Collagen Type II and a Thermo-Responsive Polymer of *N*-Isopropylacrylamide Induce Arthritis Independent of Toll-Like Receptors

A Strong Influence by Major Histocompatibility Complex Class II and Ncf1 Genes

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We established and characterized an arthritis mouse model using collagen type II (CII) and a thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PNiPAAm). The new PNiPAAm adjuvant is TLR-independent, as all immunized TLR including MyD88-deficient mice developed an anti-CII response. Unlike other adjuvants, PNiPPAm did not skew the cytokine response (IL-1 β , IFN- γ , IL-4, and IL-17), as there was no immune deviation towards any one type of immune spectrum after immunization with CII/PNiPPAm. Hence, using PNiPAAm, we studied the actual immune response to the self-protein, CII. We observed arthritis and autoimmunity development in several murine strains having different major histocompatibility complex (MHC) haplotypes after CII/PNiPAAm immunization but with a clear MHC association pattern. Interestingly, C57Bl/6 mice did not develop CII-induced arthritis, with PNiPAAm demonstrating absolute requirement for a classical adjuvant. Presence of a gene (*Ncf1*) mutation in the NADPH oxidation complex has a profound influence in arthritis and using PNiPAAm we could show that the high CIA severity in *Ncf1* mutated mice is independent of any classical adjuvant. Macrophages, neutrophils, eosino-

phils, and osteoclasts but not mast cells dominated the inflamed joints. Furthermore, arthritis induction in the adjuvant-free, eosinophil-dependent V β 12 DBA/1 mice could be shown to develop arthritis independent of eosinophils using CII/PNiPAAm. Thus, biocompatible and biodegradable PNiPAAm offers unique opportunities to study actual autoimmunity independent of TLR and a particular cytokine phenotype profile. (*Am J Pathol* 2011, 179:2490–2500; DOI: 10.1016/j.ajpath.2011.07.034)

Rheumatoid arthritis (RA) is a debilitating multifactorial disease involving complex genetic and immunological interactions. Immunization with collagen type II (CII) in an adjuvant induces polyarthritis in susceptible strains of rodents and primates, known as the collagen-induced arthritis (CIA) model, which is widely used for studying arthritis development. CIA resembles RA in several aspects, and is characterized by synovial hyperplasia, infiltration of immune cells, marginal erosions, and cartilage destruction with disruption of joint and cartilage architecture. As in RA, susceptibility to CIA is associated with certain major histocompatibility complex (MHC) class II alleles,^{1,2} H2^a and H2^e haplotypes being the most arthritis-permissive.^{3,4} However, C57Bl/6 mice with H2^b haplotype also develop arthritis,⁵ but influence of the adjuvant on arthritis

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development in these mice has not been clarified. The gene underlying the susceptibility within the H2^q haplotype is A^{q1} and the responsible MHC bound peptide is the CII₂₆₀₋₂₇₀ peptide.⁶ The peptide is conserved in CII, and consequently, severe arthritis is induced after immunization with various heterologous (eg, chick, human, bovine, or rat) CII.⁷ Murine CII differs by one amino acid, affecting MHC binding,⁶ and induction of mouse CII requires stronger adjuvant and more susceptible genetic backgrounds.⁸

The most commonly used adjuvant (complete Freund's adjuvant; CFA) to induce CIA contains bacterial derivatives, which strongly deviate the ensuing immune response.^{9,10} The use of an adjuvant such as CFA in arthritis induction is presumably to break the immune tolerance to the self-protein. The mycobacterial components, having pathogen-associated molecular patterns in CFA, activate antigen-presenting cells via pattern recognition receptors that direct T cells toward a Th1- or Th17- type immune responses characterized by the production of pro-inflammatory cytokines like IFN- γ , and IL-17.^{11,12} On the other hand, incomplete Freund's adjuvant (IFA), without any mycobacterium, deviates the immune response toward Th2 type.^{13,14} However, use of strong adjuvants precludes our understanding of the actual immune responses to the self-protein, CII and associated pathological pathways.

It is well documented that several polymeric systems act as adjuvant especially, poly(glycolide) (PGA),¹⁵ poly(lactide-co-glycolide) (PLGA),^{16,17} polyacrylic acid (PAA) and its alkyl esters,¹⁸ poloxamers,¹⁹ polyphosphazenes,²⁰ polyoxyethylene (POE),²¹ polyoxypropylene (POP), polyacrylate²², and chitosan polymers.²³ These biocompatible and biodegradable polymers have advantages over conventional adjuvants, mainly in the manipulation of degradation kinetics, by varying the concentration of monomers and cross-linkers,²⁴ thereby modulating the physicochemical properties of polymers and leading to different release profiles of an antigen. Recently, we identified a synthetic thermo-responsive polymer of *N*-isopropylacrylamide (PNiPAAm), which is biocompatible and modifiable to study autoimmunity and autoimmune diseases.²⁵ PNiPAAm has a lower critical solution temperature (LCST) of precipitation, around 32.5°C in water, and changes reversibly from hydrophilic to hydrophobic around this temperature.²⁶ This reversible phase-transition property of PNiPAAm was used for the colloid suspension formation together with CII to induce arthritis in mice. Furthermore, our recent studies demonstrated *Ncf1* (coding p47^{phox} subunit of the NADPH oxidase complex, which is a multicomponent electron carrier that is responsible for the reduction of oxygen, resulting in the production of ROS) polymorphism as one of the major genetic factors that control arthritis severity and chronicity, regulating autoimmune reactions and impaired tolerance to CII.²⁷⁻²⁹ In addition, we have also observed a high frequency of arthritis after CII immunization without any adjuvant in a transgenic mice expressing a CII-specific TCR V β 12 chain that recognizes the immunodominant glycosylated CII₂₆₀₋₂₇₀ peptide that is dependent on eosinophilic inflammation.³⁰ Hence, in the present study, we tested various mouse strains using PNiPAAm-CII immunization to find genetic

restriction of arthritis development in the absence of a classical adjuvant. Also, we analyzed adjuvant dependency of strong arthritis promoted by a mutation in *Ncf1* and determined the requirement for eosinophilic inflammation for arthritis induction in V β 12 transgenic mice. Furthermore, we studied the nature of cytokine response induced with PNiPAAm and the self-antigen, CII and compared it with immunization of CII emulsified in Freund's adjuvant(s).

Materials and Methods

Mice

Founders of B10.Q/Rhd mice were provided by Professor Jan Klein (Tübingen University, Tübingen, Germany). Breeding pairs of BALB/cJ, C57BL/6J, DBA/1J, C57BL/10ScNJ mice (*Tlr4*^{ps-del}) and TLR7 knock-out mice were from Jackson Laboratories (Bar Harbor, ME). *Tlr4*^{ps-del} mice were crossed together with B10.Q/Rhd mice to generate mice expressing arthritis-permissive MHC A^q. B10.Q mice with a mutated *Ncf1* gene (B10.Q.*Ncf1*^{*/*}) have been described previously.²⁸ The V β 12 transgenic mouse was generated on an SWR background³¹ and has been backcrossed to DBA/1J for more than 15N generations. B10.P/Rhd mice were bred at the animal facility of the division of Medical Inflammation Research at Lund University. The C3H.Q/Rhd strain was established in our laboratory by inserting the H-2^q haplotype from an older C3H.Q strain into mice of the C3H.NB background.³² Rhd indicates that these classic inbred mouse strains have been maintained in our laboratory for more than 2 decades. F1 Intercross mice QD = (B10.Q $\times$ DBA/1) F₁ and QB = (BALB/c $\times$ B10.Q) F₁ were bred in MIR, Lund University and used in this study. TLR 1, 2, 3, 5, 6, or 9 and MyD88 knock-out mice backcrossed to C57BL/6 for seven to 10 generations were obtained from Institute for Microbiology, Tumor and Cell Biology (MTC) animal facility, Karolinska Institute, Stockholm, Sweden. Most of the TLR-deficient mice were originally derived by the laboratory of Professor Shizuo Akira, Osaka University, Japan. Age-matched 8- to 12-week-old mice of both sex were used for the experiments. All animals were kept in a climate controlled environment having 12-hour light/dark cycles in polystyrene cages containing wood shavings under specific pathogen-free conditions and were fed standard rodent chow and water *ad libitum*. Animal welfare authorities (Lund-Malmö and Stockholm regions, Sweden) approved the animal experiments (permit numbers N310-07, M107-07, and N66-10).

PNiPAAm Synthesis

N-isopropylacrylamide (NiPAAm) was purchased from Acros Organics (Schwerte, Germany). Ammonium persulphate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were purchased from Sisco Research Laboratories (Mumbai, India). PNiPAAm was synthesized through free radical polymerization by using APS and TEMED. For synthesis of PNiPAAm (1%, w/v), 100 mg of NiPAAm was dissolved in 10 mL of degassed water. Initially, the solution

was bubbled with N₂ gas for 20 minutes, and free radical polymerization was initiated by adding 15 mg of APS and 19.5 μ L of TEMED. The reaction vial was immediately filled with nitrogen and tightly sealed. The reaction was stopped after 18 hours by salt induced thermal precipitation. The thermo-precipitation (40°C) and redissolution (4°C) was done several times to ensure complete removal of unreacted monomers and the polymer was freeze-dried for further use. The molecular weight of synthesized PNiPAAm was determined by gel permeation chromatography (Waters, Milford, MA) using tetrahydrofuran (THF) as an eluent and polystyrene as standard.

Arthritis Induction

CII used for immunization was from rat SWARM chondrosarcoma prepared by pepsin digestion and further purified as described earlier.³³ Several different mouse strains were tested for arthritis development after PNiPAAm-CII immunization. QD mice were immunized with PNiPAAm-Ova or PNiPAAm alone but they did not show any clinical symptoms for arthritis. CII or ovalbumin protein grade II (Sigma-Aldrich, St. Louis, MO) emulsified in complete Freund adjuvant (CFA; Difco, Detroit, MI) constituted positive and negative controls, respectively. Both CII and ovalbumin proteins (1 mg/mL) were mixed with PNiPAAm separately and 100 μ g of antigen-polymer mixture (1:1) was injected s.c. on day 0 and boosted with 50 μ g of respective antigen-polymer mixture (1:1) on day 35. Serum samples were collected on different days through the retro-orbital plexus.

Anti-CII Antibody Response

The amounts of total anti-CII IgG were determined through quantitative ELISA as described earlier.³⁴ Affinity-purified anti-CII antibody from pooled sera of CII-immunized mice or pooled sera was used as the standard. Biotinylated rat anti-mouse IgG κ (prepared in-house, clone 187.1) or mouse anti-mouse IgG2c (BD), or peroxidase-conjugated goat anti-mouse antibodies specific for IgG1, IgG2b, or IgG3 (Southern Biotech, Birmingham, AL) were used as detecting antibodies. Binding of biotinylated antibodies was revealed by extravidin peroxidase (Sigma-Aldrich). It is noteworthy that B10 mice express an IgG2a-related allele, IgG2c.³⁵ Plates were developed using ABTS (Roche Diagnostic Systems) as substrate, and measured at 405 nm (Synergy-2, BioTek Instruments, Winooski, VT). Isotype levels were measured as arbitrary units using pooled sera from arthritic mice as the standard.

Clinical Evaluation of Arthritis and Immunohistochemistry

Mice were examined for the arthritis development twice per week. Scoring of animals was done blindly using a scoring system based on the number of inflamed joints in each paw, inflammation being defined by swelling and redness.³⁶ In this scoring system each inflamed toe or knuckle gives one point, whereas an inflamed wrist or

ankle gives five points, resulting in a score of 0 to 15 (five toes + five knuckles + one wrist/ankle) for each paw and 0 to 60 points for each mouse.

For immunohistochemistry, mouse paws after decalcification for 3 to 4 weeks in an ethylenediaminetetraacetic acid (EDTA) solution containing polyvinylpyrrolidone (PVP) and Tris (pH 6.9) were frozen in OCT compound using isopentane on dry ice. The samples were stored at -80°C until cryosectioned at 10 μ m at -30°C. Sections were stained with safranin to visualize joint and cartilage destruction and mast cells. Osteoclasts were detected using histochemical staining for tartrate-resistant acid phosphatase activity.³⁷ Sections were counterstained with aniline blue (0.1%, w/v). For detection of macrophages and neutrophils, anti-mouse CD11b (M1/70) and rat anti-mouse Ly6G (RB6-8C5) antibodies were used as primary reagents.³⁸ Either streptavidin or goat anti-mouse peroxidase was used for detection. Diaminobenzidine staining was performed according to established procedures. Eosinophils were identified by staining cyanide-resistant eosinophil peroxidase activity.³⁰

Cytokine Production Assays

Mice immunized with PNiPAAm-CII were used to measure serum IL-1 β , IL-4, IFN- γ , and IL-17 levels. IFN- γ levels were determined by coating microtiter plates with anti-IFN- γ (R46-A2) in PBS overnight. After blocking with bovine serum albumin (1%, w/v) in PBS, supernatant was added to these plates. Biotinylated anti-IFN- γ -bio (AN18) was used as secondary antibody. Serum IL-4 (using 11B11 and BVD6-24G2-bio; BD Biosciences), IL-17 (using TC11-18H10 and TCH11-8H4.1-bio, BD Biosciences) and TNF- α (using purified rat anti-mouse TNF- α and biotinylated rat anti-mouse TNF- α ; BD Biosciences) levels were measured by ELISA similar to IFN- γ . Recombinant cytokines were used as standards. IL-1 β levels were measured using the assay kit supplied by eBioscience.

Neutralization of IL-5 *in Vivo*

IL-5 was neutralized *in vivo* by intraperitoneal injection of protein G purified rat monoclonal antibody, TRFK-5 as follows: days -2, 5, and 18 before/after CII immunization with 50 μ g of TRFK-5 mixed with 50 μ g of affinity purified normal rat serum IgG (control antibody). The control mice received the equivalent amount of control antibody alone.

Statistical Analyses

For calculation of arthritis susceptibility and severity, all of the mice were used. The severity of arthritis was analyzed by Mann-Whitney *U*-test and the incidence by Chi Square test using the Statview 5.0.1 version (SAS Institute, NC). Significance was considered when *P* < 0.05, for a 95% confidence interval.

Figure 1. PNIPAAm-enhanced serum cytokine production. Groups of (BALB/c × B10.Q) F1 mice ($n = 15$) were immunized with 100 μ g of rat CII emulsified with complete Freund's adjuvant (CFA-CII) or incomplete Freund's adjuvant (IFA-CII) or mixed with PNIPAAm. Sera collected on days 10 and 19 were analyzed for various cytokines, IFN- γ (A), IL-4 (B), IL-17 (C), and IL-1 β (D) as described in *Materials and Methods*. CII without any adjuvant induced low level of IL-17 (0–2.2 ng/mL) but no detectable level of other cytokines (IFN- γ , IL-4, and IL-1 β). Similarly, TNF- α levels were undetectable in all of the groups during this early phase of CIA (days 10 and 19). * $P < 0.05$; *** $P < 0.001$. Error bars denote $\pm$ SEM; n indicates number of mice in each group.

Results

Immune Response to CII and Poly(N-Isopropylacrylamide)

Poly(*N*-isopropylacrylamide) was synthesized through free radical polymerization, having weight-average molecular weight of 120 kDa. Antigen-specific immune responses were observed when the synthetic polymer of *N*-isopropylacrylamide was used as an adjuvant.²⁵ To understand the actual immune responses to an autoantigen, we immunized mice with CII mixed with PNIPAAm devoid of any classical adjuvant. We measured serum IFN- γ , IL-4, and IL-17 levels as an indicator of activation of three major T-helper cell populations (Th1, Th2, and Th17). Interestingly, all these three cytokines were found to be elevated in PNIPAAm adjuvant group (Figure 1 A–C). Similarly, we measured serum IL-1 β level as a read out for the possible involvement of inflammasome pathway when PNIPAAm was used as an adjuvant and found enhanced IL-1 β production in mice after PNIPAAm-CII immunization similar to Freund's adjuvant groups (Figure 1D). However, TNF- α levels in all of the groups during this early phase of CIA (days 10 and 19) were undetectable, suggesting the possibility of different induction kinetics for TNF- α production in these mice. Notably, significant TNF- α expression was reported in CIA mice during or after arthritis onset^{39,40} but very low levels of TNF- α were detected in CIA mouse joints between days 4 and 15⁴¹

and in QB mice, the mean day of disease onset was day 57 ± 2.7 .⁴²

Influence of Toll-Like Receptor Pathway

To understand further whether PNIPAAm adjuvant acts via the TLR pathway, we first tested TLR4^{del} mice introgressed with H-2^d haplotype. TLR4^{del} mice developed significant arthritis compared to that in WT littermate controls (Figure 2A). Despite the arthritis development, the anti-CII antibody response was low (Figure 2B), which might explain the delayed arthritis onset in TLR4^{del} mice. However, severe arthritis in TLR4^{del} mice raised the question as to whether the PNIPAAm acts as a ligand for any other toll-like receptors besides TLR4. Because all of the TLR knock-out mice are in C57Bl/6 background, we decided to check anti-CII antibody response in these mice after PNIPAAm-CII immunization. As shown in Figure 2C, all of the TLR knock-out mice including MyD88-deficient mice developed an anti-CII response.

Genetic Heterogeneity

To induce arthritis in the widely used animal model (CIA) for rheumatoid arthritis, mice are injected with either homologous or heterologous CII emulsified in complete or incomplete Freund's adjuvant. However, these adjuvants often tend to strongly change the ensuing immune response, thereby precluding our understanding of the ac-

Figure 2. Influence of TLRs on PNIpAAm-CII-induced arthritis. Groups of TLR4 deleted ($n = 8$) and WT ($n = 10$) male mice on B10.Q genetic background were immunized with 100 μ g of PNIpAAm-CII on day 0 and boosted on day 35 with 50 μ g of PNIpAAm-CII. Arthritis incidence was 88% in TLR4 deficient and 20% in wild-type littermate controls. Female mice (7–11 mice per group) did not show any significant arthritis development (data not shown). Mean clinical score of arthritis severity (A) and anti-CII IgG serum levels (B) are shown. A group of different toll-like receptor (TLR)-deficient mice on C57Bl/6 genetic background were injected with 100 μ g of PNIpAAm-CII on day 0 and boosted on day 21 with 50 μ g of PNIpAAm-CII. Serum samples collected at day 40 were used to measure anti-CII antibody levels (C). PNIpAAm alone injected mice showed negligible level of anti-CII antibody response (data not shown). Mice deficient in TLR1 ($n = 9$), TLR2 ($n = 10$), TLR3 ($n = 16$), TLR5 ($n = 9$), TLR6 ($n = 10$), TLR7 ($n = 15$), TLR9 ($n = 10$), or the adaptor molecule MyD88 ($n = 8$) were used in this experiment. Affinity purified anti-CII IgG antibodies were used as standard. Error bars indicate $\pm$ SEM.

tual immune response to the self-protein. Because we observed the adjuvant property of the synthetic PNIpAAm in B10.RIII mice,²⁵ we tested a series of inbred mouse strains for arthritis susceptibility with the polymeric adjuvant and CII to re-evaluate the MHC restriction of CII-induced arthritis. Many of the strains developed highly frequent and severe arthritis (Figure 3A and B) with a pattern similar to classical CIA^{3,7,43}; that is, mice with the MHC haplotype (H-2^q) were highly susceptible (cumulative incidence of arthritis in H-2^q versus other MHC haplotypes, $P < 0.0001$), with a more severe arthritis phenotype (mean maximal score, $P < 0.0001$) and a robust adaptive immune response (Figure 3C), whereas mice with other MHC haplotypes (H-2^p, b, d) were resistant, with low anti-CII antibody levels (d35, $P < 0.05$ and d70, $P <$

0.0001). Among H-2^q carrying mouse strains, we found two groups of PNIpAAm-CII-induced arthritis responders, high (C3H.Q and QD) and moderate (B10.Q, DBA/1 and QB). Thus, a strong MHC restriction was clearly apparent even in the absence of any classical adjuvant, which re-establishes the true MHC association of CIA. C57Bl/6 mice were earlier shown to develop arthritis after immunization with CII and CFA; but in the present study, they did not develop arthritis with PNIpAAm-CII immunization, which clearly demonstrates the absolute requirement for a classical adjuvant to induce arthritis in these mice. Furthermore, as shown in Figure 3 A and B, inject-

Figure 3. Genetic influence on PNIpAAm-CII susceptibility. Groups of mice from several different strains were injected with 100 μ g of PNIpAAm-CII on day 0 and boosted on day 35 with 50 μ g of PNIpAAm-CII. Incidence (A) of arthritis (percentage of affected mice) and arthritis severity (B) are shown. B10.Q (H-2^q; $n = 25$), DBA/1 (H-2^q; $n = 26$), C3H.Q (H-2^q; $n = 13$), QD (H-2^q; $n = 18$), QB (H-2^{q/d}; $n = 19$), B10.P (H-2^p; $n = 25$), C57Bl/6 (H-2^b; $n = 22$), and BALB/c (H-2^d; $n = 15$) mice were used in this experiment. Serum anti-CII IgG levels (C) from several different mouse strains at days 35 and 70. Mice with MHC haplotype (H-2^q) were highly susceptible (cumulative incidence of arthritis in H-2^q versus other MHC haplotypes, $P < 0.0001$) with a more severe arthritis phenotype (mean maximal score, $P < 0.0001$) and a robust adaptive immune response (C) whereas other MHC haplotypes (H-2^p, b, d) were arthritis resistant with low anti-CII antibody levels (day 35, $P < 0.05$ and day 70, $P < 0.0001$). Injecting polymer alone or with ovalbumin did not induce arthritis in the highly arthritis-prone QD mice. Pooled sera from PNIpAAm-CII mice were used as standard. Error bar indicates $\pm$ SEM; n indicates the number of mice in each group. * $P < 0.05$ and **** $P < 0.0001$.

Figure 4. Presence of a low oxidative environment significantly enhanced PNiPAAm-CII arthritis. Groups of *Ncf1* mutated (*Ncf1*^{-/-}; *n* = 17) and wild-type (WT; *n* = 16) littermate controls were immunized at the base of the tail with 100 μ g of PNiPAAm-CII on day 0 and boosted on day 35 with 50 μ g of PNiPAAm-CII. Incidence (A) of arthritis (percentage of affected mice), mean clinical score of arthritis severity (B), and serum anti-CII IgG (C) and IgG subclass (D) levels in *Ncf1*^{-/-} and WT mice at days 35 and 70 are shown. Affinity purified anti-CII IgG antibodies and pooled sera from PNiPAAm-CII mice were used as standards. Error bars indicate $\pm$ SEM; *n* denotes number of mice in each group. **P* < 0.05 and *****P* < 0.0001.

ing polymer alone or with ovalbumin did not induce arthritis in the highly arthritis-prone QD mice.

Low Oxidation Status Significantly Increases PNiPAAm-CII Induced Arthritis

The presence of low oxidative environment significantly enhanced both incidence and severity of arthritis and autoimmunity (Figure 4 A–D). Reactive oxygen species (ROS) are important in infectious immunity, but they can also play a pivotal role in the regulation of inflammatory mechanisms.⁴⁴ Reduced ROS production due to a polymorphism in the neutrophil cytosolic factor 1 (*Ncf1*) gene is associated with autoimmunity and arthritis severity in murine models with arthritis induced by CII emulsified in CFA adjuvant.²⁸ Hence, we tested *Ncf1* mutated mice with CII and PNiPAAm. Of the *Ncf1* mutated mice, 90%

developed severe arthritis (Figure 4 A and B) with a high level of anti-CII antibody response (Figure 4C), but there was no preference for any particular IgG subclass (Figure 4D). Histological examination of paws from these *Ncf1* mutated mice with safranin staining showed an extensive damage to cartilage and bone architecture compared to littermate controls (Figure 5 A and B). Similarly, we observed depletion of proteoglycan from the joint cartilage (Figure 5 C and D). Cellular infiltration was extensive in the inflamed joints that comprised of macrophages (Figure 5 E and F), neutrophils (Figure 5 G and H), and eosinophils (Figure 5 I and J), but mast cells were conspicuously absent (Figure 5 A and B). Osteoclasts were abundant in the joint (Figure 5 K and L) of the *Ncf1* mutated mice, which demonstrated the ongoing destructive process in joint cartilage.

CII-Specific V β 12T Cells Significantly Promote PNiPAAm-CII-Induced Arthritis

We have also used a TCR transgenic mouse with a V β 12 chain that has an increase in the frequency of CII-reactive cells, which recognizes the immunodominant galactosylated CII260-270 epitope. In these mice, arthritis develops after CII immunization without any requirement for an adjuvant.³⁰ Since the transgenic T cells in the V β 12 transgenic mice do still freely rearrange their endogenous TCR chain, only 5% of the T-cell repertoire recognizes CII, and the conditions of T-cell development and priming in this mouse are thus still relatively physiological.³⁰ To find out whether PNiPAAm-CII immunization can lead to a chronic progressive inflammation in the presence of CII-specific T cells, we immunized V β 12 transgenic and littermate controls. As shown in Figure 6A–C, V β 12 transgene-positive mice had a significantly higher incidence and severity of arthritis, and a greater anti-CII antibody response after PNiPAAm-CII immunization compared with the response in V β 12 transgene-negative littermate controls. However, we did not find a clear distinction of infiltrating cells in the inflamed joints of V β 12 transgene positive and negative mice (data not shown). Because adjuvant-free arthritis induction in V β 12 mice is dependent on IL-5-mediated inflammation,³⁰ we decided to test the role of IL-5 in PNiPAAm-CII-induced arthritis. To do that, we neutralized this cytokine *in vivo* with the blocking antibody TRFK-5. However, as shown in Figure 6 D and E, blocking IL-5 *in vivo* reduced neither the incidence nor the severity of the disease in the V β 12 transgenic mice significantly, showing that this cytokine has no critical role in this disease model.

Discussion

We established and characterized a new toll-like receptor (TLR)-independent arthritis murine model using collagen type II and a thermo-responsive synthetic polymer, poly(*N*-isopropylacrylamide); PNiPAAm. Biodegradable, biocompatible, and stimuli-responsive (smart) polymers have several advantages over conventional adjuvants used for development of disease models: for

Figure 5. Histology of paws from PNIPAAm-CII-immunized *Ncf1* and wild-type littermate control mice. Paws were taken from PNIPAAm-CII-immunized mice on day 62. Safranin staining for joint morphology and mast cells (**A** and **B**), and toluidine staining for proteoglycan depletion (**C** and **D**) from *Ncf1* and WT mice. Stained for RB6+ cells (**E** and **F**), Mac1+ cells (**G** and **H**), cyanide-resistant eosinophil peroxidase activity (**I** and **J**), and tartarate-resistant acid phosphatase activity (**K** and **L**) in *Ncf1* and WT mouse paws. Surrounding tissues of the joints showing the nature of infiltrating immune cells in the inflamed paw. Results shown are representative of those obtained from three to four mice in each group. Original magnifications, $\times 20$.

example, slow release of antigen over an extended period of time, the possibility for modifications using polymer chemistry, incorporation of other immunomodulatory molecules or motifs, and elimination of potentially reactive antigenic or allergenic epitopes. Moreover, synthetic polymers do not generate antibodies against themselves, but may stimulate the immune system toward other antigens, thereby acting as immunostimulators.⁴⁵ PNIPAAm is a thermo-responsive polymer having a cloud point of $\sim 32.5^{\circ}\text{C}$. Above this temperature, polymer becomes insoluble as a precipitate, and below this temperature,

polymer is in solution form. CII was mixed with the solution form of PNIPAAm and used for immunization of mice at room temperature ($\sim 25^{\circ}\text{C}$). At that temperature, PNIPAAm with CII is in solution form, and inside the body, it becomes precipitate and retains CII within polymer aggregate. Therefore, we used this useful property of PNIPAAm to carry and release antigen. PNIPAAm binds to protein mostly noncovalently and releases the protein due to weak hydrophobic interactions. Recently we evaluated the adjuvant property of PNIPAAm having *Mw* 120 kDa in mice using two different antigens.²⁵ When the CII

Figure 6. CII-specific T cells significantly enhanced PNIpAAm-CII arthritis. Groups of Vb12 transgenic (Vb12 tg; $n = 12$) and nontransgenic littermate controls (WT; $n = 21$) littermate controls were immunized at the base of the tail with 100 μg of PNIpAAm-CII on day 0 and boosted on day 35 with 50 μg of PNIpAAm-CII. Incidence (A) of arthritis (percentage of affected mice), mean clinical score of arthritis severity (B), serum anti-CII IgG (C) levels in Vb12 transgenic and nontransgenic littermate controls at days 35 and 70 are shown. Affinity purified anti-CII IgG antibodies were used as standard. IL-5 was blocked *in vivo* to address its role in PNIpAAm-CII-induced arthritis. Anti-IL-5 treatment in Vb12tg mice did not have any significant role in arthritis incidence (D) or severity (E). Vb12tg DBA/1 mice ($n = 9$ per group) was injected three times with a mixture of the IL-5–blocking antibody 50 μg of TRFK-5 mixed with 50 μg of affinity purified normal rat serum IgG or with the equivalent amount of pure normal rat IgG as control at days -2 , 5 , and 18 . At day 0, all mice were immunized with 100 μg of PNIpAAm-CII at the base of the tail. Mice were clinically scored for arthritis until day 32. Error bars indicate $\pm$ SEM; n denotes number of mice in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

was mixed with PNIpAAm, most of the CII protein retained its native configuration. We tested the adjuvant efficiency of PNIpAAm with molecular weights of 70 and 120 kDa, and found that physical adsorption and high molecular weight (120 kDa) form of moderately hydrophobic PNIpAAm seems to be more optimal to induce a significant adaptive immune response.²⁵ Although PNIpAAm does not have any known pathogen-associated molecular pattern (PAMP) ligands, it still induces a broad range of cytokine response (IL-1 β , IFN- γ , IL-4, and IL-17). Thus,

PNIpAAm offers opportunities to study the actual autoimmune responses to self-protein(s).

Activation via toll-like receptor(s) leads to immune responses to nonself structures, and many adjuvants used today in vaccinations are developed to mimic TLR ligands. At first, we used TLR4-deficient mice with H2^d haplotype to determine whether PNIpAAm-CII immunization could induce an adaptive immune response. To our surprise, these mice developed a CII-specific antibody response and severe arthritis, although with a delayed day of onset. Blocking the TLR4 with a receptor antagonist suppressed CII-induced erosive arthritis in DBA/1 mice.⁴⁶ In contrast, neither arthritis severity nor incidence was affected by TLR4 deficiency when TLR4-deficient B10.Q mice were immunized with CII and Freund's adjuvant (Kelkka et al, unpublished data). In the present study, however, we found immunization of TLR4-deficient B10.Q mice with PNIpAAm-CII-induced arthritis, suggesting that other TLRs, pathogen-recognition receptors other than TLRs, or the genetic backgrounds of the mice might have played an important role in PNIpAAm-CII-induced arthritis in TLR4-deficient mice. Hence, more in-depth studies are needed to understand this observation. However, to understand the TLR dependency of PNIpAAm adjuvant, we further tested all of the TLR-deficient mice and the adaptor protein MyD88-deficient mice for anti-CII response after PNIpAAm-CII immunization. Interestingly, all of the TLR and MyD88-deficient mice developed an anti-CII response on CII-polymer immunization, which clearly demonstrated that TLRs are redundant in this model of arthritis. Although innate immune signals mediated by TLRs help to control B cell responses,^{47,48} adjuvant enhanced antibody responses in the absence of TLR signaling have clearly been demonstrated,⁴⁹ and B cell-intrinsic TLR signals are not required for antibody production or maintenance.⁵⁰ These findings suggest that several families of pathogen-recognition receptors other than TLRs (eg, C-type lectin receptors, NOD-like receptors, and RIG-I-like receptors) are also likely to be involved in an adaptive immune response.⁵¹ In this context, it is of interest to note that PNIpAAm polymers that did not require TLRs for antibody synthesis might clearly be useful in vaccine formulations to circumvent common side effects associated with adjuvant-activated TLRs.

Recent studies have also shown that the commonly used adjuvant alum induced the release of IL-1 β , IL-18, and IL-33^{52,53} that is mediated by NALP3.⁵⁴ Hence, to check whether adjuvancy of PNIpAAm also involves the inflammasome pathway, we analyzed IL-1 β levels in the sera from PNIpAAm-CII-immunized mice and compared it with CII emulsified with Freund's adjuvant(s). PNIpAAm-CII immunization induced a level of IL-1 β production comparable to that of Freund's adjuvant groups, suggesting the possible involvement of this inflammasome pathway when PNIpAAm was used as an adjuvant. Because we observed induction of all of the major IgG subclasses when PNIpAAm was used as an adjuvant, we measured the serum IFN- γ , IL-4, and IL-17 levels as an indicator for activation of all of the three major T-helper cell populations.⁵⁵ Interestingly, we found that levels of all

three of these cytokines in PNiPAAm-CII-immunized mice were enhanced. Thus, biocompatible and biodegradable PNiPAAm can be used to study autoimmune responses without any major deviation toward any one type of immune spectrum.

Because the polymer is synthetic and has a defined structure with no strong bias in immune stimulation, we readdressed the role of MHC restriction in murine arthritis and autoimmunity using the polymer as an adjuvant. CIA susceptibility is linked to the expression of certain MHC class II alleles^{1,2} with H2^a and H2^r haplotypes being the most arthritis permissive.^{3,4} C57Bl/6 mice with H2^b haplotype have also been shown to develop arthritis,⁵ but influence of the adjuvants on arthritis development in these mice has not been clarified. Hence, we used PNiPAAm to re-evaluate the MHC association in CIA in the absence of any classical adjuvant. We found PNiPAAm-CII immunization led to a significant development of an adaptive immune response against CII, leading in turn to development of severe arthritis in various mouse strains. Interestingly, H2^a mice, not H-2^b mice, are susceptible to arthritis. Earlier we observed the development of arthritis in B10.RIII (H2^r) mice.²⁵ Taken together, MHC restriction of arthritis development in CIA has now been clearly established, and the absolute requirement for a classical adjuvant for arthritis induction in H-2^b mice is now well emphasized.

Earlier we identified *Ncf1* as an arthritis susceptibility gene in rats and mice,^{27,28} which provided evidence for the importance of the dysfunctional NADPH oxidase complex and reduced reactive oxygen species (ROS) production in arthritis development. The *Ncf1* protein (also known as p47^{phox}) is an essential organizing subunit of the NADPH oxidase complex that is responsible for the production of ROS in phagocytic cells (NOX2). Lack of oxygen radicals promotes inflammation by breaking T-cell tolerance to CII,²⁹ and the inflammation is probably mediated via CD68⁺ cells during the process of antigen presentation,⁵⁶ which in turn could affect APC-T-cell interactions and the resulting T-cell priming and activation.^{27,57} We also reported that *Ncf1*-associated, reduced oxidative environment promotes IL33⁺-dependent, T cell-mediated, adjuvant-free arthritis in mice.⁵⁸ In the present study, we found that PNiPAAm-CII immunization led to significantly enhanced incidence and severity of arthritis in the *Ncf1* mutated mice compared with wild-type littermate controls. Whether this increased arthritis development in these mice was caused by the presence of CD68⁺ cells and/or IL33⁺ T cells needs to be explored further. We did not, however, find any preponderance for one particular IgG subclass in PNiPAAm-CII "immunized" *Ncf1* mutated mice suggesting the activation of all of the T-cell helper populations, as has been observed in ROS-deficient mice, with genetic ablation of *Ncf1*.⁵⁹ Interestingly, we found all of the effector cell phenotypes (neutrophils, macrophages, eosinophils, and osteoclasts) among the infiltrating cells in the arthritic joints of PNiPAAm-CII-immunized *Ncf1* mutated mice, demonstrating active ongoing destruction processes in the articular cartilage. However, we could not find, among the infiltrating cells, any significant number of mast cells that

were also shown to be of importance in inflammatory arthritis.⁶⁰ Interestingly, there was no difference in clinical and microscopic arthritis induced with PNiPAAm-CII (in the present study) and with CFA-CII-induced arthritis in *Ncf1*-mutated mice (^{28,56} and Ibanez et al, unpublished data). This could be due to the very strong influence of reduced reactive oxygen species (ROS) levels on T cells and antigen-presenting cells.⁵⁶

To address the question as to whether the presence of CII-specific T-cell environment enhanced PNiPAAm-CII-immunized arthritis induction, as has been earlier shown in the adjuvant-free CII model,^{30,58} we used V β 12 transgenic mice. Indeed, we saw increased arthritis incidence and susceptibility in PNiPAAm-CII-immunized V β 12 transgenic mice compared with V β 12 negative littermate controls, but there was no clear difference between the infiltrating cells in the arthritic joints between these two groups of mice (data not shown). Furthermore, contrary to the IL-5- and eosinophil-dependent V β 12 transgenic adjuvant-free arthritis model,^{30,58} there was no difference in arthritis incidence and severity after IL-5 depletion in these mice. These observations suggest the involvement of a disease pathway other than eosinophilic inflammation operating in PNiPAAm-CII-immunized V β 12 transgenic mice.

PNiPAAm is a temperature-sensitive linear chain polymer with known molecular weight and chemically defined structure, whereas Freund's adjuvant is a complex mixture of nonmetabolizable mineral oil with/without mycobacterium content. PNiPAAm acts mainly through a depot effect (slow release of antigen),²⁵ and this effect is achieved at a temperature above cloud point (body temperature), whereas mineral oil in Freund's adjuvant is responsible for emulsion formation. Modifications to enhance adjuvanticity and shelf-life using polymer chemistry, formation of micro-/nanoparticles for better and specific antigen/vaccine delivery, unbiased immune response, and simplicity in preparation methods are some of the advantages of PNiPAAm over Freund's adjuvant. Thus, biocompatible and biodegradable PNiPAAm offers unique opportunities to study actual autoimmune responses to self-protein(s) in several experimental conditions. We have re-established the MHC restriction of CIA in the absence of any classical adjuvant. The presence of low oxidative environment or increased number of CII-specific T cells had significantly enhanced the incidence and severity of arthritis, and autoimmunity. Cellular infiltrates in the inflamed articular joints demonstrate the activation of several effector cell populations, resulting in the damage to the joint and bone architecture.

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