A Glucose-6-Phosphate Isomerase Peptide Induces T and B Cell–Dependent Chronic Arthritis in C57BL/10 Mice

Arthritis without Reactive Oxygen Species and Complement

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Immunization with human glucose-6-phosphate isomerase (hG6PI) protein or with several of its peptides induces arthritis in DBA/1 mice. We investigated G6PI peptide–induced arthritis in C57BL/10 mice and the effect of oxidative burst on disease. To study the arthritogenicity of G6PI peptides and its immune dependency, we used genetically modified and congenic mice on the C57BL/10 background and in vitro T- and B-cell assays. hG6PI325–339 peptide induced arthritis in C57BL/10 mice. The disease was associated with major histocompatibility complex class II and was dependent on T cells, B cells, and complement C5. Th1 and Th17 cells primed with the hG6PI325–339 peptide cross-reacted with the murine G6PI protein. The severity of the disease increased in mice carrying a mutation in Ncf1 (Ncf1*/*), which abolishes the NADPH oxidase 2 complex oxidative burst. Ncf1*/* mice developed arthritis also on immunization with the mouse G6PI325–339 peptide and in the absence of C5. The antibody responses to the G6PI protein and peptides were minimal in both Ncf1*/* and wild-type mice. Herein is described G6PI peptide as the first peptide to induce arthritis in C57BL/10 mice. The differences between the wild-type and Ncf1*/* mice suggest that an alternative complement-independent arthritogenic pathway could be operative in the absence of oxidative burst. (Am J Pathol 2013, 183: 1144–1155; http://dx.doi.org/10.1016/j.ajpath.2013.06.019)

Glucose-6-phosphate isomerase (G6PI) is a ubiquitously expressed cytosolic enzyme that has an essential role in glycolysis. It can also be secreted and function as an extracellular signaling molecule, in which context it is known as tumor autocrine mobility factor, neuroleukine, or maturation factor.¹

G6PI became relevant in arthritis research when it was found that T-cell receptor (TCR) transgenic K/BxN mice on a mixed non-obese diabetic (NOD) and B6 background developed spontaneous arthritis.² TCR transgenic T cells activated B cells to produce arthritogenic antibodies²,³ that bound to the G6PI deposited on the cartilage surface of the joints.¹ B cells contributed to the disease by producing antibodies and presenting the antigen to T cells.⁵

Immunization with human recombinant G6PI protein induced arthritis in DBA/1,⁶ C3H.Q, and C57BL/10.Q (B10.Q) mice.⁷ All of these strains carried major histocompatibility complex class II (MHC II) of haplotype q, which together with the genetic background determined the outcome of the disease.⁷ Using published information about arthritogenic T-cell epitopes in DBA/1 mice and predicting the binding motif to Aq molecules, Iwanami et al⁸ selected G6PI peptides that were thought to bind Aq molecules. One of the peptides, human (h) G6PI325–339, induced arthritis in (B10.Q) mice.⁷ Supported by the Swedish Research Council, the K.A. Wallenberg Foundation, the European Community Framework Programme under grant agreements NEURINOX (Health-F2-2011-278611) and MASTER-SWITCH (Health-F2-2008-223404), and the Innovative Medicine Initiative program Be the Cure.

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congenic C57BL/10.Q/rhd (B10.Q) mice express MHC II fragment from the P/J strain on chromosome 17; MHC II expressed MHC II H2-Ar encoded by a congenic fragment from chromosome 17; and C57BL/10.RIII/rhd (B10.RIII) mice a mutated the RIII strain on chromosome 17. B10.Q mice with have been described previously.10,11 B10.Q defective C5 molecule.12 The mice were kept and bred in generations. Congenic B10.Q.C5.NOD mice contain a fragment C5. We also tested hG6PI325-339 peptide Using congenic and knockout mice, we found that the disease was the most effe...DBA/1 mice in an IL-17—dependent manner.8 Bruns et al9 confirmed this discovery in a hypothesis-free screening of the full G6PI protein and found two additional G6PI peptides that induced arthritis in DBA/1 mice.

We tested all of these peptides in B10.Q mice to determine whether peptide—induced arthritis depended on similar pathogenic pathways as other protein—induced arthritis models such as collagen—induced arthritis (CIA) and G6PI protein—induced arthritis. We found that hG6PI1325—339 peptide was the most efficient inducer of arthritis in B10.Q mice. Using congenic and knockout mice, we found that the disease was dependent on MHC II, T and B cells, and complement factor C5. We also tested hG6PI1325—339 peptide—induced arthritis on a mutated strain of B10.Q mice, which was previously found in this laboratory to be susceptible to CIA and other autoimmune disease models.10 These mice carry a mutation in the Ncf1 gene (B10.Q.Ncf1+/−, named Ncf1+/−/ mice), which leads to a defective NADPH oxidase 2 (NOX2) complex—dependent oxidative burst.11 Ncf1+/−/ mice developed severe arthritis after immunization with hG6PI1325—339 peptide and the corresponding homologous self—peptide (murine G6PI1325—339 peptide) and induced activation of autoreactive T cells. Contrary to B10.Q mice, Ncf1+/−/ mice developed hG6PI1325—339 peptide—induced arthritis without complement C5. The induced antibody response in both Ncf1+/−/ and wild—type (WT) mice was minimal, with barely detectable response to the G6PI protein and peptides. Thus, we established a new peptide—induced arthritis model operating through a T and B cell—dependent pathway but without antibody effector mechanisms.

Materials and Methods

Animals

All mice used were genetically controlled and shared the C57BL/10 background. The MHC congenic C57BL/10.P/rhd, C57BL/10.Q/rhd, and C57BL/10.RIII/rhd strains originated from the Jan Klein mouse colony (Tübingen, Germany) and were maintained in our laboratory. C57BL/10.P/rhd (B10.P) mice express MHC II H2—A8 encoded by a congenic fragment from the P/J strain on chromosome 17; MHC II congenic C57BL/10.Q/rhd (B10.Q) mice express MHC II H2—A8 encoded by a fragment from the DBA/1 strain on chromosome 17; and C57BL/10.RIII/rhd (B10.RIII) mice express MHC II H2—A8 encoded by a congenic fragment from the RIII strain on chromosome 17. B10.Q mice with a mutated Ncf1 mutation (Ncf1m1Jm1J, denoted as Ncf1m7m) have been described previously.10,11 B10.Q μMT mice were generated by backcrossing the original μMT founder to B10.Q mice for >10 generations. TCR knockout animals (Tcrbim1Jm1J;mom) were purchased from The Jackson Laboratory (Bar Harbor, ME) and backcrossed to B10.Q mice for >10 generations. Congenic B10.Q.C5.NOD mice contain a fragment from NOD.Q mice on chromosome 2 that harbors the defective C5 molecule.12 The mice were kept and bred in a climate—controlled specific pathogen—free (Federation of Laboratory Animal Science Associations II) environment with 12—hour light—dark cycles, housed in polystyrene cages containing wood shavings, and provided with standard rodent chow and water ad libitum in the animal house of the Division of Medical Inflammation Research, Karolinska Institutet (Stockholm, Sweden). All experiments were performed in 8— to 10—week—old mice under standard conditions (littermates, scored blindly by A.P., mixed in cages, and age— and sex—matched). Experiments were approved by the Stockholm Ethical Committee under license Nos. M107/07, M109/07, and N66/10.

Peptides and Proteins

All peptides were purchased from Schafer—N ApS (Copenhagen, Denmark). Peptides used for arthritis induction and in vitro stimulation of T cells were dissolved in dimethyl sulfoxide (Prolabo; VWR International, Ltd., Leicestershire, UK) and 1.2 mol/L urea (Bio—Rad Laboratories, Inc., Hercules, CA). The aliquoted stocks at 21 mg/mL were stored at −80°C until use. From the stock, the peptides were then diluted in PBS or complete medium for in vitro stimulation of cells. The peptides used for MHC binding and enzyme—linked immunosorbent assay (ELISA), N—terminal biotinylated and otherwise, were dissolved in dimethyl sulfoxide, 1.2 mol/L urea, and 9 mmol/L Tris (2—carboxyethyl) phosphine at a concentration of 21 mg/mL and stored in 10—μL aliquots at −80°C until use. Recombinant human (h) and mouse (m) G6PI proteins were a gift. Rat type II collagen (CII) was purified from Swarm rat chondrosarcoma as previously described.13 Murine class II—associated invariant chain peptide (CLIP) (sequence) was diluted in PBS and stored at 4°C until use. The rat CII356—370 peptide (H—GIAGF/KGEOGKGETG—OH) was synthesized, purified, and characterized as previously described.14 Both CII and CII peptide were dissolved and stored in 0.1 mol/L acetic acid at 4°C.

Arthritis Induction

Arthritis was induced by injecting an emulsion composed of complete Freund’s adjuvant (Difco Laboratories, Inc., Detroit, MI) and 10 μg peptides or 300 μg protein diluted in PBS (about 5 mmol/L final concentration for both). The total volume of emulsion injected was 150 μL, divided into two intradermal injections of 75 μL each at the two sides of the base of the tail. Arthritis development was monitored using a macroscopic scoring system; one point was given for each swollen or red toe or joint, and five points for a swollen ankle or wrist, for a maximum score of 60 points per mouse.

Histology

At day 30, paws were dissected and fixed in 4% paraformaldehyde and decalcified in solution containing EDTA, polyvinylpyrrolidone, Tris HCl, and potassium HCl until the
bone was dissolved. Paws were then dehydrated and embedded in paraffin. Sections (5 µm thick) were cut and stained with H&E.

In Vitro Stimulation

Lymph nodes were conferred to a single cell suspension; 10^9 cells per well were plated in 96-well cell culture plates (Nunc; Thermo Fisher Scientific, Inc., Waltham, MA) and cultured for 96 hours in Dulbecco’s modified Eagle’s medium (Gibco; Life Technologies Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, GE Healthcare Bio-Sciences Corp., Piscataway, NJ), 10 mmol/L HEPES, penicillin/streptomycin, and 5 and 0 µmol/L β-mercaptoethanol (Sigma, St. Louis, MO). Cells were stimulated using various concentrations of hG6P1325-339, mG6P1325-339, hG6P1 protein, or mG6P1 or were left unstimulated.

Anti-Cytokine ELISA

Anti-cytokine ELISAs were performed by coating MaxiSorp plates (Nunc) with the specific purified antibody in PBS overnight at 4°C: 5 µg/mL TC11-18H10 (BD Biosciences, San Jose, CA) for IL-17 and 5 µg/mL R46A2 for interferon (IFN)-γ. After blocking with 2% powdered fat-free milk in water and washing with PBS plus 0.1% Tween (Sigma), the supernatant from cell culture and recombinant cytokine as standard were added to the plates for 2 hours at room temperature. For IL-17 cytokine, the standard was purchased from e-Bioscience (San Diego, CA), and for IFN-γ, homemade supernatants from concanavalin A–stimulated lymph node cells were used. After washing, the biotinylated detection antibody was added for 1 hour at room temperature: 0.5 µg/mL TC11-8H4.1 (BD Biosciences) for IL-17 and 0.6 µg/mL AN18.17.26 (Mabtech AB, Nacka Strand, Sweden) for IFN-γ. After washing, the biotinylated antibody was detected using europium-labeled streptavidin diluted in assay buffer (PerkinElmer, Inc., Waltham, MA) for 30 minutes at room temperature. After a final wash, the enhancement solution (PerkinElmer) was added, and the luminescence emitted was measured using a Wallac Victor 1420 multilabel counter (Molecular Devices Corp.). The assay was performed in duplicate, and the mean value was reported.

MHC Peptide-Binding Assay

A peptide-binding assay was used to indirectly measure the affinity of binding of peptides to MHC by determining the ability of the peptides to inhibit binding of biotinylated CLIP peptide to purified soluble recombinant empty Aβ MHC molecules, as described previously.15 The mix of MHC molecules (final concentration 0.8 µmol/L), biotinylated murine CLIP peptide (final concentration 3 µmol/L), and the competitive peptide at various concentrations was incubated for 48 hours at room temperature in PBS and protease inhibitors cocktail (Complete; Roche Diagnostics) in U-shaped PP-microplates (Greiner Bio-One, Stonehouse Gloucestershire, UK). The amount of biotinylated CLIP peptide bound to MHC was then quantified via ELISA. Each mix was washed three times with PBS. The amount of protein was quantified using the DC protein assay (Bio-Rad Laboratories) according to the manufacturer’s instructions.

Avidin (Thermo Scientific Pierce Protein Biology Products, Rockford, IL) (10 µg/mL) and approximately 0.16 µmol/L recombinant mG6P1 and hG6P1 proteins were diluted in PBS and rat CII in carbonate buffer [15 mmol/L Na2CO3, 35 mmol/L NaHCO3, and 11 mmol/L mgCl2 (pH 9.6) with 1 mol/L NaOH]. The proteins were then coated onto MaxiSorp plates (Nunc) for 2 hours at 37°C. After blocking with 2% fat-free milk powder for 45 minutes at 37°C, the plates were washed in PBS and 0.1% Tween (Sigma). Biotinylated peptides were diluted to a concentration of 250 µg/mL in dimethyl sulfoxide and then further diluted in carbonate buffer to a final concentration of 0.5 µg/mL (approximately 0.27 µmol/L). The final concentrations of dimethyl sulfoxide, urea, and Tris (2-carboxymethyl) phosphine in the carbonate buffer were 0.2% v/v, 28.5 µmol/L, and 21.4 µmol/L, respectively. The biotinylated peptides or no peptide control in carbonate buffer were added to the wells coated with avidin for 2 hours at room temperature. The plates were washed, and plasma from immunized mice diluted in PBS was added to the plates at various concentrations for 2 hours at room temperature. After washing, peroxidase-conjugated anti-immunoglobulin antibodies were diluted in PBS, 0.1% bovine serum albumin, and 0.1% Tween and added to the plates for 1 hour at room temperature. To detect the antibody, horseradish peroxidase–conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added to the plates. The horseradish peroxidase was detected using ABTS [2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)] (Roche Diagnostics GmbH, Mannheim, Germany) as substrate. Absorbance was measured at 405 nm using a Wallac Victor 1420 multilabel counter (Molecular Devices Corp.). The assay was performed in duplicate, and the mean value was reported.
The Netherlands) in triplicate for 2 hours at room temperature to link the MHC molecule to the plate. After washing in PBS plus 0.1% Tween (Sigma), the biotinylated CLIP bound to MHC was detected using europium-labeled streptavidin diluted in assay buffer and enhancement solution according to the manufacturer’s instructions (PerkinElmer). The luminescence emitted was read using a Wallac Victor 1420 multilabel counter (Molecular Diagnostics).

The mean of the triplicates is given. The concentration of peptide that prevented 50% of the biotinylated CLIP from binding was defined as IC_{50} value.

**Statistical Analysis**

Statistical analysis was performed using commercially available software (PRISM version 5.0c; GraphPad).

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**Figure 1** Arthritogenicity of various hG6PI peptides in B10.0 and B10.0.Ncf1^+/+^ mice. Three previously identified arthritogenic hG6PI peptides and the full recombinant hG6PI protein were tested in B10.0 mice and B10.0.Ncf1^+/+^ mice. A–D: Incidence of disease is indicated in parenthesis in the key as number of sick animals divided by the total number of mice in the experiment. Comparisons were performed using the U test. E and F: Anti-hG6PI protein IgG and anti-rCII IgG detected in plasma of immunized animals at day 14 after immunization. For the anti-hG6PI IgG ELISA, the standard used was pooled plasma; titers are represented as arbitrary units per milliliter. In the anti-rCII IgG arm, serum with known concentration of anti-CII Ig was used as standard. * * * P < 0.05, groups connected by a bar; ** *P < 0.01; *** P < 0.001 between the anti-hG6PI IgG titers of mice immunized with the protein versus those immunized with the peptides; mice with the same genotype but different immunization were compared. No difference between the genotypes was detected. All comparisons were performed using the Kruskal-Wallis test followed by Dunn’s comparison test.

**G–J:** Representative images of healthy front paws (G and I) and arthritic back paws (H and J) of hG6PI_{325-339}–immunized mice. K–M: Histology (H&E staining) of paws from naïve and hG6PI_{325-339} peptide–immunized mice. A representative of each genotype is shown. Histologic data represent qualitative assessment of the inflammatory state of the joints. *Arrows* in L and M indicate infiltrating cells in synovium and bone. Original magnification, ×10. si, sinovium; ta, tattus; ti, tibia.
Only biological replicates are given.

Results

hG6PI325-339 Peptide—Induced Arthritis in B10.Q Mice, Which Is Enhanced in Absence of Oxidative Burst

To test whether B10.Q mice were susceptible to arthritis induced by hG6PI peptides, we selected three hG6PI peptides identified as arthritogenic in DBA/1 mice. Two peptides, hG6PI35-95 (H-FNGEKINYTEGRAVL-OH) and hG6PI469-483 (H-EGNRPTNSIVFTKLT-OH), as well as the full recombinant hG6PI protein, induced very mild disease in both B10.Q and B10.Q Ncf1-/- mice (Figure 1, A, C, and D), whereas only hG6PI325-339 peptide (H-IWYINCFGETCHAML-OH) induced disease with high severity and incidence (Figure 1, B and G–J). A similar molar concentration of protein and peptides was administered. The harmful effect of a dysfunctional NOX2-dependent oxidative burst, caused by a mutation in the Ncf1 gene (Figure 1B), was apparent only in severe hG6PI325-339 peptide—induced arthritis, consistent with previous observations in other induced and spontaneous arthritis models. No significant effect of the mutated Ncf1 was observed in arthritis induced by the G6PI protein or the G6PI peptides, inducing only mild arthritis (Figure 1, A, C, and D).

Immunization with hG6PI peptides generated only barely detectable titers of anti-hG6PI protein IgG, in contrast to immunization with G6PI protein, which induced high titers (Figure 1E). The anti-hG6PI IgG titers in individual mice were not associated with arthritis. Anti-CII antibodies are present in serum samples from many patients with rheumatoid arthritis, and their presence was checked in the G6PI-immunized animals (Figure 1F). The antibody response to CII was detectable but at low levels and did not reflect arthritis severity.

Histopathologic analysis of the paws from mice immunized with hG6PI325-339 peptide 30 days earlier showed that B10.Q Ncf1-/- mice developed severely inflamed synovium and erosive destruction of bone and cartilage (Figure 1L), whereas B10.Q mice developed mild disease with only moderate synovium infiltration (Figure 1M).

hG6PI325-339 Peptide—Induced Arthritis Is MHC Dependent

To determine the MHC association of hG6PI325-339 peptide—induced arthritis, we used congenic B10 mice expressing MHC of different haplotypes. Both B10.Q (H-2q) and B10.P (H-2p) mice were susceptible to hG6PI325-339 peptide—induced arthritis, whereas B10.RIII (H-2r) mice were resistant (Figure 2). As in the B10.Q mice, also in the susceptible B10.P strain, the Ncf1 mutation increased the severity of the disease (Figure 2, A and B). In conclusion, hG6PI325-339 peptide induces arthritis in B10 mice expressing MHC of q or p, but not r, haplotype.

Immunization with hG6PI325-339 Peptide Induces Priming of Th17 and Th1 Autoreactive T Cells

To check the antigen specificity and cytokine profile of T cells primed by immunization with hG6PI325-339 peptide, 30 days after immunization, draining lymph node cells were stimulated in vitro using the same peptide or the homologous mG6PI325-339 peptide, the full hG6PI protein, or the full mG6PI protein. Lymph node cells stimulated with hG6PI325-339 peptide produced high amounts of IL-17 and IFN-γ (Figure 3, A and B, respectively) but no IL-4 (data not shown). In contrast, very low cytokine production was detectable after repeat stimulation with mG6PI325-339 peptide (Figure 3, A and B).
hG6PI protein stimulated high cytokine production (Figure 3, C and D). mG6PI protein stimulated low but significant production of IFN-γ and IL-17 (Figure 3, C and D), indicating that immunization with hG6PI325-339 peptide leads to priming of cross-reactive T cells to the murine protein.

Compared with B10.Q cells, B10.Q.Ncf1 */* cells produced a similar level of cytokines in response to human and murine G6PI325-339 peptides and G6PI proteins (Figure 3, A–D).

Similar results were observed at days 7 and 13 after disease induction (data not shown).

B10.RIII mice were resistant to hG6PI325-339 peptide-induced arthritis (Figure 2C). Nevertheless, both Ncf1 */* and Ncf1 WT mice on a B10.RIII background mounted a moderate Th1 response to hG6PI325-339 peptide (Figure 3, E and F). B10.P mice were susceptible to hG6PI325-339 peptide-induced arthritis (Figure 2B), and their lymph node cells responded to the same peptide and to a lesser extent to the murine peptide, similarly as B10.Q cells (Figure 3, G and H). No difference between Ncf1 */* and Ncf1 WT mice was observed.

In conclusion, the susceptible B10.Q and B10.P strains could prime Th1 and Th17 cells that were specific for hG6PI325-339 peptide and cross-reacted with mG6PI protein.

**B and T Cells and Complement Are Necessary for Development of hG6PI325-339—Induced Arthritis**

To determine the dependency of hG6PI325-339-induced arthritis from the adaptive immune system, we used B10.Q

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**Figure 3** Immunization with hG6PI325-339 peptide induced priming of Th1 and Th17 cells. A–D: At 30 days after hG6PI325-339 immunization, lymph nodes from B10.Q and B10.Q.Ncf1 */* mice were stimulated in vitro using various concentrations of hG6PI325-339 peptide, mG6PI325-339 peptide, mG6PI protein, or hG6PI protein for 96 hours before quantifying IL-17 and IFN-γ in the supernatant via ELISA. *P < 0.05 and **P < 0.01 comparing human versus murine peptide or protein between the same genotypes. All comparisons are pair comparisons performed using the Kruskal-Wallis test followed by Dunn’s comparison posttest. Four or five mice per group were used. IL-17 and IFN-γ levels after stimulation with medium only were below detection limit. The experiment was repeated twice and yielded similar results. E–H: At 30 days after immunization with hG6PI325-339 peptide, inguinal lymph nodes from B10.RIII and B10.RIII.Ncf1 */* mice (E and F) or B10.P and B10.P.Ncf1 */* mice (G and H) were stimulated in vitro with 10 μmol/L human or murine G6PI325-339 peptide for 96 hours before quantifying IL-17 and IFN-γ in the supernatant via ELISA. *P < 0.05 comparing the groups as indicated by the bar using the Kruskal-Wallis test followed by Dunn’s comparison posttest. Seven or eight mice per group were used for the B10.RIII strain, and four or five mice per group for the B10.P strain.
cytokines were detected in T cell–deficient mice (Figure 5, C and D), suggesting that T cells were the major producers of IFN-γ and the exclusive producers of IL-17 in this assay.

**hG6PI_{325-339}**—Peptide Immunization Induces Low Titters of Anti-hG6PI_{325-339} Peptide IgG

Although anti-hG6PI protein IgG was detectable in low titers in plasma from hGPI_{325-339}–immunized animals (Figure 1E), anti-hG6PI_{325-339} peptide IgG titers were undetectable at both days 14 and 30 after immunization (data not shown). To ascertain the presence of anti-hG6PI_{325-339} peptide IgG, plasma from pooled immunized Ncf1/+ mice was concentrated using centrifugal filter units and tested via ELISA on congenic mice deficient in either B or T cells. Both B cell– and T cell–deficient mice on B10.Q.Ncf1/+ background demonstrated resistance to hG6PI_{325-339} peptide–induced arthritis (Figure 4, A and B).

To understand the role of complement in this disease, mice deficient in C5 due to a congenic fragment from the NOD strain on the B10.Q background were immunized with hG6PI_{325-339} peptide. Mice deficient in C5 were not susceptible to the disease, whereas C5 WT littermates were (Figure 4C). When C5-deficient mice carried the Ncf1 mutation, they were as susceptible to arthritis as were C5-sufficient Ncf1/+ mice (Figure 4D). Although B and T cells are necessary for disease in Ncf1/+ mice, C5 does not seem to be essential.

Lymph node cells from hG6PI_{325-339} peptide–immunized mice (Figure 4) were stimulated again in vitro with hG6PI_{325-339} peptide, and IL-17 and IFN-γ production were measured. T cells from B cell– and C5-deficient mice (Figure 5, A, B, E, and F) produced similar levels of cytokines as did their WT littermates, suggesting that neither B cells nor C5 are necessary for priming of hG6PI_{325-339} peptide–specific Th1 and Th17 cells. Very low levels of both

![Image](image-url)
Concentrated serum samples were tested via ELISA for anti-hG6PI protein and peptide immunoglobulin (IgG) in serum from naïve animals (Figure 6A). Similarly, very low concentrated plasma from immunized hG6PI325-339-immunized animals. Two separate pools of serum, each derived from five Ncf1+/− mice immunized with hG6PI325-339 in complete Freund's adjuvant, were concentrated using a filtration column. The same procedure was used on pooled serum from five naïve Ncf1+/− mice. Concentrated serum samples were tested via ELISA for anti-hG6PI protein and peptide immunoglobulin (A) and for anti-mG6PI protein and peptide (B). Background optical density from avidin only was 0.2, as the signal from the naïve serum toward the peptides.

plates coated with avidin and biotinylated peptides. Low anti-hG6PI325-339 peptide IgG titers were detectable in concentrated plasma from immunized Ncf1+/− mice but not in plasma from naïve animals (Figure 6A). Similarly, very low titers of anti-mG6PI325-339 peptide IgG were detectable in plasma from immunized mice but not in plasma from naïve mice (Figure 6B). Both anti-hG6PI325-339 IgG and anti-mG6PI325-339 peptide IgG were of IgG2b and IgG2c isotypes (data not shown). Low levels of anti-mG6PI protein IgG were also detected (Figure 6B).

A Single Amino Acid Difference between Human and Murine G6PI325-339 Peptide Determines Capacity to Induce Arthritis

mG6PI325-339 peptide (H-IWYINCYGCETHALL-OH) differs from human peptide by only two amino acids (underlined in the sequence). We wanted to test whether immunizing the mice with mG6PI325-339 peptide could induce arthritis. The murine peptide induced substantial but mild arthritis in B10.Q,Ncf1+/− mice and failed to induce arthritis in B10.Q mice (Figure 7, A and B). Of the two amino acids that differ between human and murine peptide, of particular interest is the amino acid in position 331, a phenylalanine (F) in the human peptide and a tyrosine (Y) in the murine peptide. A comparison with CII256-270 peptide suggests that position 331F/Y is in the P4 position, resembling 263F in CII peptide. Thus, we tested two peptides that differ only in position 331 by removing the last two amino acids from both the human and murine peptides. To further increase the solubility of the peptide and narrow down the arthritogenic region of the human peptide, we also removed the first two hydrophobic amino acids in the N-terminal. As a result of these modifications, we obtained the hG6PI326-337W326A peptide (H-AYINCYG) and the mG6PI326-337W326A peptide (H-AYINCG) (Figure 7C). hG6PI326-337W326A peptide induced severe arthritis in B10.Q,Ncf1+/− mice but only mild arthritis in B10.Q mice (Figure 7, A and B). mG6PI326-337W326A peptide induced very mild arthritis with low incidence (Figure 7, A and B). Because mG6PI326-337W326A and hG6PI326-337W326A peptides differed only in the amino acid at position 331, we concluded that the difference in arthritogenicity between the human and murine peptides relies mainly in the phenylalanine to tyrosine substitution at position 331.

In addition, the N- and C-terminal hydrophobic regions of hG6PI325-339 peptide (IWYINCYGCETHALL) influence
peptide mG6PI326-337W326A, the affinity of the CLIP to soluble Aq MHC molecules was measured by adding one of the truncated forms of hG6PI 325-339 peptide in which the amino acids at position 331 are essential to induce disease. We then evaluated the binding affinity of hG6PI 325-339 peptide to Aq MHC II using an in vitro competition assay with CLIP peptide. hG6PI325-339 peptide bound with good affinity to MHC (IC50, about 30 µmol/L), 10-fold less efficiently than CLIP (IC50, 2 µmol/L) but about 5-fold more efficiently than rCII256-270 peptide (H-GIGFKEQGPKGETG-OH) (IC50, 120 µmol/L) (Figure 8A). The truncated form of hG6PI325-339 peptide in which the hydrophobic residues at the N- and C-terminals were removed (hG6PI326-337W326A) bound MHC II with similar efficiency as did rCII, with IC50 about 110 µmol/L (Figure 8A). mG6PI325-339 peptide had a lower affinity to MHC than did the human counterpart, with IC50 about 110 µmol/L (Figure 8B). In the shortened murine peptide mG6PI326-337W326A, the affinity was even lower, with IC50 of >400 µmol/L (Figure 8B).

In conclusion, hG6PI325-339 peptide bound to murine MHC II Aq with higher affinity than did murine peptide. The hydrophobic residues at the N- and C-terminals strengthened the binding affinity. hG6PI325-339 peptide bound with higher affinity than did rCII256-270, the immunodominant epitope in CIA.

**Discussion**

Herein we characterize a mouse model of arthritis induced by immunization using a peptide from the hG6PI protein. The peptide hG6PI325-339 has previously been reported to be arthritogenic in DBA/1 mice.8,9 Whereas in DBA/1 mice more severe arthritis developed in response to the G6PI protein than to the peptide,9 in B10.Q mice the opposite occurred. That B10.Q mice were susceptible to hG6PI325-339 peptide-induced disease indicates that there was no strong tolerance in the mice against this peptide. Processing of the protein could lead to different peptide pools in B10.Q than in DBA/1 mice, ie, arthritogenic in DBA/1 mice and mildly arthritogenic in B10.Q mice. It is also possible that processing of the protein generates tolerogenic peptides in one strain but not in the other.

hG6PI325-339-induced arthritis was dependent on the MHC II haplotype; similar q and p haplotypes enabled susceptibility to the disease, but the r haplotype did not, consistent with observations for hG6PI protein—induced arthritis (Figure 2).7 Restimulated lymph node cells from hG6PI325-339 peptide-immunized B10.RIIII mice produced low amounts of IFN-γ and IL-17, which suggests that priming against the peptide occurred in vivo and that the peptide could bind to MHC of the r haplotype, consistent with the observation that immunization of B10.RIIII mice with the hG6PI protein could induce anti-hG6PI antibodies.7 Arthritis resistance of B10.RIIII mice could be due to

**Figure 8**  In vitro binding assay of hG6PI325-339 peptide and its modifications, as well as mG6PI325-339. A and B: Inhibition of binding of biotinylated CLIP to soluble Aq MHC molecules was measured by adding one of the following peptides: hG6PI325-339, hG6PI326-337, W326A, hG6PI327-337 Y327A, rCII256-270, mCLIP, mG6PI325-339, or mG6PI326-337, W327A. The assay was performed in triplicate. Values represent the means.

**Figure 9** Immunization with mG6PI325-339 peptide—induced Th1 and Th17 cells priming in B10.Q.Ncf1+/* mice. A and B: At 30 days after mG6PI325-339 immunization, inguinal lymph nodes from B10.Q (black bars) and B10.Q.Ncf1+/* (white bars) animals were stimulated in vitro with the same peptide for 96 hours before quantifying IL-17 and IFN-γ in the supernatant via ELISA. *P < 0.05 comparing the groups as indicated by the bar using the Kruskal-Wallis test followed by Dunn’s comparison posttest. Ten mice per group were used.
several mechanisms of tolerance such as induction of T regulatory cells or too weak Th1 and Th17 responses elicited by the immunization (Figure 3).

Both the q and p haplotypes are permissive of hG6PI325-339–induced arthritis. They behave differently in CIA, in which q is permissive and p is not.18 The difference between q and p haplotypes consists of only four amino acids in the β chain,19 which slightly influences the binding affinity of rCII356-270, the immunodominant peptide of CII.15 It is speculated that the lower affinity of H-2q to rCII356-270 is the molecular reason for the resistance of H-2p—bearing mice to arthritis.15 Inasmuch as H-2β binds to the hG6PI325-339 peptide with higher affinity than to rCII356-270, it is likely that H-2β also binds to the hG6PI356-339 peptide with sufficient affinity to enable development of arthritis. This is supported in that hG6PI356-339 peptide—specific T cells are primed in B10.P mice (Figure 3), indicating efficient antigen presentation of the peptide in vivo.

Insofar as MHC II binding and its relation to arthritis development, hG6PI325-339 and rCII356-270 peptides share the same residues in the same positions that are crucial for MHC II binding of rCII356-270.8,15 Iwami et al15 have suggested that the 328, 331, and 334 positions in hG6PI325-339 peptide would be the anchor amino acid of the peptide to MHC. In the hG6PI325-339 peptide, these positions correspond to isoleucine, phenylalanine, and glutamic acid. The same amino acids are found in the rCII356-270 peptide, at the same distance, and isoleucine and phenylalanine are essential for binding of the rCII356-270 peptide to MHC.15 Inasmuch as the arthritogenic hG6PI325-339 peptide and the low arthritogenic mG6PI325-339 peptide differ by only two amino acids, one in position 331, we could confirm that substitution of the phenylalanine 331 in hG6PI325-339 peptide for the tyrosine in mG6PI325-339 peptide decreased the binding affinity of the peptide to MHC. This result suggests that the difference in arthritogenicity between human and murine G6PI325-339 peptide could be explained by their different binding affinity to MHC, which is strongly influenced by the amino acid in position 331. hG6PI325-339 peptide binds MHC with higher affinity than rCII356-270 peptide does, which could explain in part why the hG6PI325-339 peptide is so strongly arthritogenic and the rCII356-270 peptide is not. As in the comparison between Aq and Ap binding to rCII356-270 peptide and also in the comparison of MHC II binding of hG6PI325-339 and mG6PI325-339 peptides, the higher affinity of MHC II and peptide is associated with arthritis development, whereas the lower affinity is associated with arthritis resistance.

Despite the sequence similarity between hG6PI325-339 and rCII356-270 peptides, no priming of anti-rCII or rCII356-270 peptide T cells could be detected in hG6PI325-339–immunized animals, as well as very low anti-CII IgG titers (Figure 1 and data not shown), which suggests that no cross-reactivity between the two peptides occurs in vivo.

Indeed, very low antibody titers against both hG6PI and rCII were measured in animals immunized with hG6PI325-339 peptide (Figure 1). Low antibody titers in hG6PI325-339 peptide—induced arthritis represent a major difference between this model and the CIA, hG6PI protein—induced and K/BxN models, where instead high titers of pathogenic anti-rCII or anti-hG6PI antibodies are detected.27,28 All four models are dependent on B cells27,28,29 and are mediated by C522–24 and antibody.3,25 Low levels of anti-hG6PI325-339 peptide antibodies can be detected in the serum of hG6PI325-339–peptide–immunized animals only when the serum is concentrated (Figure 6). Low levels of mG6PI protein have been detected as described previously in DBA/1 mice.6,8,9 It is unlikely that low levels of specific antibodies against mG6PI protein contribute to the pathogenesis in particular because serum samples containing anti-hG6PI antibodies are not arthritogenic.21 It is also possible that B cells are essential for arthritis development through functions other than antibody production such as antigen presentation, as recently described for hG6PI protein—induced arthritis,5,8,9 although it was earlier suggested that B cells are pathogenic in the G6PI model through its antibody-producing function.5

Similar to CIA, K/BxN, and hG6PI protein arthritis models, hG6PI325-339 peptide—induced arthritis is dependent on T cells.2,6 Immunization with the hG6PI325-339 peptide primed Th1 and Th17 T cells but not Th2 cells (Figure 3 and data not shown). This is consistent with the observation that IL-17 production is important in arthritis development after immunization with both hG6PI325-339 peptide and the full protein.8,26 T cells from mice immunized with hG6PI325-339 peptide responded also to the murine protein and moderately to the peptide (Figure 3 and data not shown), as observed previously in DBA/1 mice after immunization with the hG6PI325-339 peptide and the full protein.6,8,9 This suggests that other epitopes of the murine proteins could be presented to activated T cells in vivo and mediate the disease. The G6PI protein and peptide of human origin stimulated a higher response than did the murine counterparts, which suggests a higher MHC II affinity to peptides of human origin and/or limited cross-reactivity on the TCR level with the corresponding mouse peptide.

One of the major differences between B10.Q.Ncf1 */+ and Ncf1 WT mice is the capacity of Ncf1 */+ mice to prime autoreactive Th1 and Th17 cells against the autologous mG6PI325-339 peptide and therefore to develop arthritis (Figures 7 and 9). This is consistent with previous observations that tolerance to autologous proteins is more easily broken in Ncf1 */+ mice compared with Ncf1 WT mice.27,28 Ncf1 */+ macrophages, but not Ncf1 WT macrophages, can prime arthritogenic autoreactive Th1 cells and induce arthritis.29,30 In Ncf1 */+ mice, T cells produce higher amounts of IFN-γ, TNF-α,29 IL-17,31 and IL-516 on immunization. Similarly in rats, a single-nucleotide polymorphism in Ncf1 that decreases reactive oxygen species production increases arthritogenicity of the cells.32 Several T cell signaling pathways and functions are regulated by redox balance; however, their dependency on NOX2 complex—derived reactive oxygen species is not clear. Among those, the most studied are several molecules downstream of the TCR signaling pathway that have been
associated with rheumatoid arthritis and are redox-sensitive: protein phosphatases, protein tyrosine kinases, and the linker for activation of T cells. Another candidate in T cell regulation is the kynurenine pathway in the tryptophan catabolism, which suppresses T cell activation and is not functional in the absence of reactive oxygen species. In addition to modulating the activity of T effector cells, it has been shown recently how defects in Ncf1 impair development and functionality of T regulatory cells. Together, these observations corroborate the hypothesis that NOX2-dependent reactive oxygen species affects T cell activity. The precise molecular mechanisms behind this effect are still under investigation.

Compared with Ncf1 WT mice, Ncf1+/+ mice developed more severe arthritis on both B10.Q and B10.P backgrounds and were susceptible to the disease in lack of C5 (Figures 2 and 4). The latter result suggests that in Ncf1+/+ mice a different arthritogenic pathway is operative that overrules the C5-dependent pathway observed in Ncf1 WT mice, and it is T cell— and B cell-dependent. One possible explanation is that NOX2 deficiency leads to such a hyperinflammatory milieu that other pathways can efficiently replace the C5-dependent pathway. As noted in NOX2-deficient humans and rodents, leukocytes produce higher amounts of proinflammatory cytokines and lower amounts of anti-inflammatory cytokines, which lead to pathologic conditions of chronic inflammation such as autoimmune diseases. In this scenario, the possibility that other antibody-mediated pathways, such as direct Fc receptor binding, are sufficient to sustain activation of Ncf1+/+ leukocytes is still valid, and it has thus far not been tested. The dispensability of C5 in Ncf1+/+ mice also indicates how B cells may be essential for disease development through functions that are not antibody mediated. This hypothesis is strengthened by the low antibody titers observed in hG6PI325-339 peptide—induced arthritis and discussed above.

It is possible that both the antibody-dependent and antibody-independent B cell functions are active in human disease. It is therefore important to study the pathologic mechanisms in hG6PI325-339 peptide—induced arthritis in both Ncf1+/+ and Ncf1 WT mice because they can reveal different and new B cell—dependent pathways that lead to rheumatoid arthritis.

In conclusion, hG6PI325-339—induced arthritis in B10.Q mice is a T cell— and B cell—dependent disease that is exacerbated by a mutation in Ncf1. Because different B cell—dependent pathways mediate arthritis in Ncf1 mutated and WT mice, this model is valuable for understanding the various functions of B cells in the pathogenesis of arthritis.

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