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The Rheumatoid Arthritis-Associated Autoantigen hnRNP-A2 (RA33) Is a Major Stimulator of Autoimmunity in Rats with Pristane-Induced Arthritis

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A single intradermal injection of the mineral oil pristane in susceptible DA.1F rats induces erosive arthritis closely mimicking rheumatoid arthritis (RA). Pristane-induced arthritis (PIA) is driven by autoreactive T cells but no autoantigen has been identified to date. We therefore analyzed B and T cell responses to autoantigens potentially involved in the pathogenesis of RA, including IgG, citrullinated proteins, stress proteins, glucose-6-phosphate isomerase, and heterogeneous nuclear ribonucleoprotein (hnRNP)-A2 (RA33). IgG and IgM autoantibodies to hnRNP-A2 were detectable in sera of pristane-primed DA.1F rats already 1 wk before disease onset, reached maximum levels during the acute phase, and correlated with arthritis severity. Apart from rheumatoid factor, autoantibodies to other Ags were not observed. CD4+ lymph node cells isolated 10 days after pristane injection produced IFN-γ but not IL-4 in response to stimulation with hnRNP-A2, whereas none of the other candidate Ags elicited cytokine secretion. Surprisingly, hnRNP-A2 also stimulated lymph node cells of naive animals to produce inflammatory cytokines in a MyD88-dependent manner. Furthermore, hnRNP-A2 was highly overexpressed in the joints of rats injected with pristane. Overexpression coincided with the appearance of anti-RA33 Abs and preceded the onset of clinical symptoms of PIA by several days. Taken together, these data suggest hnRNP-A2 to be among the primary inducers of autoimmunity in PIA. Therefore, this Ag might play a pivotal role in the pathogenesis of PIA and possibly also human RA. The Journal of Immunology, 2007, 179: 7568–7576.

Rheumatoid arthritis (RA) is a chronic inflammatory and highly destructive joint disease characterized by persistent synovial inflammation, cartilage degradation, and bone erosion. Serologically, the presence of serum autoantibodies in the majority of patients with RA is a characteristic feature of this disorder that distinguishes it from other arthritides with a nonautoimmune pathogenesis such as reactive arthritis or osteoarthritis. Remarkably, autoantibodies such as rheumatoid factor (RF) and Abs to citrullinated proteins can be found in sera of patients several years before the onset of clinically overt disease (1, 2).

Among the various animal models of arthritis, pristane-induced arthritis (PIA) in the rat most closely mimics human RA as it fulfills many of the clinical criteria of RA, including a symmetrical involvement of peripheral joints and the presence of RF (3, 4), the destruction of cartilage and bone, and a chronic disease course. PIA constitutes one of several types of rat adjuvant arthropathies that are usually triggered by a single intradermal injection of an immunostimulatory agent (adjuvant). A variety of arthritogenic adjuvants have been identified, including polysaccharides, glycolipids (5), and nonimmunogenic oils such as squalene (6), avridine (7), or pristane (3). In contrast to immunization with cartilage-derived proteins like type II collagen or cartilage oligomeric matrix protein, this mode of arthritis induction shows that joint-specific disease can also be elicited by nonspecific stimulation of the immune system.

Pristane (2,6,10,14-tetramethylpentadecane) is a mineral oil that is also used for producing ascites in mice and has been shown earlier to induce arthritis in susceptible mouse strains (8). PIA in rats is a T cell-driven disease that can be transferred by CD4+ T lymphocytes (9) and is amelioriated by treatment with Abs to the αβ TCR during initial development as well as during the chronic stage (3). The disease is strongly associated with certain MHC haplotypes (3) and dependent on MHC class II-restricted T cells (9). However, pristane does not contain peptides that could bind to MHC molecules. Pristane could possibly bind to other cell surface receptors such as CD1 (10), but because the arthritogenicity is
dependent on MHC class II (9), a direct pathogenic role of CD1-restricted T cells is unlikely. This would suggest that the disease is caused by non-Ag-specific stimulation of the immune system. It has been hypothesized that pristane elicits a polyclonal activation of already primed self-reactive T cells that cannot be completely counteracted by regulatory T cells (11).

Unlike murine PIA, wherein Abs and cellular responses against a variety of Ags including joint-specific proteins, heat shock proteins (HSP), and ubiquitously expressed Ags have been detected (12), no (auto)immune target has thus far been identified in rat PIA (11). Murine PIA is, however, clearly different from PIA in rats. It is induced by i.p. administration of pristane, which leads to a more systemic inflammation and a delayed onset of disease. Moreover, in mice pristane induces autoimmune reactivities via, presumably, immune responses to microbial Ags because it cannot be established under special pathogen-free conditions (13). In contrast, injection of pristane-containing mineral oil in rats induces arthritis also in a germfree environment, demonstrating that microorganisms are not involved in the pathogenic process (14).

The strict dependence of rat PIA on MHC class II argues for an Ag-driven disease process. Nevertheless, classical immunological approaches have until now failed to detect a T or B cell response toward joint Ags like collagen II, cartilage oligomeric matrix protein, or HSPs that have been suggested to be among the primary immune targets in adjuvant arthritis as well as in RA (11, 15, 16).

In an attempt to characterize autoantigens of potential pathogenic relevance, we investigated humoral and cellular reactivity in rats with PIA by using a large panel of more systemically expressed candidate autoantigens known to be associated with RA and other systemic autoimmune diseases, such as citrullinated fibrinogen, HSPs, glucose-6-phosphate isomerase (G6Pi), and the heterogeneous nuclear ribonucleoprotein (hnRNP)-A2, also known as the RA33 autoantigen (17). The data obtained suggest hnRNP-A2 to be a primary and early target of the autoimmune response in PIA, both at the B cell level and the T cell level. Moreover, hnRNP-A2 stimulated the innate immune system to secrete inflammatory cytokines like TNF-α and IL-6 also in non-pristane-primed cells in a MyD88 signaling-dependent manner. Thus, hnRNP-A2 appears to be a promising candidate for an autoantigen that might be directly involved in the pathogenesis of an arthritis model that is not dependent on immunization with an exogenous Ag.

Materials and Methods

Animals

Congenic rat strains DA.1F, DA.1U, DA.11, and DA (originating from Zentralinstitut für Versuchstierzucht, Hannover, Germany) were bred and maintained in the animal facility of the Section for Medical Immunology, Lund, Sweden. Experiments were performed on age- and sex-matched rats. C57/HeN and CeHHeL-Lpr mice were obtained from Harlan-Winkelmann C57BL/6 MyD88™ (where ko stands for knockout) mice were a gift of Dr. S. Akira, Department of Host Defense, Osaka University, Osaka, Japan. All experiments were approved by the local (Malmö/Lund, Sweden) ethical committee (license M70-04).

PIA induction and evaluation of arthritis

Arthritis was induced by an intradermal injection at the base of the tail with 150 μl of pristane oil (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich). Arthritis development was monitored in all four limbs using a macroscopic scoring system as previously described (9). Briefly, one point was given for each swollen or red interphalangeal joint, one point was given for each swollen or red metacarpophalangeal joint, and five points were given for a swollen ankle (the maximum score per limb and rat was 15 and 60, respectively).

Ags

Both recombinant and natural hnRNP-A2 derived from HeLa cells was used throughout this study. As the recombinant Ag, the A2 or B1 splice variant of hnRNP-A2, which differs from hnRNP-A2 by a 12-aa insertion close to the N terminus (hnRNP-A2/B1), was expressed as a His-tagged fusion protein and purified by Ni-NTA affinity chromatography (Quiagen) followed by polyethylene B-Sepharose adsorption (Bio-Rad) and anion exchange chromatography as described (18).

Natural hnRNP-A2 was highly purified from HeLa nuclei (obtained from the Computer Cell Culture Center, University of Mons-Hainaut, Mons, Belgium) by heparin-Sepharose and cation exchange chromatography as previously described (17). Purity was >95% as assessed by gel electrophoresis and immuno blotting. Endotoxin content was determined by the limphytolytic amebaecyte lysate assay (BioWhittaker). Polyethylene B-agarose (Sigma-Aldrich) was used to further remove LPS.

G6Pi from rabbit muscle was obtained from Sigma-Aldrich. Recombinant mycobacterial hsp65, mouse hsp60, rat hsp70, and hamster grp78 were obtained from StressGen Biotechnologies. Fibrinogen and deaminated fibrinogen were prepared as described (19). Con A and LPS as control stimulants were obtained from Sigma-Aldrich.

Ab detection

Sera were taken from naive DA.1F rats on days 4, 5, 8, 12, 13, 16, 20, 25, 40, and 78 after intradermal injection of 150 μl of pristane. IgM- and IgG-RF was detected by ELISA (Hycor Biomedical). Anti-RA33 autoantibodies were detected by ELISA (IMTEC) and immunoblotting using the natural or recombinant Ag as described (18). For detection of autoantibodies to HSPs, 10 μg of recombinant mouse hsp60, mycobacterial hsp65, rat hsp70, and hamster hsp78, respectively, were loaded on SDS minigels (BioRad), separated electrophoretically, and transferred to nitrocellulose membranes essentially as described (20). A mAb to hsp60 (clone LK1; Stressgen Biotechnologies) served as positive control.

Additionally, sera were analyzed by line immunoassay (Innogenetics) for the presence of autoantibodies against autoantigens associated with other rheumatic autoimmune diseases (such as Sm, Ro, La, topoisomerase I, centromere protein B, ribosomal P protein, and histones). Human autoimmune sera containing defined Ab specificities served as positive controls (as described (20).

Alkaline phosphatase-conjugated goat anti-rat IgG and IgM Abs (Southern Biotechnology Associates), diluted 1/1,000, were used as secondary Abs in immunoblotting and line immunoassays; HRP-conjugated goat-anti-rat IgG and IgM Abs (Southern Biotechnology Associates), diluted 1/2,000, served as secondary Abs in ELISAs.

T cell stimulation assays and cytokine detection

Single cell suspensions were prepared from draining inguinal lymph nodes and spleens of female rats before or 10 days after intradermal injection of 500 μl pristane. CD4+ T cells were isolated through negative selection; cells were incubated with an Ab-cocktail containing mouse-anti-rat-His48 (anti-granulocyte), mouse-anti-rat-OX33 (anti-B cell), mouse-anti-rat-RP3 (anti-neutrophiles), mouse-anti-rat-ED2/ED3 (anti-macrophages), and mouse-anti-rat-OX8 (anti-CD8+ T cells) (BD Biosciences, San Diego, CA). Ab-bound cells were removed from the suspension by binding to petri dishes coated with rabbit-anti-mouse-IgG (Southern Biotechnology Associates). The purity of recovered CD4+ T cells ranged between 90 and 95%, irradiated (1,800 rad) splenocytes from naive rats were used as APCs. Cells were cultured for 72 h in duplicate or triplicate in 96-well tissue culture plates in the presence of the Ags in a total volume of 200 μl (6 × 10^5 total lymph node cells/splenocytes or 4 × 10^4 CD4+ T cells together with 16 × 10^4 irradiated APCs, respectively). Ags were used at 10 and 30 μg/ml. Quantitative measurement of IFN-γ, IL-4, and TNF-α in cell supernatants was performed using the EBioscience rat IFN-γ protocol and the rat IL-4 and TNF-α OptEIA ELISA set (BD Biosciences). Detection limits were 15 pg/ml for IFN-γ, 1.6 pg/ml for IL-4, and 13 pg/ml for TNF-α. In addition, for determination of TNF-α an alternative ELISA protocol was used with anti-rat TNF-α mAb SB/230499/GR as the coating Ab and biotin-conjugated anti-rat TNF-α mAb S54/230499/GR for detection; Abs as well as the TNF-α standard (190499) were provided by Dr. S. Poole (Division of Endocrinology, National Institute for Biological Standards and Control, Hertfordshire, U.K.). Both TNF-α ELISA protocols gave comparable results.

Murine splenocytes were isolated from 8- to 12-wk-old mice and cultured as described above. At 48 h, supernatants were removed and measured for IL-6 concentration by an ELISA obtained from BD Biosciences.
Histological examinations

Paws from naive and arthritic rats 25 and 75 days after pristane injection were collected and decalcified with EDTA. Serial paraffin-embedded tissue sections of hind and front paws were analyzed by H&E staining and tartrate-resistant acid phosphatase (TRAP) staining (leukocyte acid phosphatase kit; Sigma-Aldrich) for the identification of osteoclasts (21) and by toluidine blue staining for visualizing cartilage. To assess the expression of hnRNP-A2 by immunohistochemistry, cryostat sections from synovial tissue of rat paws were incubated for 60 min with the anti-hnRNP-A2 mAb 10D1 as described (20). After rinsing and blocking of endogenous peroxidase, sections were incubated for 30 min with a biotinylated horse anti-mouse IgG Ab followed by incubation with the Vectastain ABC reagent (Vector Laboratories) for another 30 min using diaminobenzidine (Sigma-Aldrich) as substrate, leading to a brown staining of hnRNP-A2-expressing cells. Finally, slides were counterstained with hematoxylin (Merck).

Analysis of protein expression by Western blotting

After removal of skin, muscles, and tendons, synovial tissue from hind and front paws of naive rats and rats 6, 9, 13, and 20 days after pristane injection was mechanically homogenized in Schreiber buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1.5 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 0.1 mM EGTA, and 20% glycerol) containing 5 mM benzamidine, 10 mM N-ethylmaleimide, and 0.08 mM PMSF as protease inhibitors. Rat spleens, lymph nodes, thymuses, brains, lungs, livers, hearts, and kidneys were homogenized in the same buffer as described (20). The tissue extracts were centrifuged for 20 min at 38,000 × g, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Recombinant hnRNP-A2 served as positive control. Membranes were blocked overnight at 4°C and incubated for 1 h at room temperature under constant shaking with mAb 10D1, diluted 1/2,000 in blocking buffer. After washing, the membranes were incubated for 1 h at room temperature with a HRP-conjugated rabbit anti-mouse secondary Ab (DakoCytomation) diluted 1/2,000 in blocking buffer. Finally, immunostained proteins were visualized using an ECL detection kit (Amersham Biosciences).

Analysis of mRNA expression by real-time PCR

Total RNA was isolated from paws of naive and arthritic animals using RNeasy columns (Qiagen), and cDNA was synthesized using a First-Strand cDNA Synthesis kit (GE Healthcare). Rat hnRNP-A2 was amplified in triplicate in a 25-µL real-time PCR (SYBR Green containing ROX passive reference dye; Bio-Rad) in a Prism 7700 sequence detector (Applied Biosystems). GAPDH was used as a reference gene. Primer3 software was used for primer design. The following primer sets were used: rat hnRNP-A2, 5'-ctgaaaattgaggacacacca-3' (sense) and 5'-acaacgcacctgtgtaact-3' (antisense); GAPDH, 5'-tgcaaggggacctga-3' (sense) and 5'-atgcatggcgctggcaga-3' (antisense). The fragment amplified by rat hnRNP-A2 primers is contained in both hnRNP-A2 and -B1. Results are given as n-fold induction of hnRNP-A2 expression upon pristane injection over expression in paws from naive animals.

Statistical analysis

Unpaired Student’s t test was used for statistical analyses of cytokine and Ab levels as well as for expression analysis. In all experiments a p < 0.05 was considered significant. Welsh correction was applied to groups with unequal variances. Correlation tests were performed using the parametric Pearson’s correlation and the nonparametric Spearman correlation as appropriate.

Results

RF is increased during acute phase of PIA

Serum levels of IgG-RF and IgM-RF were analyzed by ELISA in DA.1F rats before pristane injection (naive) as well as during the acute phase (day 25 after pristane injection) and the chronic phase (day 78) of PIA (Fig. 1). Both IgG-RF and IgM-RF were present in naive animals, although IgG-RF levels were notably lower than IgM-RF levels (p < 0.002). IgG-RF levels increased significantly (p < 0.0001) during the development of arthritis into acute phase, while the increase of IgM-RF reached only borderline significance (p = 0.0546). Both IgG- and IgM-RF levels decreased subsequently, and levels during the chronic relapsing phase were no longer significantly different from the levels observed in naive animals. Neither IgG- nor IgM-RF levels correlated with disease severity during the acute phase or the chronic phase.

FIGURE 1. Rheumatoid factor production in naive and arthritic DA.1F rats. IgG-RF (A) and IgM-RF (B) were measured by ELISA. IgG-RF was significantly elevated during acute phase (day 25 after pristane injection) compared with chronic phase of arthritis (day 78) (p = 0.0019) and naive animals (p < 0.0001). In contrast, IgM levels were higher at baseline but did not change significantly during the progression of PIA. All experiments show mean ± SEM of at least 20 sera. ***p < 0.0001. *, p < 0.05

FIGURE 2. Anti-RA33 IgG autoantibodies in naive rats and rats with PIA. A. Anti-RA33 levels in DA rats with different MHC haplotypes. IgG autoantibodies were determined by ELISA in sera of DA.1F (RT1I), DA.1U (RT1u), DA.11 (RT11), and DA (RT1B) rats during the acute phase of arthritis (day 25 after pristane injection). DA.1F rats showed the highest levels and were used for additional experiments. All bars show mean ± SEM of at least eight sera per group. B. Anti-RA33 levels in DA.1F rats during the course of PIA. Levels around the onset of PIA (days 12–15 postinjection) were already highly and significantly above the levels of naive animals (p = 0.0002) and peaked during the acute phase at the disease maximum (day 25). Anti-RA33 levels during chronic phase (day 78) were not significantly different from the levels measured in naive animals. All experiments show mean ± SEM of at least 12 sera per group. C. Immunoblot analysis of anti-RA33 IgG autoantibodies in sera of DA.1F rats with PIA drawn during the acute phase (day 25 after pristane injection). lanes 1-8) and the chronic phase of arthritis (days 78, lanes 9–25) and sera of naive animals (lanes 26–32) using recombinant hnRNP-A2. Control lanes (c) were stained with the mAb 10D1 that specifically recognizes hnRNP-A2. D. Pooled sequential sera of 7 DA.1F rats analyzed on days 0, 5, 10, 12, and 17 after pristane injection.
HnRNP-A2 (RA33) is a major B cell autoantigen in PIA

IgG autoantibodies to hnRNP-A2 were detected by immunoblotting and ELISA in all four of the rat strains investigated. The immune response to hnRNP-A2 was particularly associated with the RT1^d (MHC) haplotype (DA.1F), because mean levels were highest in the strain carrying this haplotype (Fig. 2A) and all animals tested (n = 22) showed anti-RA33 Ab (Fig. 3A). Therefore all subsequent studies were performed with DA.1F rats. Of note, DA.1F rats also developed the most severe arthritis of the four strains investigated, whereas arthritis was comparatively milder in strain RT1^av1 (DA), which showed the lowest incidence of anti-RA33 Ab.

None of the sera from the naive animals tested showed a level of anti-RA33 IgG Abs >11 arbitrary U/ml, which was chosen as the cutoff level (mean value of sera from naive animals ± 2 SD: 4.1 ± 7.11 U/ml). Already near the onset of clinical arthritis symptoms the anti-RA33 levels were highly and significantly above the levels of naive rats (p = 0.0002) and increased further during the progression of the disease, peaking at the acute phase (around day 25 after pristane injection) with levels ranging between 17 and 47 U/ml. Remarkably, during the acute phase of PIA anti-RA33 levels correlated with disease severity (r = 0.60, p = 0.038). During the chronic phase (day 78) anti-RA33 levels were no longer significantly different from those in naive rats (Fig. 2B); however, sera remained reactive with the blotted Ag (Fig. 2C).

When the time courses of pooled sera obtained from seven animals were analyzed by immunoblotting, anti-RA33 Abs were seen already on day 5 (Fig. 2D). To further investigate the kinetics of anti-RA33 autoantibody induction, individual time courses were analyzed in three rats; blood was taken on days 0, 4, 8, 12, 16, 20, 25, and 40 after pristane injection and the IgG and IgM subtypes of anti-RA33 were measured by ELISA. Increases of both IgM and IgG Abs occurred between days 4 and 8 and preceded the clinical signs of arthritis, which were first observed around day 12 (Fig. 3). Interestingly, anti-RA33 Abs peaked around day 12 immediately before the appearance of the first symptoms (i.e., paw swelling), declined thereafter, and increased again during the acute phase. This second increase was more pronounced for IgM Abs, which peaked again around day 25.

Autoantibodies to HSPs, G6P1, and citrullinated Ags are absent in PIA

Sera from DA.1F rats with acute PIA were tested by immunoblotting to autoantibodies to other RA candidate autoantigens, including HSPs from rat (hsp70), mouse (hsp60), hamster (grp78), and mycobacteria (hsp65), G6P1, fibrinogen, and deiminated fibrinogen; additionally sera were tested by ELISA for Abs to cyclic citrullinated peptide, which are considered the most specific marker Abs for RA (19, 22, 23). Furthermore, sera were analyzed by immunoblotting and line immunoassay for the presence of autoantibodies associated with other rheumatic autoimmune diseases such as systemic lupus erythematosus, systemic sclerosis, or poly/dermatomyositis. Apart from single reactivities to hsp60 (Fig. 4) and hsp65 and two weak reactivities to the SmB Ag, no positive results were obtained for any of the tested Ags (Table I).

Arthritic rats exhibit T cell reactivity to hnRNP-A2

Cells were isolated from draining inguinal lymph nodes of naive DA.1F rats and DA.1F rats 10 days after pristane injection (2–5 days before the onset of arthritis symptoms) and exposed to eight
different candidate Ags (Fig. 5A). Upon stimulation with natural or recombinant hnRNP-A2, a pronounced and dose-dependent IFN-γ secretion was seen in all cultures derived from rats with PIA, whereas cells from naive rats did not show an increase in IFN-γ secretion upon stimulation with natural hnRNP-A2. However, a weak response to recombinant hnRNP-A2 was seen that was presumably caused by bacterial contaminations in the Ag preparation (Fig. 5B). Also, purified CD4+ T cells significantly increased their IFN-γ secretion upon stimulation with both natural and recombinant hnRNP-A2 (Fig. 5C). Remarkably, none of the other candidate Ags tested, including four HSPs, G6P, fibrinogen, and deaminated fibrinogen could elicit a significant IFN-γ production (Fig. 5A). IL-4 was not detectable upon coculture with hnRNP-A2 or any of the other candidate autoantigens (data not shown).

HnRNP-A2 activates innate immunity in a MyD88-dependent manner

HnRNP-A2-stimulated cells secreted large amounts of TNF-α, which is also produced by Th1 cells. Surprisingly however, a TNF-α response was also seen in nonprimed cells from naive rats (Fig. 6A) while IFN-γ was not detectable, indicating that the source of TNF-α was not T cells but most likely monocytes/macrophages. Because these cells might be stimulated via TLR engagement, we further addressed this issue by measuring cytokine responses in splenocytes from MyD88-deficient and wild-type mice (Fig. 6B). Although wild-type cells produced high amounts of TNF-α and IL-6 upon coculture with hnRNP-A2, neither cytokine was detectable in the supernatants of MyD88-deficient cells. The lack of secretion of inflammatory cytokines in MyD88−/− mice argues for a direct activation of one or more TLRs by this Ag. The TLR-activating function of host-derived compounds like HSPs has often been attributed to trace LPS contamination (24, 25).

Although wild-type cells produced high amounts of TNF-α and IL-6 upon coculture with hnRNP-A2, neither cytokine was detectable in the supernatants of MyD88-deficient cells. The lack of secretion of inflammatory cytokines in MyD88−/− mice argues for a direct activation of one or more TLRs by this Ag. The TLR-activating function of host-derived compounds like HSPs has often been attributed to trace LPS contamination (24, 25).

![FIGURE 5. IFN-γ production of lymph node cells and CD4+ T cells upon in vitro exposure to candidate autoantigens. Total lymph node cells and purified CD4+ cells were isolated from naive rats and DA.1F rats 10 days after pristane injection and incubated for 72 h with 20 μg/ml Ag. A, Stimulation with natural (nat.) or recombinant (rec.) hnRNP-A2 elicited strong IFN-γ production (measured by ELISA) in lymph node cells from rats primed with pristane, whereas none of the other candidate autoantigens could trigger IFN-γ production that was significantly different from that of medium control. B, In naive animals natural hnRNP-A2 did not stimulate IFN-γ secretion while a small increase was observed in response to recombinant hnRNP-A2, which was probably caused by minor bacterial contaminations in the Ag preparation. C, Stimulation with natural or recombinant hnRNP-A2 elicited IFN-γ secretion also in purified CD4+ T cells isolated from pristane-primed animals. All experiments show mean ± SEM of at least six animals. ***, p < 0.0001; **, p < 0.005; *, p < 0.02.](http://www.jimmunol.org/)

Histological examination of PIA and synovial overexpression of hnRNP-A2

Previous investigations had revealed overexpression of hnRNP-A2 in synovial tissues of RA patients (18) and TNF-α transgenic mice (20). To study the expression in joints of DA.1F rats, tissue sections of arthritic and naive animals were analyzed by immunohistochemistry and immunoblotting. In parallel, a detailed histological analysis was performed. H&E-staining (Fig. 7, A–C) of the tarsal area of the hind paws did not show any signs of pathological alterations in the joints of naive rats, whereas pronounced inflammation with the appearance of subchondral pannus tissue was already visible during the acute phase of PIA (Fig. 7B). The chronic phase (day 78) was characterized by full-blown, massive subchondral inflammation with the appearance of newly formed vessels (Fig. 7C). Cartilage changes were analyzed by toluidine blue staining (Fig. 7, D–F). Already during the acute phase the loss of proteoglycans became evident (Fig. 7E), particularly at sites where the cartilage was invaded by the pannus, and increased during the progression to the chronic phase (Fig. 7F). TRAP staining (Fig. 7, G–I) revealed multinucleated TRAP+ cells attached to the bone at the site of erosion during the acute phase (Fig. 7H), indicating the presence of activated osteoclasts. The number of osteoclasts was further increased in the chronic phase, where almost complete destruction of the subchondral bones was observed (Fig. 7I). No TRAP+ cells were found in the joints of naive DA.1F rats (Fig. 7G).

To study the expression of hnRNP-A2, sections were stained with the mAb 10D1 (Fig. 7, J–L). Although the expression of hnRNP-A2 was hardly detectable in the synovial cells of naive rats (Fig. 7J), pronounced overexpression was observed already during the acute phase of PIA in the majority of synoviocytes of the infiltrating inflammatory pannus tissue (Fig. 7K). Increased expression was also seen in most of the chondrocytes of articular cartilage when compared with naive rats in which only ~30% of chondrocytes expressed hnRNP-A2. During chronic phase, virtually all synoviocytes expressed hnRNP-A2, especially at the sites where the invading pannus tissue directly attaches to cartilage and bone (Fig. 7L). During the acute and chronic phases of PIA, pronounced expression was also seen in multinucleated cells close to the cartilage-pannus junction, presumably representing (TRAP+) osteoclasts.

To control for the specificity of hnRNP-A2 expression, we additionally investigated the expression of a structurally and functionally closely related protein, hnRNP-A1, which shows ~70% identity with hnRNP-A2 (26). This analysis revealed hnRNP-A1 to be overexpressed in a similar manner as hnRNP-A2 (not shown). However, autoantibodies to hnRNP-A1 were infrequently detected and, when so, mostly in anti-RA33 positive sera; a similar observation had previously been made in TNF-α transgenic mice (20).

Immunoblotting of protein extracts from rat joints and other organs confirmed the immunohistochemical findings because only weak expression was seen in the joints of naive rats, whereas the injection of pristane highly increased the expression of hnRNP-A2.
were incubated for 48 h with 20 ng/ml Ag. Incubation with natural (nat.) or recombinant (rec.) hnRNP-A2 elicited strong TNF-α production in pristane-primed as well as in naïve animals, whereas none of the other candidate autoantigens under investigation could trigger TNF-α production that was significantly above that of medium control. All experiments show mean ± SEM of at least five animals. ***p < 0.0001; **p < 0.01.

and its major splice variant, hnRNP-B1, in joints of DA.1F rats (Fig. 8A). Higher expression was already seen 6 days after pristane injection and thus preceded the onset of clinical symptoms of PIA by ~1 wk. Interestingly, the hnRNP-A2-specific mAb stained a third band, in addition to the hnRNP-A2 and -B1 bands, which migrated in SDS-PAGE with a slightly higher apparent Mr. This band may represent a novel isoform of hnRNP-A2 or -B1, respectively, that might be alternatively spliced or posttranslationally modified, an issue to be investigated in future studies. Strong constitutive expression of hnRNP-A2/B1 was observed in brain, thymus, lymph nodes, spleen, lung, and liver while no expression could be detected in kidney and heart (data not shown), confirming observations previously made in mice (20). Apart from joints, no apparent differences in expression levels were observed between naïve and arthritic animals.

The up-regulation of hnRNP-A2 in joints already several days after pristane injection was mirrored by increases in mRNA levels (Fig. 8B). Real-time PCR analysis demonstrated high induction of hnRNP-A2 mRNA already at day 6 after pristane injection (p = 0.033), which was further increased at day 9 (p = 0.019), day 13 (p = 0.0011), and day 20 (p = 0.0023).

Discussion

Although it is now clearly established that RA constitutes an autoimmune disease driven by dysregulated immune processes, the role of autoantibodies and autoreactive T cells in the pathogenesis of this disorder is still not fully understood. Elucidation of the initial pathogenic pathways is hampered by the lack of spontaneous animal models of arthritis. For obvious reasons, models in which arthritis is induced by immunization with a defined Ag, such as collagen or G6PI, are of limited usefulness. A more suitable and attractive model for studying the very early events is PIA in the rat, because here arthritis appears to be driven by true autoimmune processes initiated by the nonimmunogenic molecule pristane.

However, the cellular and molecular mechanisms leading to PIA have not yet been clarified. T cells transferring PIA are MHC class II restricted, seem to mediate their arthritogenic function in the joints via secretion of proinflammatory cytokines such as IFN-γ and TNF-α (9), and are assumed to be self-reactive T cells primed to one or more autoantigens. Because only peripheral joints are attacked in PIA, the inflammatory response and possibly also immune recognition, at least at some stage, appear to be joint specific. However, attempts to find autoimmunity to collagen II or other cartilage components in rats with PIA have, to date, failed (11).

In the present study we show that rats with PIA exhibit a profound response, both on the humoral and cellular level, to hnRNP-A2 (RA33), an abundant mRNA binding protein that shows a predominantly nuclear localization and exerts multiple functions, including the regulation of alternative splicing and the transport of mRNA (27, 28). This mimics the situation in RA, where autoimmune responses have also been found to be predominantly directed to ubiquitous Ags such as IgG, citrullinated proteins including fibrin and vimentin, or hnRNP-A2, whose expression is not restricted to the joint. Therefore, the observed immunological responses against hnRNP-A2, which is a systemically occurring protein that is also targeted by both autoantibodies and autoreactive T cells of patients with RA (18, 29, 30), together with the presence of RF, indicate that PIA is indeed a model that in its immunological aspects is quite close to human disease.

In contrast, neither humoral nor cellular responses to any of the other systemically expressed candidate Ags investigated could be detected. This was somewhat unexpected in the case of HSPs, because these molecules have been described to be among the immunodominant targets in adjuvant arthritis and murine PIA (31). Similarly as in previous investigations using murine arthritis models (32, 33), we could not detect any B or T cell reactivities to citrullinated Ags, which form major and disease-specific Ags in RA. In patients with RA, Abs to citrullinated proteins are closely linked to the presence of the shared epitope, a pentameric sequence found in RA-associated HLA-DR alleles (34, 35). Thus, a possible cause for the absence of autoimmunity to citrullinated peptides in mouse models and rat PIA may be that citrullinated epitopes cannot be efficiently presented by murine and rat MHC class II molecules. Furthermore, no autoimmune reactivity to G6PI was observed that has been identified as an arthritogenic T and B cell autoantigen in the KRN × NOD TCR-transgenic mouse model (36) and in genetically unaltered mice when systemically administered in CFA (37, 38). However, G6PI does presumably not play a major pathogenic role in RA, because autoantibodies are rarely seen in patients with RA and are not specific for this disease (39–41). Finally, no autoantibodies to other autoantigens associated with systemic human autoimmune diseases (including Sm, U1 RNP, Ro, La, etc.) were detected. Thus, aside from anti-hnRNP-A2 autoimmunity, the only other autoimmune response observed in PIA was RF. However, in contrast to the anti-hnRNP-A2 response, neither IgG- nor IgM-RF was related to the severity of the disease.
With respect to the question of why hnRNP-A2 forms such a preferred target in this model of destructive arthritis, two findings of the current study allow partial answers. First, even before the actual onset of clinical symptoms of PIA, massive cellular overexpression of hnRNP-A2 was observed in the rats’ synovial tissue. This is in line with similar findings in RA patients (18) and also in the joints of TNF-α/H9251 transgenic mice that suffer from a severe destructive inflammatory polyarthritis (20). These mice spontaneously developed an autoantibody to hnRNP-A2, and immunization with recombinant hnRNP-A2 aggravated the disease, suggesting that autoimmune reactions to hnRNP-A2 may contribute to the inflammatory and destructive processes in PIA. Second, we made the surprising observation that hnRNP-A2 is able to directly stimulate innate immunity at least in vitro, eliciting the secretion of inflammatory cytokines such as TNF-α and IL-6 apparently in a MyD88-dependent manner. It has been shown recently that RNA sequences within ribonucleoprotein complexes can stimulate the TLRs 3, 7, and 8 and directly activate innate immunity (48, 49). HnRNP-A2 is an mRNA binding protein that exerts multiple functions, including regulation of alternative splicing and transport of mRNA (27, 28). It is associated with many different (pre-) mRNAs (50–54) and binds with high affinity to the telomeric sequence TTTAGG as well as to its RNA counterpart UUUAGG (51, 55). Even though the hnRNP-A2 preparations used did not contain detectable amounts of RNA or DNA, the involvement of nucleic acids cannot be completely ruled out because they might associate with the protein in vitro. In contrast, several other endogenous proteins like HSPs (56) and fibrinogen (57) have been implicated with TLR ligation without the involvement of associated nucleic acids.

Although the details of the binding principles of hnRNP-A2 to TLRs remain to be determined, these two observations may allow us to partly explain how immunization with a nonimmunogenic oil could possibly trigger joint-specific disease. The sudden and selective presence of an immune stimulatory agent such as pristane in the lymph nodes almost certainly leads to T cell proliferation and local inflammation (58). Loss of tolerance to a protein such as hnRNP-A2, which is highly expressed in lymphoid tissues, seems to be one of the consequences. Activated autoreactive and potentially arthritogenic T and B cells would then migrate to the joints where they presumably induce a mild local inflammation recognizing the weakly expressed hnRNP-A2. Subsequent up-regulation of hnRNP-A2 (and many other proteins) could set in motion a


