN treatment. IL1Ra expression was markedly induced in the *Ikk ϵ ^{-/-}* mice, as well as the wild type mice. In this model IL1 is quintessential to the manifestation of arthritis.^{22 26} Treatment of KRN transgenic mice with anti-IL1 receptor antibodies inhibits arthritis and the transfer of K/BxN serum does not induce arthritis in IL1R1^{-/-} mice.²² Hence, the elevation of IL1Ra in interferon-treated mice and the local increase in articular IL1Ra transcripts suggests a role for this protein as a mechanism of interferon responses.

The novel observation that IKK ϵ deficiency and IFN β synergise suggests an interesting therapeutic approach to RA. Because only low doses of IFN β are required, problems with side effects that have plagued clinical trials in RA would be minimised. IKK ϵ blockade could potentially suppress host defence due to its effect on type I interferon production, but this

problem could be alleviated by treating with exogenous IFN. The data suggest that coadministration of an IKK ϵ inhibitor and low dose IFN β could modulate innate immune responses and decrease tissue damage in autoimmune diseases.

Acknowledgements

We are grateful to Patty Charos, Sasha Gardner, Emily Grauel and Katharyn Topolewski for technical assistance.

Funding: This work was supported by grants from the US National Institutes for Health (NIH), including R01 AI067752.

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Figure 1.

Adult K/BxN mice were bled and the sera pooled. Mice were injected on day 0 with 150 μ l of pooled K/BxN sera intraperitoneally. Additional treatments are indicated below. The ankle thickness was serially measured with a calliper. A. Transfer of arthritis is attenuated by polyinosinic polycytidylic acid (poly(I:C)). The control group (squares) received injections of normal saline on days 0 and 7. The pretreatment group (circles) received 200 μ g of poly(I:C) intraperitoneally on day 0 and normal saline on day 7. The post treatment groups (triangles) received normal saline on day 0 and 200 μ g of poly(I:C) intraperitoneally on day 7. The means of 10 mice per group (standard error of the mean (SEM)) pooled from three experiments are shown. The day 0 poly(I:C) treatment significantly reduced paw swelling ($p < 0.01$), but the day 7 treatment had no clinical effect. B. Interferon (IFN) α/β R-deficient mice have an accentuated course of arthritis. Adult IFN α/β R^{-/-} and 129Sv/Ev mice were injected intraperitoneally on day 0 with 150 μ l of pooled K/BxN sera. The means of 19 mice per group (SEM) pooled from four experiments are shown. IFN α/β R^{-/-} mice develop significantly more severe paw swelling than 129Sv/Ev control mice ($p < 0.05$). C. IFN α/β R^{-/-} mice are refractory to poly(I:C). Control

IFN α / β R^{-/-} mice (squares) received normal saline and treated mice (circles) received 200 μ g of poly(I:C) intraperitoneally on day 0. The means of six or seven mice per group (SEM) pooled from two experiments are shown. There was no significant difference in the poly(I:C)-treated group. D. Daily IFN β diminishes arthritis. Groups were injected with 1000 IU IFN β (circle) or normal saline (square) daily. The means of 16 mice per group (SEM) pooled from three experiments are shown. The interferon therapy significantly reduced paw swelling ($p < 0.01$).

Figure 2.

Ikbke^{-/-} mice are less sensitive to arthritis induction and respond to polyinosinic polycytidylic acid (poly(I:C)). A. Adult homozygous and heterozygous *Ikbke*^{-/-} and *Ikbke*^{+/+} mice were injected intraperitoneally on day 0 with 150 μ l of pooled K/BxN sera. Ankle thickness was measured with a calliper. The means of 10 or 11 mice per group (standard error of the mean (SEM)) pooled from two experiments are shown. *Ikbke*^{-/-} mice develop significantly less paw swelling than heterozygous littermate control mice ($p < 0.05$). B. Adult *Ikbke*^{-/-} mice were injected intraperitoneally on day 0 with 150 μ l of pooled K/BxN sera and 200 μ g of poly(I:C) or normal saline (NS). Ankle thickness was measured with a calliper. The means of six or seven mice per group (SEM) pooled from two experiments are shown. Poly(I:C) reduces paw swelling in *Ikbke*^{-/-} mice ($p < 0.05$).

Strain \ Score	<i>IkBke</i> ^{-/-} (n = 11)	<i>IkBke</i> ^{+/-} (n = 6)	P
Inflammation	0.18 ± 0.12	1.33 ± 0.49	0.04
Erosion	0.36 ± 0.24	1.67 ± 0.56	0.04
Cartilage damage	1.18 ± 0.42	1.67 ± 0.56	0.44

Figure 3.

IkB kinase (IKK) $\epsilon^{-/-}$ mice develop less joint destruction than control mice. *IkBke* $^{-/-}$ (A and C) and *IkBke* $^{+/-}$ (B and D) mice were injected intraperitoneally on day 0 with 150 μ l of pooled K/BxN sera and killed on day 10. The hind limbs of all mice were removed and one knee from each mouse was prepared for histological scoring with haematoxylin and eosin and safranin O staining. Magnification 40 $\times$. Shown are the average inflammation and erosion scores (standard error of the mean (SEM)). * $p < 0.05$ by Mann–Whitney U test.

Figure 4.

IkB kinase (IKK) ϵ null mice are sensitive to low dose interferon (IFN) β . Adult wild type (A) and IKK ϵ null (B) mice were injected intraperitoneally on day 0 with 150 μ l of pooled K/BxN sera. Groups of mice were injected with 1000 IU IFN β , 500 IU IFN β or normal saline daily. Ankle thickness was measured with a calliper. The means of seven mice per group (standard error of the mean (SEM)) pooled from two experiments are shown. IKK ϵ null mice significantly responded to daily 500 IU IFN β ($p < 0.01$), whereas the wild type mice responded to the 1000 IU daily dose of IFN β ($p < 0.01$).

Figure 5.

Gene expression profiles of IkB kinase (IKK) ϵ null and wild type arthritic joints. A. Four mice per group were injected on day 0 with 150 μ l of pooled K/BxN sera and killed on day 4. Gene expression in pooled wrist RNA from IKK ϵ deficient and control mice was assessed by quantitative PCR. Shown are the mean fold inductions from four experiments (standard error of the mean (SEM)). B. Four mice per group were injected on day 0 with 150 μ l of pooled K/BxN sera and treated daily with normal saline (NS) or 1000 IU interferon (IFN) β and killed on day 1 or 4. The mRNA levels of interleukin 1 receptor antagonist (sIL1Ra) were quantified by quantitative PCR. Fold induction is relative to uninjected control mice. Shown are the averages from three experiments (SEM). C. IL1Ra is elevated in the sera of IFN β -treated mice. Four mice per group were injected with 150 μ l of K/BxN serum and treated

500 IU/day IFN β after day 0. IL1Ra and IL10 levels in the sera on day 9 were measured by ELISA and Luminex respectively. * $p < 0.01$ by analysis of variance (ANOVA) and Bonferroni post hoc analysis.