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Inhibition of Inflammation and Bone Erosion by RNA Interference–Mediated Silencing of Heterogeneous Nuclear RNP A2/B1 in Two Experimental Models of Rheumatoid Arthritis

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Objective. **The nuclear protein heterogeneous nuclear RNP A2/B1 (hnRNP A2/B1) is involved in post-transcriptional regulation of gene expression. It is constitutively expressed in lymphoid organs and highly up-regulated in the synovial tissue of patients with rheumatoid arthritis (RA), who may also generate autoantibodies to this protein. This study was undertaken to investigate the potential involvement of hnRNP A2/B1 in the pathogenesis of autoimmune arthritis, by silencing hnRNP A2/B1 expression in 2 animal models of RA.**

Methods. **Collagen-induced arthritis (CIA) and the K/BxN serum–transfer model were used as animal models of RA. Efficient silencing of hnRNP A2/B1 was achieved using a liposome-based carrier system for delivery of small interfering RNAs. Expression of hnRNP A2/B1 was analyzed by flow cytometry, reverse transcription–quantitative polymerase chain reaction, Western blotting, and immunohistochemistry. The number of osteoclasts was determined by tartrate-resistant acid phosphatase staining. Cytokine levels and anticollagen antibody levels were measured by enzyme-linked immunosorbent assay.**

Results. **Efficient silencing of hnRNP A2/B1 was achieved in all lymphoid organs. In both experimental models, the incidence and severity of arthritis were largely reduced and bone erosion was not detectable as compared to the control groups. Down-modulation of hnRNP A2/B1 significantly interfered with the production of proinflammatory cytokines from monocyte/macrophages, but not from T cells. Consistent with these findings, production of T cell cytokines was not impaired when cells were restimulated in vitro with type II collagen. Furthermore, levels of anticollagen antibodies were not affected by hnRNP A2/B1 silencing.**

Conclusion. **Our findings suggest that hnRNP A2/B1 has an important role in regulation of the innate immune system, especially at the level of monocyte/macrophage activation. Therefore, down-modulation of hnRNP A2/B1 seems to affect primarily the effector phase of autoimmune arthritis.**

Rheumatoid arthritis (RA) represents the most common systemic autoimmune disease. Chronic inflammation eventually leads to devastating systemic and local bone loss with irreversible functional disability and reduced life expectancy (1,2). The invasion of immune cells such as monocytes, neutrophils, T cells, and B cells into the synovial membrane is a hallmark of the disease, and causes persistent overproduction of proinflammatory cytokines, driving chronic inflammation in the affected joints (3). This vicious circle of invading cytokine-producing immune cells activating resident synoviocytes that subsequently produce a plethora of cytokines and chemokines provokes increased

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generation of osteoclasts. Activated fibroblasts and bone-resorbing osteoclasts derived from monocytes drive cartilage degradation and bone erosion, finally leading to irreversible destruction of the joints (4,5).

Another characteristic of RA is the occurrence of autoantibodies that are largely specific for the disease, such as rheumatoid factor and antibodies to citrullinated proteins. These autoantibodies are found in approximately two-thirds of RA patients, and are associated with more aggressive disease and unfavorable disease outcome (6,7). Autoreactive B and T cells directed against targets associated with nucleic acids are also observed, but to a lesser extent than in systemic lupus erythematosus (SLE) (8). The most specific reactivity is directed against heterogeneous nuclear RNP (hnRNP) A2/B1, a member of the hnRNP family of multifunctional intracellular DNA and RNA binding proteins which are involved in multiple biologic processes including the processing, transport, and translation of messenger RNA (mRNA), cell proliferation, transcription, and signal transduction (9).

Autoantibodies against hnRNP A2/B1 can be found in 20–30% of RA patients and in a similar proportion of patients with SLE, but rarely occur in other systemic autoimmune diseases or arthritic disorders (10,11). Moreover, autoreactive T cells have been found in ~50% of RA patients, and recently a major T cell epitope was characterized (12,13). Notably, autoreactivity against hnRNP A2/B1 has also been detected at both the B and T cell levels in 2 established animal models of RA, namely TNF-transgenic mice (14) and rats with pristane-induced arthritis (15). Moreover, pristane-induced arthritis could be transferred by splenocytes reactivated in vitro with hnRNP A2/B1 or nucleic acids associated with this protein, suggesting a pivotal role of hnRNP A2/B1 in the pathogenesis of inflammatory arthritis (15,16).

To address the pathogenetic role of hnRNP A2/B1, we performed a small interfering RNA (siRNA)–mediated functional knockdown in 2 different arthritis models reflecting the initial and the effector phase of RA. In both models down-regulation of hnRNP A2/B1 impaired inflammation and joint destruction and reduced production of proinflammatory cytokines by cells of the mononuclear phagocyte system, suggesting a crucial role of hnRNP A2/B1 in the induction of inflammatory autoimmune responses that may eventually lead to overt arthritis and severe joint damage.

MATERIALS AND METHODS

Small interfering RNAs. All siRNAs used in these experiments were purchased from Dharmacon. The following sequences of siRNA duplexes were used: mouse hnRNP A2/B1, 5′-UCUUCUGUGGUUUCAAAGC-3′; control sequence, 5′-UAAGGCUAUGAAGAGAUAC-3′; human hnRNP A2/B1, 5′-GAGTCCGCGATGGAGAGAG A-3′; and control sequence, 5′-UGGUUUACAUGUCGACUAA-3′.

Preparation of siRNA–lipoplexes for in vivo application. The cationic liposome RPR209120/DOPE was prepared as previously described (17,18). To enhance gene silencing efficiency, carrier DNA derived from a pcDNA3 plasmid (Invitrogen) was added to the siRNA at a 1:1 ratio (weight/weight). DNA and siRNA were then dissolved in 0.9% NaCl solution and mixed with an equivalent volume of saline liposome suspension. The liposome RPR209120/DOPE suspension (60 nmoles per mouse) and the nucleic acids (10 μg siRNA and 10 μg carrier DNA) were then mixed and incubated at room temperature for 30 minutes before intravenous injection.

Preparation of siRNA–transfection complexes for in vitro application. Fifty nmoles siRNA was mixed with Opti-MEM medium (Gibco), and 5 μl of Lipofectamine LTX transfection reagent (Invitrogen) was mixed with 245 μl of Opti-MEM and incubated for 5 minutes at room temperature. Thereafter, Lipofectamine was mixed with hnRNP A2/B1–specific siRNA, control siRNA, or with Opti-MEM only and incubated for 20 minutes at room temperature. After removal of 500 μl of Opti-MEM from the cells, 500 μl of the siRNA–Lipofectamine complexes was added. Cells were incubated for 72 hours and then prepared for use in different analyses.

Animals. DBA/1 mice and C57BL/6 mice were obtained from The Jackson Laboratory. All animal experiments were approved by the animal ethics committee of the Austrian Ministry of Science (GZ 66.009/0277-II/3b/2010).

Induction of collagen-induced arthritis (CIA). DBA/1 mice were immunized subcutaneously with bovine type II collagen (MD Biosciences) emulsified in Freund's complete adjuvant (100 μg per mouse). After 21 days animals were boosted intraperitoneally with 100 μg bovine type II collagen dissolved in phosphate buffered saline (PBS). Mice were randomized into 3 groups (10 animals per group) and injected intravenously in the eye venous plexus with hnRNP A2/B1–specific siRNA or control siRNA lipoplexes (0.5 mg/kg) or with PBS 1 day prior to the booster immunization, followed by weekly applications as described previously (17,18). Mice were evaluated for arthritis symptoms using a semiquantitative scoring system as described previously (19). Animals were killed on day 60 after disease induction.

Induction of serum-transfer arthritis. C57BL/6 mice were injected with 150 μl K/BxN serum intraperitoneally on day 1 and on day 3. Clinical scoring was started immediately after the first serum application, using a semiquantitative scoring system similar to the CIA scoring system described above (19). Small interfering RNA–lipoplex vehicles were injected intravenously in the eye venous plexus 3 times, once every third day starting 1 day before serum transfer, in hnRNP A2/B1–specific siRNA–treated, control siRNA–treated, and PBS-treated mice (n = 7 animals per group). The animals were killed on day 10 after the first serum application.

Evaluation of joint inflammation and local bone destruction. Hind paws were prepared and fixed in formalin overnight, and then bones were decalcified in 14% EDTA solution. Serial paraffin sections (2 μm) were stained with hematoxylin and eosin (H&E) and toluidine blue or were stained for tartrate-resistant acid phosphatase (TRAP) expression. TRAP staining was performed as previously described (20). In H&E-stained sections, areas of inflammation and bone erosion were analyzed using an Axioscope 2 microscope (Carl Zeiss MicroImaging) and an Osteomeasure Analysis

System (OsteoMetrics), which allows quantification of areas in histologic sections. All 4 joints were analyzed, and at least 8 slides per mouse were scored. The sum of the areas of inflammation for each single mouse was determined, and an average score was calculated. The same sections were also analyzed for quantification of erosions. In addition, the number of osteoclasts in TRAP-stained serial sections was counted.

Cell cultures. Draining lymph nodes were harvested and passed through a nylon mesh. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 m*M* L-glutamine in 96-well plates (1×10^6 cells/well) and stimulated with 100 μg/ml bovine type II collagen for 72 hours. Supernatants were then collected and stored for subsequent cytokine analysis. Synovial tissue was obtained from the knee joints, cut into small pieces, and cultured in 2-cm petri dishes. After 48 hours of incubation (at 37°C in 5% CO_2), cytokines were measured in the culture supernatants.

In vitro transfection of the rheumatoid synovial cell line MH7A. For in vitro transfection experiments, MH7A cells were plated into 6-well plates (6×10^4 cells/well) in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1% nonessential amino acids without antibiotics. At 30% confluency, cells were transfected with hnRNP A2/B1–specific siRNA, control siRNA, or medium only complexed to Lipofectamine LTX reagent (Invitrogen) and incubated for 3 days. Cell lysates were analyzed for hnRNP A2/B1 expression by Western blotting and reverse transcription–quantitative polymerase chain reaction (RT-qPCR).

Quantitative PCR. Total RNA from cells and organs was isolated using an RNeasy kit according to the recommendations of the manufacturer (Qiagen). RNA (1 μg) was reverse transcribed using an Omniscript RT kit (Qiagen). RT-qPCR was performed using LightCycler technology (Roche Germany) and a Fast Start SYBR Green I kit for amplification and detection. The expression of the target molecule was normalized to GAPDH, and expression of the gene of interest (hnRNP A2/B1 or hnRNP A1) was calculated as the difference in its expression versus expression in cells/organs from animals not treated with siRNA or treated with control siRNA. The following primer sets were used: for hnRNP A2/B1, 5′-TGGCTTTGTTACTTTTGATGA-3′ (forward) and 5′-TTCTGTTACCTCTGGGCTCTC-3′ (reverse); for hnRNP A1, 5′-GCATAGGATGTGCCAACAATC-3′ (forward) and 5′-GGAGAAGCCATTGTCTTCGCA-3′ (reverse); for GAPDH, 5′-TGGCATTGTGGAAGGGCTCATGA-3′ (forward) and 5′-ATGCCAGTGAGCTTGCCGTTCAG-3′ (reverse).

Western blotting. Mouse organs were mechanically homogenized in Schreiber buffer (20 m*M* HEPES, pH 7.9, 0.4*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol; 1 m*M* EDTA, 0.1 m*M* EGTA, and 20% glycerol) containing 5 m*M* benzamidine, 10 m*M* *N*-ethylmaleimide, and 0.08 m*M* phenylmethylsulfonyl fluoride as protease inhibitors. The tissue extracts were centrifuged at 38,000*g* for 10 minutes, and supernatants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes.

Membranes were blocked overnight at 4°C, followed by incubation for 1 hour with the anti–hnRNP A2/B1 monoclonal antibody (mAb) 10D1 (12) (diluted 1:3,000 in blocking buffer) or the anti–hnRNP A1 mAb 4B10 (1:10,000 dilution; Santa Cruz Biotechnology). After washing, membranes were incubated for 1 hour at room temperature with a horseradish peroxidase (HRP)–conjugated rabbit anti-mouse antibody (Dako Cytomation) diluted 1:2,000. Finally, immunostained proteins were visualized using an ECL detection kit (Amersham Biosciences).

Flow cytometry. Spleens were cut into small pieces and passed through a nylon mesh; whole blood was obtained by tail vein puncture. T cells, B cells, and monocytes were isolated from splenocyte or whole blood cell suspensions by flow cytometric cell sorting on a FACSAria (BD Biosciences) using mAb specific for CD3, B220, or F4/80 (DakoCytomation). Subsequently, the different cell populations were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed, and resuspended in ice-cold 90% methanol to allow permeabilization. Cells were then incubated with an anti–hnRNP A2/B1 mAb (Santa Cruz Biotechnology) for 30 minutes at 4°C; after washing, a phycoerythrin-labeled anti-mouse IgG mAb (Santa Cruz Biotechnology) was used for detection and quantification.

Detection of cytokines and anti–type II collagen antibodies. Serum samples were obtained from anesthetized mice by puncture of the tail vein. Supernatants from isolated and cultured draining lymph node cells or synovium were collected and stored at −20°C. The cytokines interferon-γ (IFNγ), tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-6, IL-12, IL-17A, IL-4, and IL-23 in serum and culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA; all from R&D Systems).

Anti–type II collagen IgG, IgG1, and IgG2a antibodies were measured by ELISA in serum obtained on day 30 and 50. Wells were coated overnight with 0.5 mg/ml bovine type II collagen (MD Biosciences) dissolved in carbonate buffer, pH 9.4 and subsequently blocked with 3% gelatin (Sigma) dissolved in PBS. After repeated washing with PBS containing 0.05% Tween 20, wells were incubated for 1 hour with 100 μl serum diluted 1:10,000 in PBS, 1% gelatin, 0.05% Tween 20. After extensive washing, wells were incubated for 1 hour with 100 μl HRP-conjugated goat anti-mouse IgG, IgG1, or IgG2 (Southern Biotech). After further washing, the color reaction was allowed to develop using tetramethylbenzidine as substrate and stopped by adding 50 μl of 2*M* H_2SO_4.

Statistical analysis. Arthritis scores and grip strength scores are presented as the mean ± SEM. The area under the curve (AUC) was used as the relevant summary measure describing the time course of these variables. Analysis of variance models incorporating a random factor that identifies individuals within the same experiments were used to compare AUCs between different groups. Within each set of experiments pairwise comparisons were adjusted using the method of Bonferroni and Holm. The significance of differences between 2 groups was determined by unpaired *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Expression of hnRNP A2/B1 predominantly in lymphoid tissue and cells of the mononuclear phagocyte system. In order to gain more insight into a possible role of hnRNP A2/B1 in immune regulation, we first investigated its expression in the lymphoid organs (spleen, lymph nodes, and thymus) and immune cells of untreated DBA/1 mice, by immunoblotting (Figure 1A). The data confirmed the previously reported high expression of hnRNP A2/B1 in the spleen and thymus (14) and showed that hnRNP A2/B1 is also abundantly expressed in draining

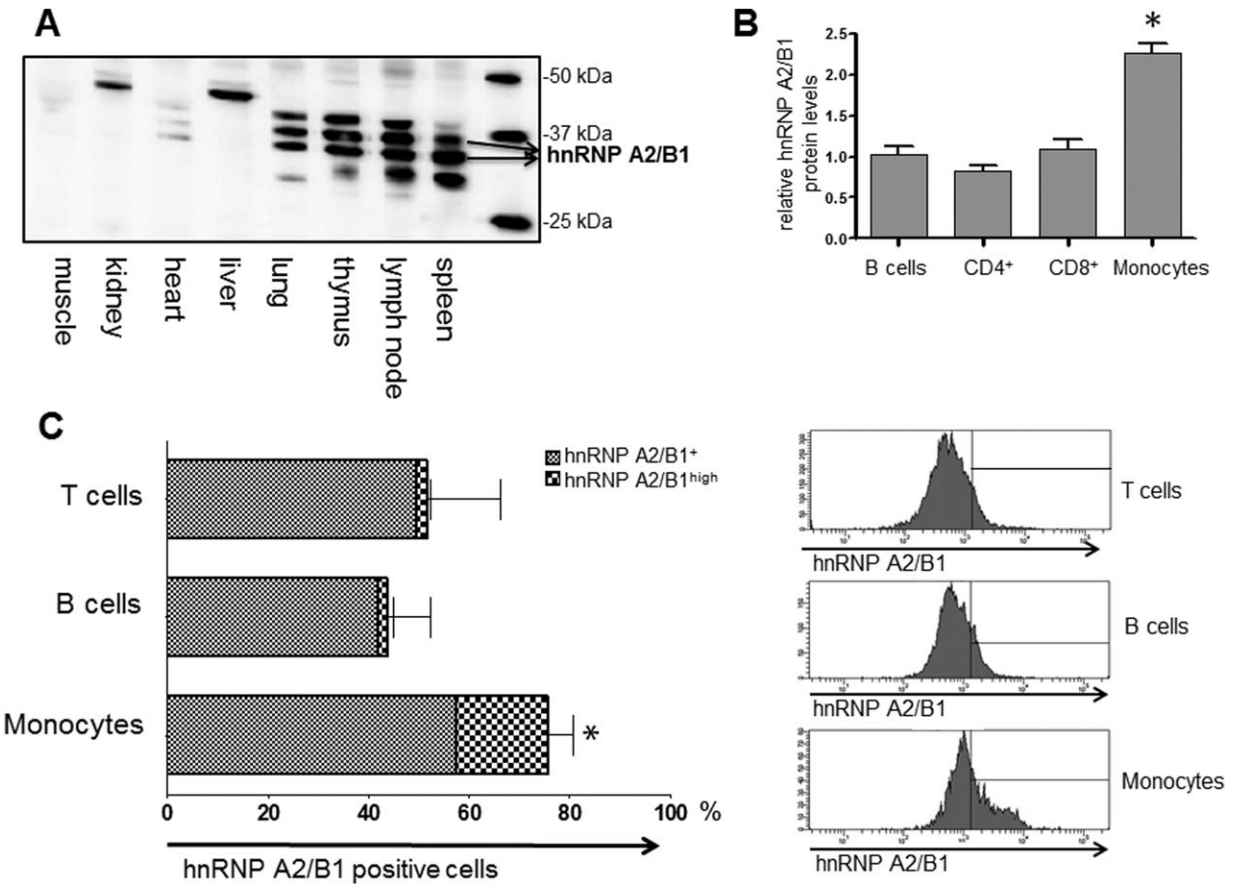

Figure 1. Expression pattern of heterogeneous nuclear RNP A2/B1 (hnRNP A2/B1) and its splice variants in mouse organs and cells of the immune system. **A,** Western blot analysis showing expression patterns of hnRNP A2/B1, the smaller splice variant hnRNP B0 (visible below the A2 band), and the larger variant hnRNP B2 (visible above the B1 band) in different organs of untreated DBA/1 mice, particularly in lymphoid tissue. A monoclonal antibody recognizing hnRNP A2/B1 and all known splice variants was used for immunodetection. A representative immunoblot of 3 independent experiments is shown. **B,** Protein levels of hnRNP A2/B1 in B cells, CD4+ T cells, CD8+ T cells, and monocytes isolated from mouse spleens, determined by Western blot analysis. Results are representative of 3 independent experiments. Bars show the mean ± SEM. $* = P \leq 0.05$ versus all other groups. **C,** Flow cytometric analysis of hnRNP A2/B1 expression in different immune cells. Splenocytes of untreated DBA/1 mice were stained with a phycoerythrin-labeled anti–hnRNP A2/B1 antibody. The cutoff for very high specific staining of hnRNP A2/B1 was set as above channel 5×10^3, leaving 99% of the CD3+ and B220+ cell populations below this threshold, but including a significant proportion of F4/80+ cells inside this interval gate termed "hnRNP A2 high." Results are representative of 5 separate experiments. Bars show the mean ± SEM. $* = P \leq 0.05$ versus T cells and B cells.

lymph nodes. HnRNP A2 and its longer splicing variant hnRNP B1 were expressed at a similar level in the lymph nodes, spleen, and thymus. The larger 38-kd hnRNP B2 variant was expressed mainly in the thymus and lymph nodes, while the smaller 30-kd variant hnRNP B0 was predominantly expressed in the lymph nodes and spleen. Furthermore, strong expression of hnRNP A2/B1/B2 was also observed in the lungs, whereas these proteins were hardly detectable in other organs and skeletal muscle, confirming previous observations (14).

In the liver and kidney, a 40-kd protein, which might represent another hitherto uncharacterized splicing variant or a posttranslationally modified isoform, was detected. Apart from this one, no unidentified proteins were detected with the mAb used. In addition, expression of hnRNP A2/B1 in immune cells isolated from the spleen was analyzed by immunoblotting (Figure 1B) and flow cytometry (Figure 1C). The protein was predominantly expressed in monocyte/macrophages with ~80% of cells staining positive for hnRNP A2/B1, among which 20% showed high expression. Expression was less prominent in the B and T cell compartment, where 40–50% of the cells expressed hnRNP A2/B1 with <5% showing high expression.

Silencing of hnRNP A2/B1 by RNA interference in vitro and in vivo. To address the role of hnRNP A2/B1 in the pathogenesis of inflammatory arthritis, we studied

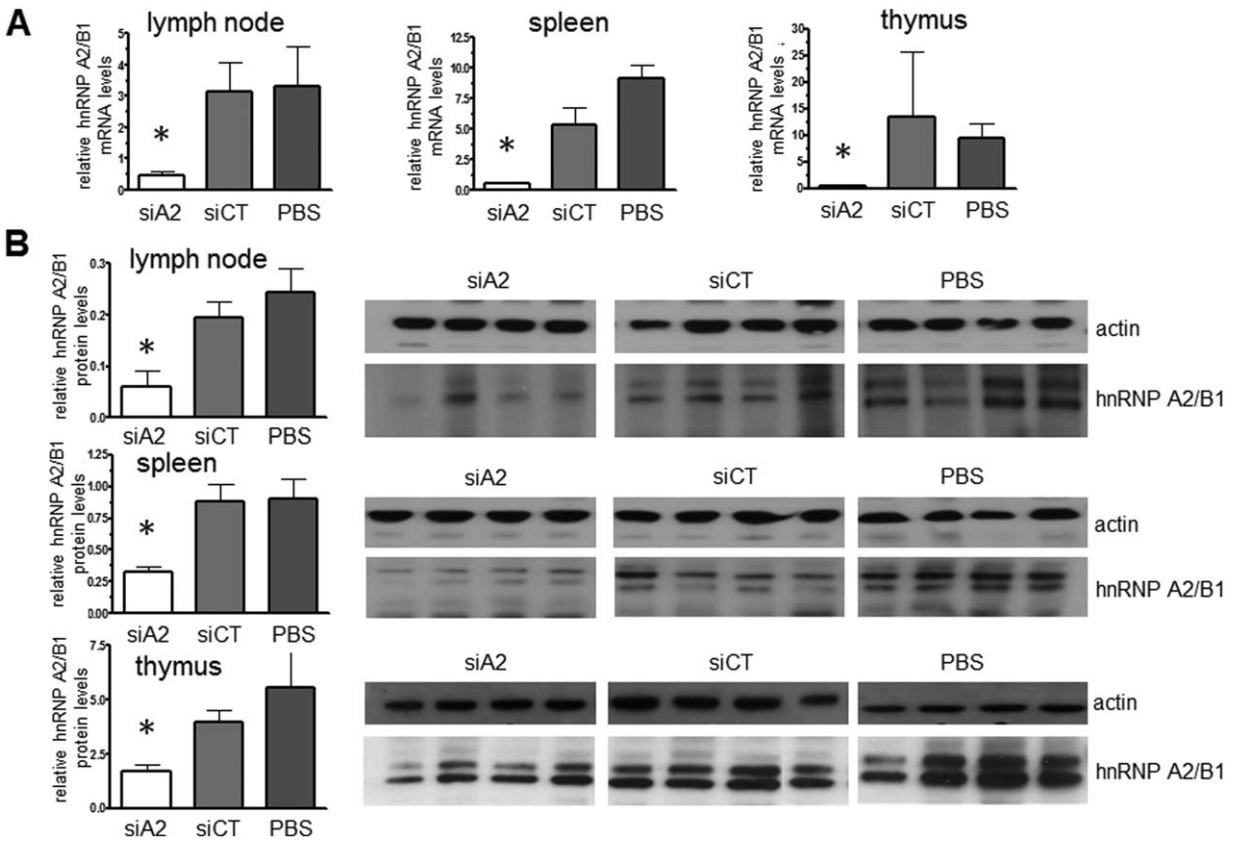

Figure 2. Inhibition of heterogeneous nuclear RNP A2/B1 (hnRNP A2/B1) expression by in vivo application of hnRNP A2/B1–specific small interfering RNA (siA2). **A,** Expression of hnRNP A2/B1 mRNA. Total RNA was obtained from the lymphoid organs of mice treated with hnRNP A2/B1–specific siRNA, control siRNA (siCT), or phosphate buffered saline (PBS). Reverse transcription–quantitative polymerase chain reaction analysis was performed to quantify hnRNP A2/B1 expression, using GAPDH as a control. **B,** Analysis of hnRNP A2/B1 expression at the protein level by immunoblotting, using the monoclonal antibody 10D1 for the detection of hnRNP A2/B1 and β-actin staining as a loading control. Bars show the mean ± SEM (n = 10 mice per group). $* = P \leq 0.05$ versus mice treated with control siRNA and mice treated with PBS, by analysis of variance.

the effects of hnRNP A2/B1 down-regulation in mice with CIA. Since a knockout strain is not available, we performed a functional knockdown by siRNA-mediated silencing, taking advantage of the fact that siRNAs preferentially accumulate in lymphoid organs and in cells of monocytic origin, particularly in inflammatory Ly6C^{high} monocytes when formulated with the cationic liposome RPR209120/DOPE (21). Furthermore, monocytic cells not only in peripheral blood and spleen, but also in the inflamed joints, can be targeted efficiently with siRNA–lipoplexes (18,22).

To demonstrate the efficiency of siRNA-mediated silencing, we first investigated the expression of hnRNP A2/B1 in the rheumatoid synovial cell line MH7A, which we transfected with siRNA targeting hnRNP A2/B1 or a control siRNA. Between 50% and 70% down-regulation of hnRNP A2/B1 expression was observed both at the protein level and the mRNA level (data not shown). Silencing was even more pronounced in the lymphoid organs of DBA/1 mice treated with hnRNP A2/B1–specific siRNA or control siRNA (Figures 2A and B). To assess the specificity of hnRNP A2/B1 silencing, we additionally analyzed the expression of hnRNP A1, which is the most closely related relative of hnRNP A2/B1, showing ~70% sequence homology. No change in hnRNP A1 protein expression in the thymus, spleen, or lymph nodes was observed (data not shown).

Reduction in the incidence and severity of CIA by silencing of hnRNP A2/B1 in DBA/1 mice. Arthritis was induced in DBA/1 mice by immunization with bovine type II collagen. One day before animals received the booster injection, lipoplexes containing hnRNP A2/B1–specific siRNA or control siRNA were applied intravenously, followed by weekly applications as described above. Animals injected with PBS or control siRNA developed clinical signs of arthritis, such as paw swelling and reduced grip strength, 3 weeks after the second immunization. In contrast, symptoms of arthritis were

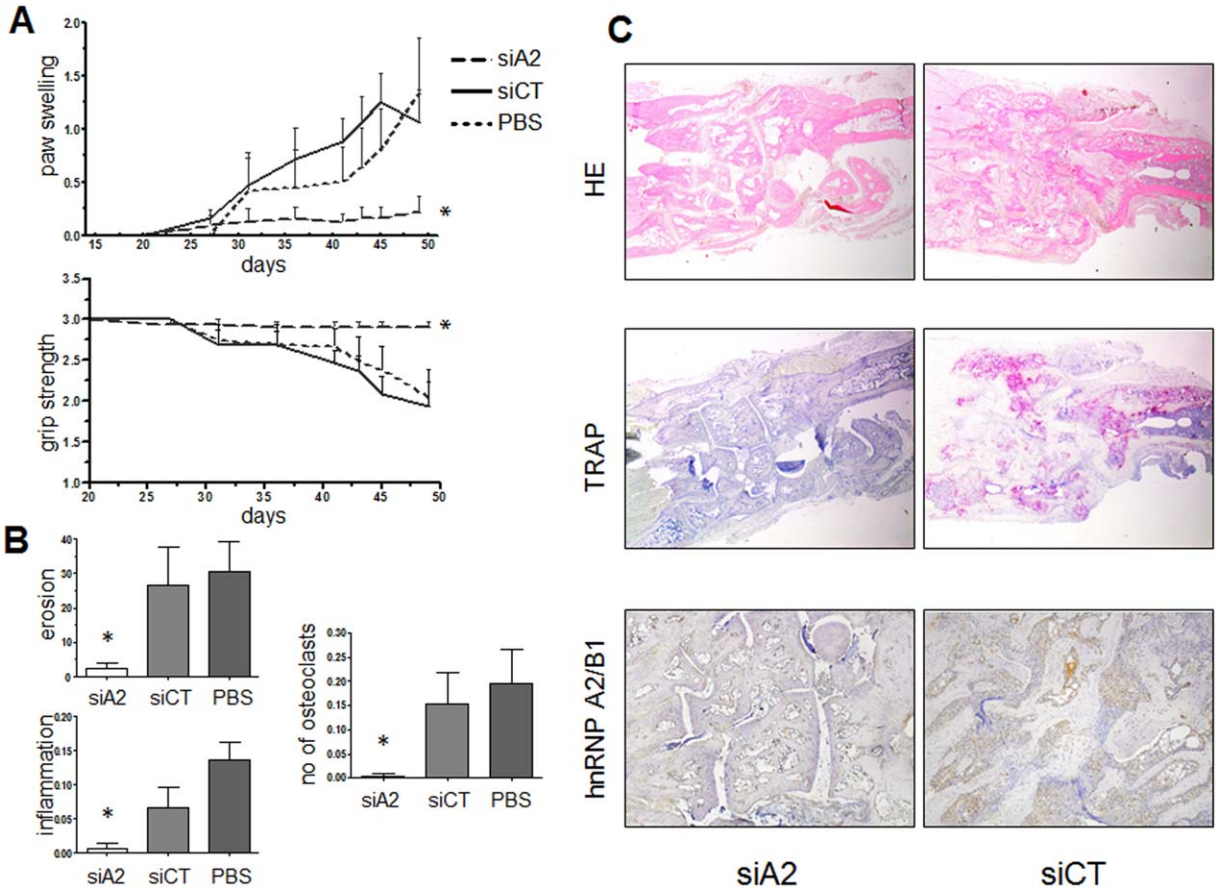

Figure 3. Diminished inflammation and bone erosion in mice with collagen-induced arthritis (CIA) after silencing of heterogeneous nuclear RNP A2/B1 (hnRNP A2/B1) expression by systemic administration of hnRNP A2/B1–specific small interfering RNA (siA2) lipoplexes. Mice were immunized with bovine type II collagen and received a booster injection on day 21. The first intravenous injection of hnRNP A2/B1–specific siRNA was performed 3 days before the booster immunization, followed by weekly applications. Control mice were injected with either phosphate buffered saline (PBS) or a control siRNA (siCT). **A,** Paw swelling and grip strength. Values are the mean ± SEM (n = 10 animals per group). The area under the curve was assessed. $* = P \leq 0.05$ versus mice treated with control siRNA and mice treated with PBS, by post hoc analysis of variance. **B,** Quantitative histomorphometric analysis of the extent of erosion per bone area, inflammation per bone area, and number of osteoclasts per bone area in the tarsal area of the hind paws. Bars show the mean ± SEM (n = 10 animals per group). $* = P \leq 0.05$ versus control siRNA and PBS. **C,** Representative findings from histologic assessment of the hind paws of hnRNP A2/B1–specific siRNA–treated and control siRNA–treated animals obtained 8 weeks after the induction of CIA. Top, Hematoxylin and eosin (H&E) staining. No signs of inflammation or bone erosion were seen in sections from hnRNP A2/B1–specific small interfering RNA–treated mice, in contrast to control siRNA–treated animals. Middle, Tartrate-resistant acid phosphatase (TRAP) staining. Purple staining indicates osteoclasts. Bottom, Staining for hnRNP A2/B1. Cells expressing hnRNP A2/B1 (brown) are predominantly found in the pannus areas of inflamed joints. Original magnification × 100 for H&E and TRAP staining; × 200 for hnRNP A2/B1 staining.

strongly reduced in animals treated with hnRNP A2/B1–specific siRNA. This reduction was already significant 2 weeks after disease onset and became more and more pronounced as disease progressed (Figure 3A). In fact, in the hnRNP A2/B1–specific siRNA-treated group only 20% of the animals developed disease as compared to 70% and 80%, respectively, in the 2 control groups. Nine weeks after disease induction mice were killed and histomorphometric analysis of the hind paws was performed. In mice treated with PBS or control siRNA, severe joint inflammation characterized by invading inflammatory cells, increased generation of bone-resorbing osteoclasts, and bone and cartilage destruction was observed. In contrast, mice treated with hnRNP A2/B1–specific siRNA showed little or no inflammation, almost no signs of bone erosion, and few, if any, osteoclasts. Furthermore, we observed hardly any expression of hnRNP A2/B1 in the joints of hnRNP A2/B1–specific siRNA–treated animals in comparison to control siRNA–treated mice (Figures 3B and C).

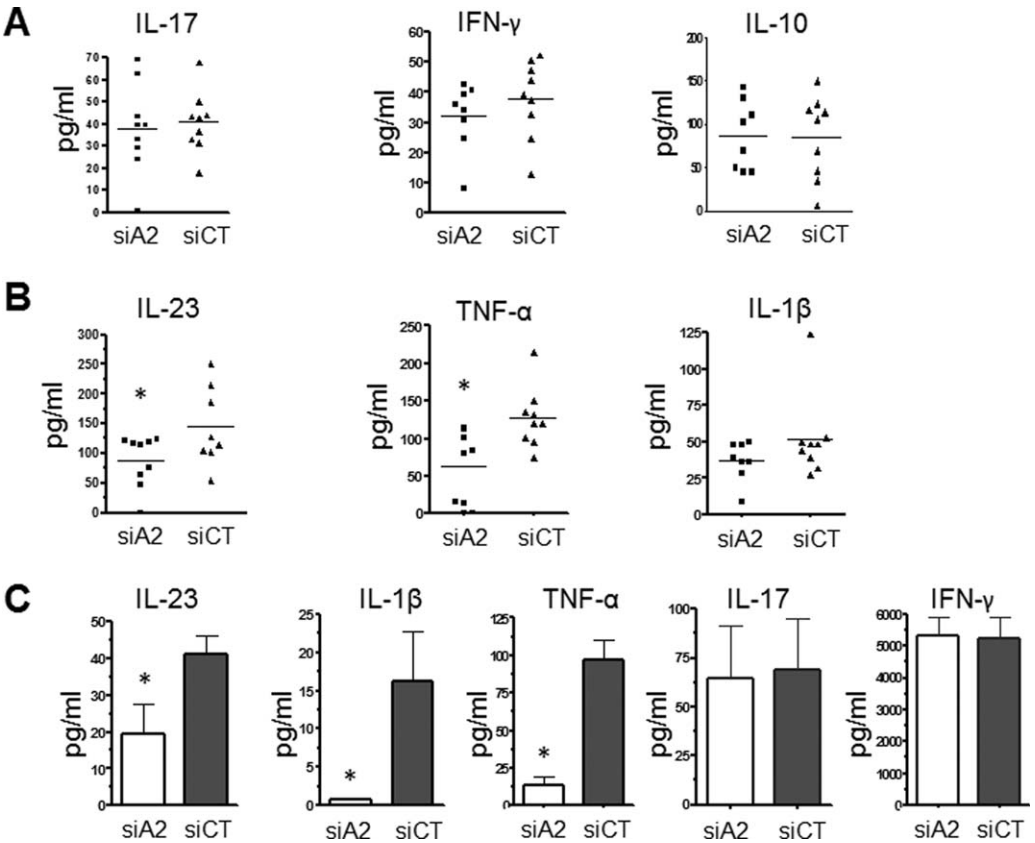

Figure 4. Impaired in vitro cytokine production in mouse synovial macrophages and in cells of draining lymph nodes after systemic application of heterogeneous nuclear RNP A2/B1 (hnRNP A2/B1)–specific small interfering RNA (siA2) lipoplexes. Synovial tissue was isolated from the knee joints of hnRNP A2/B1–specific siRNA–treated or control siRNA (siCT)–treated DBA/1 mice (n = 10 animals per group) and cultured for 24 hours. Subsequently, levels of **A,** interleukin-17 (IL-17), interferon-γ (IFNγ), and IL-10, and **B,** IL-23, tumor necrosis factor (TNF), and IL-1β in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA). In **A** and **B,** symbols represent individual mice; horizontal lines show the mean. * = $P \leq 0.05$ versus control siRNA, by Student's *t*-test. **C,** Draining lymph nodes of hnRNP A2/B1–specific siRNA–treated and control siRNA–treated mice were collected 8 weeks after induction of collagen-induced arthritis, and isolated lymphoid cells were exposed to bovine type II collagen (20 μg/ml). After 24 hours of culture, cytokines in the supernatants were measured by ELISA. Bars show the mean ± SEM (n = 10 animals per group). * = $P \leq 0.05$ versus control siRNA, by Student's *t*-test.

Effect of silencing of hnRNP A2/B1 on the expression of cytokines of the mononuclear phagocytic system. To elucidate the molecular mechanism of arthritis inhibition caused by hnRNP A2/B1 silencing, we investigated cytokine production at the site of inflammation. To this end, synovial tissue obtained from mice treated with hnRNP A2/B1–specific siRNA or control siRNA was cultured overnight, and cytokines in the supernatants were measured by ELISA. No differences were found between hnRNP A2/B1–specific siRNA– and control siRNA–injected animals for cytokines specifically produced by T lymphocytes, such as IL-17, IFNγ, and IL-10 (Figure 4A). However, when analyzing cytokines typically produced by monocyte/macrophages we found a significant reduction in TNF and IL-23 levels, while IL-1β production did not differ between the 2 groups (Figure 4B).

Since macrophages are crucially involved in the pathogenesis of CIA and hnRNP A2/B1 is most strongly expressed in cells of the monocyte/macrophage lineage, we next analyzed the cytokine expression pattern in the draining lymph nodes. Cells were isolated from draining lymph nodes, restimulated with type II collagen, and analyzed for the production of various cytokines by ELISA (Figure 4C). The results were similar to those obtained with synovial cells. We found significantly reduced IL-23, TNF, and IL-1β levels in the supernatants of draining lymph nodes from the hnRNP A2/B1–specific siRNA–treated group as compared to draining lymph nodes obtained from control siRNA–treated mice. In contrast, IL-17 and IFNγ levels did not differ between the 2 groups. These results provide evidence functional impairment of monocyte/

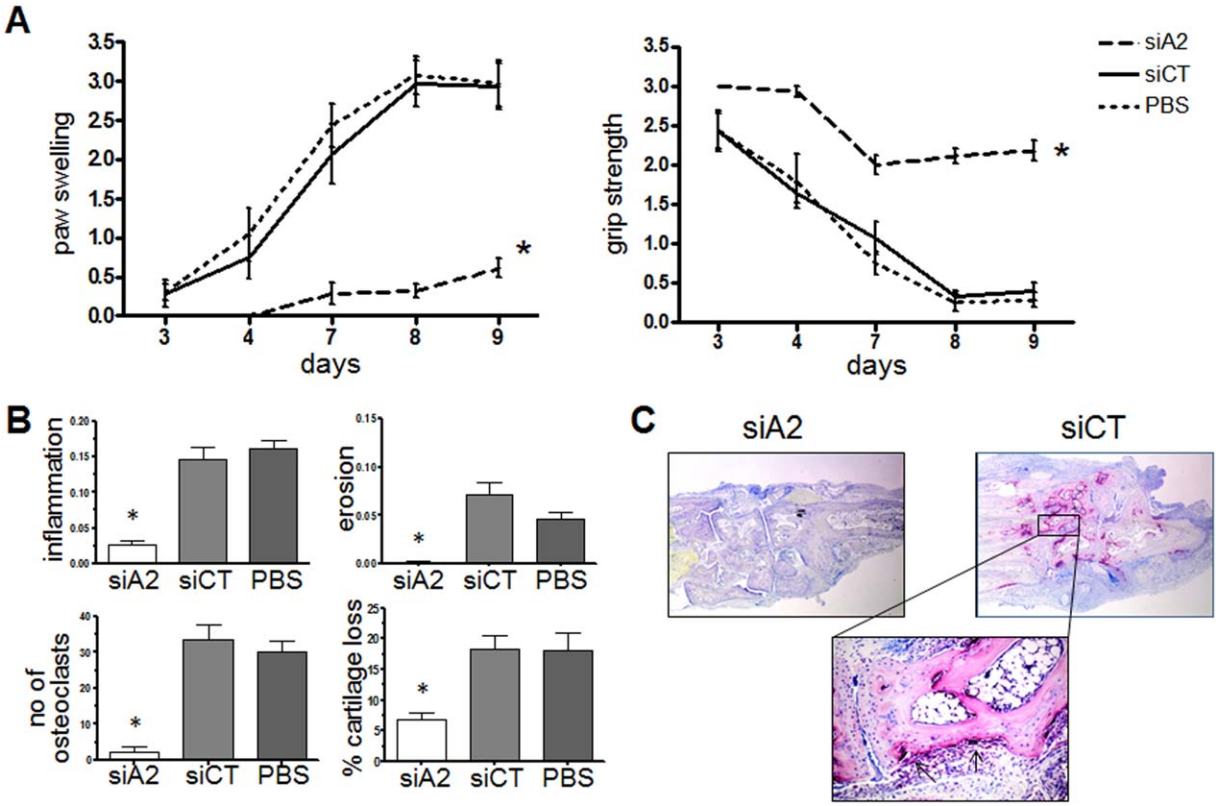

Figure 5. Inhibition of arthritis in the K/BxN serum–transfer model after systemic application of heterogeneous nuclear RNP A2/B1(hnRNP A2/B1)–specific small interfering RNA (siA2) lipoplexes. **A,** Clinical assessment of paw swelling and grip strength after induction of K/BxN serum–transfer arthritis in C57BL/6 mice. Intravenous injection of hnRNP A2/B1–specific small interfering RNA lipoplexes was performed every third day. Control animals were injected with either a nontargeting control siRNA (siCT) or phosphate buffered saline (PBS). Bars show the mean ± SEM (n = 7 animals per group). * = $P \leq 0.05$ versus control siRNA–treated and PBS-treated animals. **B,** Histomorphometric assessment of the extent of inflammation, bone erosion, number of osteoclasts, and percent of cartilage loss in the tarsal area of the hind paws of hnRNP A2/B1–specific small interfering RNA–treated, control siRNA–treated, and PBS-treated animals. Bars show the mean ± SEM (n = 7). The area under the curve was assessed. * = $P \leq 0.05$ versus control siRNA and PBS, by analysis of variance. **C,** Representative hematoxylin and eosin and tartrate-resistant acid phosphatase (TRAP) staining of the hind paws of hnRNP A2/B1–specific small interfering RNA–treated and control siRNA–treated mice obtained 10 days after arthritis induction. **Arrows** indicate TRAP-positive multinucleated osteoclasts seen in deep erosive caverns in control siRNA–treated mice. Original magnification × 100. The lower panel shows a higher-magnification view (×400) of the boxed area. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.39223/abstract.

macrophages caused by hnRNP A2/B1 silencing, leading to strongly diminished arthritis development.

Silencing of hnRNP A2/B1 does not affect generation of anticollagen antibodies. To analyze the effect of hnRNP A2/B1 silencing on the B cell response, serum levels of collagen-specific IgG1 and IgG2a antibodies were measured by ELISA. No differences in antibody levels could be detected between the 3 differently treated groups (hnRNP A2/B1–specific siRNA, control siRNA, and PBS) either on day 30 or on day 50 (data not shown), indicating that the adaptive immune system driving the anticollagen response was not functionally impaired by hnRNP A2/B1 silencing.

Inhibition of arthritis by silencing of hnRNP A2/B1 in antibody–mediated K/BxN serum–transfer arthritis. Since silencing of hnRNP A2/B1 seemed to particularly affect the mononuclear phagocyte system and obviously had little or no effect on B and T cell function, we next focused on the effector phase of erosive arthritis. This phase is characterized by severe joint inflammation caused by invading immune cells, overproduction of proinflammatory cytokines, and aberrantly activated osteoclasts, eventually leading to bone destruction. Infiltrating monocyte/macrophages play a pivotal role in this process, on the one hand due to their strong local cytokine and chemokine production, and on the other hand because of their ability

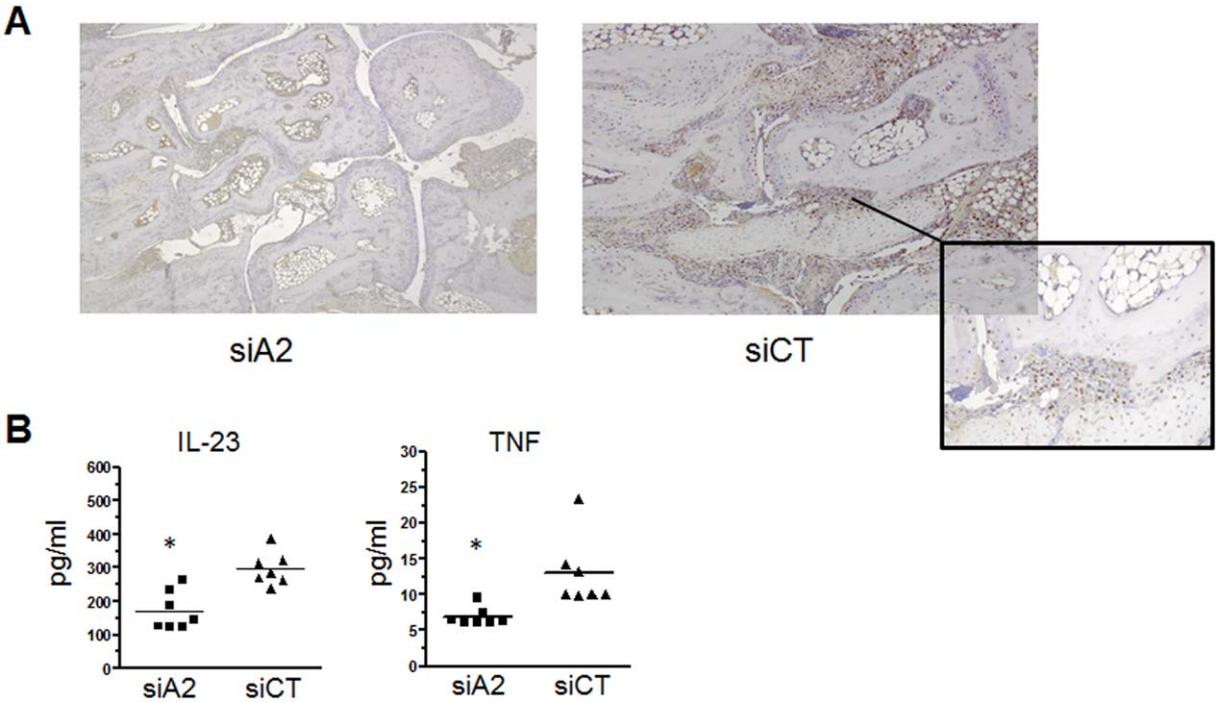

Figure 6. Inhibition of expression of heterogeneous nuclear RNP A2/B1 (hnRNP A2/B1) in the joints and reduction in the production of proinflammatory cytokines in the K/BxN serum–transfer model after systemic application of hnRNP A2/B1–specific small interfering RNA (siA2) lipoplexes. **A,** Immunohistochemical analysis of hnRNP A2/B1 expression in the joints of animals treated with hnRNP A2/B1–specific siRNA or control siRNA (siCT). Original magnification × 100. The lower panel on the right shows a higher-magnification view (×200) of the indicated area. **B,** Levels of interleukin-23 (IL-23) and tumor necrosis factor (TNF) in the sera of hnRNP A2/B1–specific small interfering RNA–treated and control siRNA–treated mice, measured by enzyme-linked immunosorbent assay. Symbols represent individual mice; horizontal lines show the mean (n = 7 animals per group). $* = P \leq 0.05$ versus control siRNA, by Student's *t*-test. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.39223/abstract.

to differentiate into bone-resorbing osteoclasts. In order to examine the effect of hnRNP A2/B1 silencing on the late erosive effector phase of arthritis, we used the K/BxN serum–transfer arthritis model, which is completely independent of the adaptive immune system and, importantly, also of the DBA/1 genetic background (23,24).

For arthritis induction, K/BxN serum was administered intraperitoneally to hnRNP A2/B1–specific siRNA–, control siRNA–, and PBS-treated C57BL/6 mice 1 day after the first intravenous siRNA application, followed by a second serum injection 3 days later. Lipoplexes were injected intravenously every third day, for a total of 3 times before mice were killed. Mice treated with control siRNA or PBS developed severe clinical arthritis, whereas the incidence and severity of arthritis were strongly reduced in mice treated with hnRNP A2/B1–specific siRNA lipoplexes (Figure 5A). Histologic examination of the joints showed almost no signs of inflammation and bone erosion in hnRNP A2/B1–specific siRNA–treated mice. In contrast, the 2 control groups developed massive inflammation and severe bone erosion; moreover, a large number of osteoclasts were observed, whereas these cells were hardly detectable in the joints of hnRNP A2/B1–specific siRNA–treated animals (Figures 5B and C). Also, in this arthritis model we did not observe hnRNP A2/B1 expression in the paws of hnRNP A2/B1–specific siRNA–treated animals in comparison to the control mice (Figure 6A). Similar to the findings in mice with CIA, we observed that IL-23 and TNF levels were significantly reduced in the sera of hnRNP A2/B1–specific siRNA–treated mice as compared to control siRNA–treated animals (Figure 6B). Thus, silencing of hnRNP A2/B1 during the effector phase of the disease, without involvement of the adaptive immune system, was sufficient to almost completely abrogate the development of arthritis.

DISCUSSION

The nuclear protein hnRNP A2/B1 represents one of the most thoroughly investigated members of the hnRNP family and has been shown to be involved in essential biologic processes related to transcription, maturation, transport, and translation of mRNA (9),

including cellular proliferation and regulation of the cell cycle (25,26). Consistent with these findings, increased expression of different hnRNPs has been observed in various types of cancer (27–30). Concerning possible involvement of hnRNP A2/B1 in immune regulation, its recently suggested role as a coactivator of the serine/threonine protein kinase Akt1 during the mitochondrial stress response is of particular interest. It was demonstrated that phosphorylation of hnRNP A2/B1 by Akt1 is indispensable for activation of proinflammatory transcription factors such as NF-κB, NF-AT, and CREB that interact to form the enhanceosome complex (31,32). Thus, hnRNP A2/B1 seems to be an essential coactivator for proinflammatory transcription factors under conditions leading to mitochondrial alterations and dysfunctions, which are also implicated in pathologic inflammatory processes (33). Consistent with these findings, a role of hnRNP A2 in ERK pathway activation was recently described. Up-regulation of hnRNP A2 in a hepatocellular cancer line caused down-regulation of a dominant-negative isoform of A-Raf via an alternative splicing event, leading to activation of the Raf-MEK-ERK signaling pathway (34).

Its high constitutive expression in lymphoid organs and cells of the monocyte/macrophage lineage demonstrated here and in previous studies (14) may be considered as a further indication of the potential involvement of hnRNP A2/B1 in the regulation of immune responses and inflammation. This is supported by the pronounced overexpression seen in the inflamed synovial tissue of patients with RA and in several animal models of arthritis, which was most prominent in macrophages of the synovial lining layer (12,14,15). It is conceivable that aberrant expression at sites of inflammation might lead to loss of tolerance and generation of autoreactive T cells and autoantibodies against hnRNP A2/B1 that have been described in RA (8,10,12,13) and, notably, also in 2 animal models of arthritis, namely TNF-transgenic mice (14) and rats with pristane-induced arthritis (15), suggesting potential pathogenetic involvement of these autoimmune responses. However, in the 2 models used in the present study such autoreactivities were not observed, at least not during the acute phase of the disease (Herman S, et al: unpublished observations). This is not surprising, given that in CIA a strong autoimmune response to type II collagen is prevalent and in serum-transfer arthritis disease is induced by passive immunization with arthritogenic antibodies.

Hence, these data suggest that hnRNP A2/B1 is involved in the regulation of inflammation and play a crucial role in the pathogenesis of inflammatory arthritis. This is further and strongly supported by the finding in this study that silencing of hnRNP A2/B1 by siRNA technology protected almost completely against the development of arthritis in 2 different mouse models of RA. Thus, histomorphometric analysis revealed a nearly complete absence of joint inflammation and bone erosion, both in CIA and in K/BxN serum–transfer arthritis. Importantly, the number of osteoclasts was not increased in hnRNP A2/B1–specific siRNA–treated mice, in contrast to mice treated with control siRNA or PBS. These observations indicated inhibition of function in effector cells, particularly in macrophages and monocyte-derived osteoclast precursor cells, which are responsible for inducing joint damage via the release of proinflammatory cytokines and osteoclast-activating factors. Macrophages act as key players in local and systemic inflammation in RA, producing a plethora of proinflammatory cytokines and recruiting leukocytes to the inflamed joint. Moreover, macrophages are the only cells capable of transforming into bone-resorbing osteoclasts.

In both the serum-transfer model and in CIA the absence of inflammatory cells and osteoclasts in the joints of animals treated with hnRNP A2/B1–specific siRNA was obviously independent of B or T cell responses because neither the levels of anticollagen antibodies nor the T cell recall response was affected by silencing of hnRNP A2/B1, which shows much lower expression in these 2 cellular compartments than in monocytes or macrophages. Furthermore, serum levels and in vitro production of proinflammatory cytokines typically secreted by macrophages, such as TNF and IL-23, were significantly lower in hnRNP A2/B1–silenced mice than in control animals, while levels of the T cell cytokines IFNγ and IL-17 were not diminished.

Taken together, the data obtained in 2 experimental models of RA allow us to conclude that hnRNP A2/B1 has essential involvement in the pathogenesis of erosive arthritis and presumably also other inflammatory diseases. Apart from its established roles in pre-mRNA splicing and regulation of translation, hnRNP A2/B1 seems to play a pivotal role in the activation of the innate immune system, presumably via interaction with NF-κB and MAP kinase signaling pathways (32,34). Our data appear to support these findings and suggest a new and important role of this multifunctional protein in immune regulation as well as involvement in the pathogenesis of inflammatory diseases. The pronounced autoimmune responses to anti–hnRNP A2/B1 repeatedly observed in RA and several arthritis models (10,12–15) indicate a dual role of this protein in the pathogenesis of autoimmune arthritis, acting on the one hand as a proinflammatory immune regulator, and on the other hand as an autoantigen inducing potentially arthritogenic autoimmune responses. Hence, specific targeting of hnRNP A2/B1 in cells of the mononuclear phagocyte system might constitute an interesting novel concept for the treatment of

RA and other chronic inflammatory conditions, a notion deserving further investigation.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Steiner had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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