**Interleukin 1 receptor antagonist mediates the beneficial effects of systemic interferon beta in mice: implications for rheumatoid arthritis**

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Rheumatoid arthritis (RA) is an immune-mediated disease characterised by systemic and synovial inflammation, resulting in bone and cartilage destruction. Cytokine-modulating therapies have been effective in some but not all patients. In addition to approaches aimed at reducing the levels of proinflammatory cytokines, administering anti-inflammatory cytokines has been studied. Interferon β (IFNβ) is such a factor with immunomodulatory effects and its expression is increased in RA synovium. IFNβ can inhibit the expression of proinflammatory cytokines such as interleukin 1β (IL-1β) and tumour necrosis factor (TNF), and increase anti-inflammatory factor production, such as IL-10 and IL-1 receptor antagonist (IL-1Ra). Despite the promising in-vitro and animal model results trials with systemic IFN therapy in RA did not demonstrate efficacy by clinical, radiographic, biochemical and histological parameters.

We previously reported the increased sensitivity to IFNβ therapy in mutant mice that had a genetic disruption in a gene associated with the proinflammatory response to type I IFN. Surprisingly, systemic IFNβ treatment did not demonstrate efficacy by clinical, radiographic, biochemical, and histological parameters.

METHODS

Mice

KRN T-cell receptor 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), 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, Il1tm transgenic, Il10−/−, Il1rn−/−, and NOD/Lt mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The mice were bred and maintained under standard conditions in the University of California, San Diego Animal Facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols receive previous approval by the institutional review board.
Serum transfer and arthritis scoring
Arthritic adult K/BxN mice were bled and the sera were pooled. Recipient mice were injected with 150 μl intraperitoneally on day 0. Some groups of mice also received IFNβ (Chemicon (3.6×10^7 U/mg or 1000 U=28 ng)) intraperitoneally, or normal saline (NS). 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 caliper (Mitutoyo, Kawasaki, Japan) in mm.13

Histology
Whole knee joints and hind paws were fixed in 10% formalin, decalcified, trimmed and embedded. Sections were prepared from the tissue blocks and 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, erosion and cartilage damage.13

Gene expression
The wrists of mice were snap frozen and pulverised as a pool. Total RNA was isolated using the PerfectPure RNA Fibrous Tissue Kit (5 PRIME, Gaithesburg, Maryland, USA) and complementary DNA was prepared with the qScript cDNA SuperMix kit (Quanta, Gaithesburg, Maryland, USA). The messenger RNA levels for matrix metalloproteinase 3 (MMP3), IFN regulatory factor 7 (IRF7), C-X-C motif chemokine 10 (CXCL10)/interferon gamma-induced protein 10 (IP-10), IL-6, IFNβ and IL-1Ra were quantified by real time reverse transcriptase PCR using commercially designed and prepared primer and probe sets (Applied Biosystems, Foster City, California, USA) on a Bio-Rad MiQ software (version 5.0). Significance was assessed using area under the curve analysis, statistics

Statistics
Significance was assessed using area under the curve analysis, analysis of variance for multiple comparisons and the Mann-Whitney U test was used for pairwise comparisons using Prism software (version 5.0).

RESULTS
Regulation of passive K/BxN arthritis by IL-1Ra and IL-10
We previously reported that genetic deficiency in IFNα/β accelerated arthritis in K/BxN serum transfer arthritis, and C57BL/6 mice had an attenuated clinical course when treated with IFNβ.7 We noted that diminished arthritis in IFNβ-treated mice correlated with higher serum levels of IL-1Ra and mRNA levels of sIL-1Ra in arthritic paws.7 Although IL-10 has been implicated as a mediator of the anti-inflammatory response to IFNβ, serum levels of IL-10 in IFNβ-treated mice were similar to controls. To assess further the relative contributions between IL-1Ra and IL-10 we examined the effects of the constitutive production of IL-1Ra and genetic disruption in the Il1rn gene (figure 1). The IL-1Ra overexpressing transgenic mice had a very mild clinical course and minimal damage after 10 days by histology. Histology scores for IL-1Ra transgenic mice averaged 0.3±0.2, 0.3±0.2 and 0±0 compared with 3.7±0.8, 3.7±0.2 and 3.0±0 for wild-type mice for inflammation, bone erosion and cartilage damage, respectively (n=6 per group, p<0.001). In contrast the IL-1Rn-deficient mice had more severe paw swelling than wild-type littermates (p<0.01) (figure 1B). IL-10 deficiency also increased arthritis severity (p<0.01) (figure 1C).

Il1rn^−/− mice are refractory to IFNβ therapy whereas Il10^−/− mice are responsive
Our previous studies suggested that IL-1Ra, but not IL-10, is regulated by IFNβ in passive K/BxN.7 Because this model is exquisitely dependent on IL-1, we hypothesised that the protective effect of IFNβ is mediated by IL-1Ra. The effect of IFNβ on arthritis in Il1rn^−/− mice was determined to test this possibility. Unlike wild-type mice (figure 2A) the Il1rn^−/− mice did not clinically respond to IFNβ treatment (figure 2B). However, the IL-10 null mice responded to daily IFNβ injections (p<0.05 treated compared with controls) (figure 2C). Synovial inflammation, bone erosion and cartilage damage were also unaffected by IFNβ treatment in Il1rn^−/− mice. Treatment with IFNβ significantly improved the histological scores in wild-type littermates (p<0.01) (figure 3). The IL-10^−/− mice treated with IFNβ had a reduction in their histology score, with means of 0.4±0.2, 1±0.3 and 0.4±0.2 for inflammation, bone erosion and cartilage damage, respectively, compared with 1.8±0.5, 3.0±0.0 and 2.0±0.5 for NS-treated mice (n=5/group, p<0.05).

Enhanced expression of proinflammatory cytokine and MMP3 transcripts in Il1rn^−/− mice
IFNβ treatment reduced the levels of IL-6 and MMP3 mRNA transcripts and modestly increased the IFN response gene transcription (IRF7 and IP-10) in wild-type mice compared with NS (p<0.01) (figure 4). In contrast IFNβ treatment did not reduce the

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Synovial samples post-IFNβ treatment have increased IL-1Ra and decreased IL-10 by immunostaining

During a previous phase I trial, patients were treated for 12 weeks with purified natural fibroblast IFNβ, which was injected intraperitoneally on day 0 with 150 μl of pooled K/BxN sera and with 1000 IU IFNβ, or normal saline (NS) daily. Ankle thickness was measured daily with a caliper. Shown are the means ±SEM from (A) 10 wild-type (WT) mice/group, (B) 13 II1m mice/group and (C) 10 II10−/− mice/group from two to three pooled experiments each. WT and II10−/− mice significantly responded to daily 1000 IU IFNβ (p<0.05), whereas the II1m−/− mice did not.

DISCUSSION
IFNβ was developed as a therapeutic agent in autoimmune diseases due to its anti-inflammatory activities. Similar to other

transcription of these genes commonly associated with inflammatory arthritis in IL-1Ra-deficient mice. The II1m−/− mice treated with IFNβ demonstrated a small increase in IRF7 and IP-10 mRNA transcription, indicating at least a partial biological response to the treatment. Notably, IL-1Ra null mice with arthritis also had approximately a twofold change of IL-10 mRNA expression compared with uninjected mice.

Persistently elevated IL-6 serum levels in II1m−/− IFNβ-treated mice
Control arthritic mice had increases in the levels of IL-6 in their sera (figure 5). The level of IL-6 was lower in IFNβ-treated wild-type and II10−/− mice, but not II1m−/− mice. Both the wild-type and the II10−/− mice had increases in the levels of serum IL-1Ra in response to IFN treatment. Serum levels of IL-1Ra in II1m−/− mice were below the sensitivity of the assay.
biological therapies, this treatment has not been uniformly effective. In preclinical studies IFNβ demonstrated efficacy in rodent and non-human primate models of RA. Daily systemic administration of IFNβ reduced paw swelling, decreased serum levels of anticollagen antibodies and improved histological scores in DBA/1 mice with collagen-induced arthritis (CIA). Similarly, systemic treatment with recombinant IFNβ in thersus monkeys with CIA resulted in remarkable clinical improvement and decreased serum levels of C-reactive protein. Despite the early promise therapeutic trials in with IFNβ treatment in RA had a minimal effect on synovial histology or clinical manifestations of RA and non-human primate models of RA. Daily systemic administration of IFNβ or indirect means. Although CIA is largely a T-cell-dependent, yet still responds to IFNβ treatment. The IL-1Ra-deficient mice were, however, refractory to treatment. These results suggest that IL-1Ra is the predominant active mediator for improvement with IFNβ treatment in this model of arthritis.

Investigating the complexities of the in-vivo activities of IFNβ might lead to optimising its use clinically either as a single agent or in combination with other therapeutic options. As the IFN receptors are widely expressed, the effects of IFNβ might span both the adaptive and innate immune compartments by direct or indirect means. Although CIA is largely a T-cell-dependent model, an earlier report suggested that the effects of IFNβ were not limited to T-cell-dependent mechanisms, but were also mediated by stromal cells and osteoclasts. We thus utilised the K/BxN model in these studies as this model was not B or T-cell dependent, yet still responds to IFNβ treatment.

Figure 3 Persistent joint inflammation and damage in Il1m−/− mice despite interferon β (IFNβ) treatment. (A) Wild-type (WT) and Il1m−/− mice were injected intraperitoneally on day 0 with 150 µl of pooled K/BxN sera and treated with daily injections of normal saline (NS) or 1000 IU IFNβ intraperitoneally. On day 10 the mice were killed, the hind limbs were removed and one ankle from each mouse was prepared for histological scoring. Representative sections with Safranin O staining are shown at 40× magnification. (B) Shown are the average inflammation, erosion and cartilage damage scores ±SEM (six mice/group). *p<0.01 by analysis of variance with Bonferroni comparison.

Figure 4 Gene expression profiles of interleukin 1 receptor antagonist null and wild-type arthritic joints. Three mice per group were injected on day 0 with 150 µl of pooled K/BxN sera and then received daily injections of 1000 U of interferon β (IFNβ) or normal saline. The wrist joints were removed on day 4, snap frozen and pulverised. RNA was isolated from pooled wrists using commercial kits and the relative levels of gene expression were assessed by quantitative PCR and normalised to actin. The fold induction is compared with the relative level of messenger RNA expression of un.injected control mice. Shown are the averages of replicates pooled from two experiments +SEM. The amount of Il1m transcript was below detection (b.d.) in the Il1m−/− mice. IRF7, IFN regulatory factor 7; IL-10, interleukin 10; MMP, matrix metalloproteinase.

noted that IL-10 and IL-1Ra-deficient mice developed accelerated paw swelling compared with wild-type controls. However, the benefit of IFNβ treatment in our experiments was not due to IL-10, as IL-10 null mice responded to IFNβ. The IL-1Ra-deficient mice were, however, refractory to treatment. These results suggest that IL-1Ra is the predominant active mediator for improvement with IFNβ treatment in this model of arthritis. Despite the lack of efficacy in RA, IFNβ has proved effective in some cases of multiple sclerosis. The murine experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis is dependent on the production of both IL-17 and IFNγ by T helper cells for the full manifestation of disease and responds to IFNβ treatment by reducing IL-17. The most pathogenic T cells in this murine model are those that co-express IL-17 and IFNγ. This effect is not limited to the EAE model as IFNβ also reduces IL-17 production by human T lymphocytes in vitro. Both in humans and mice IL-1β contributes to the priming environment that is conducive to T cells developing an IL-17-secreting phenotype (Th17). Notably, the K/BxN passive transfer model is T-cell and IL-17 independent and the effects on adaptive immunity as a mechanism explain our results.

IL-1Ra is only a modestly effective therapeutic agent in RA. Other IL-1-directed therapies, including monoclonal antibodies, soluble IL-1 receptors and caspase 1 inhibitors, also show limited benefit. The minimal efficacy of systemic IFNβ in RA might be related to the limited role of IL-1 on the clinical manifestations of this particular disease. Alternatively, a reduction in the priming of Th17 cells due to IL-1Ra treatment might be circumvented by redundant priming mechanisms or the expression of IL-17 in synovial mast cells. The increase in IL-1Ra expression...
observed in RA synovium and in murine arthritis could thus explain the divergent results in the two species. IL-1 is a pivotal cytokine in passive K/BxN arthritis, and the disease is markedly attenuated by deficient IL-1R signalling. The increase in IL-1Ra in RA patients would not have the same impact on clinical signs and symptoms.

Reduced IL-10 in the human synovial samples after IFNβ treatment was consistently observed and could also contribute to a reduction in the immunosuppressive effect of IFNβ. The effect on IL-10 represents another difference between RA and passive K/BxN arthritis. Although IL-10-deficient mice had an exacerbated clinical course, they still responded to IFNβ treatment.

The data on IFNβ therapy in RA focus on systemic therapy. It is possible that higher concentrations or continuous delivery, such as local gene therapy in the synovium, might activate additional mechanisms that possibly suppress synovial inflammation in RA. Frequent dosing might be required to sustain the activity of intracellular molecular signalling pathways responsible for regulating IFNβ-induced gene expression. Still, IFNβ at the dosage used had a biological effect, as noted by altered IL-1Ra and IL-10 expression in the synovium of RA patients.

Alternatively, IFNβ might be more useful as part of combination therapy in RA. Our previous studies suggested that the concurrent modulation of kinases, such as IKKε in the IFN response pathway, in combination with IFNβ therapy, can enhance the anti-inflammatory response and augment potency. Those studies showed that IKKε deficiency suppressed chemokine expression, and that low-dose IFNβ provided synergistic benefit. This approach could leverage the anti-inflammatory effects of both IKKε and IFNβ.

In conclusion, our data suggest that IFNβ acts through IL-1Ra induction for its anti-inflammatory effects in passive K/BxN arthritis. This mechanism, although effective in mice, has less utility in RA. Alternative approaches, such as local gene therapy or combination therapy with specific kinase inhibitors, could overcome these limitations.

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Competing interests None.

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