# Antigen-Specific B Cells Are Required as APCs and Autoantibody-Producing Cells for Induction of Severe Autoimmune Arthritis[1]

**Shannon K. O'Neill,* Mark J. Shlomchik,§ Tibor T. Glant,† Yanxia Cao,‡ Paul D. Doodes,* and Alison Finnegan[2]*‡**

**B cells play an important role in rheumatoid arthritis, but whether they are required as autoantibody-producing cells as well as APCs has not been determined. We assessed B cell autoantibody and APC functions in a murine model of autoimmune arthritis, proteoglycan (PG)-induced arthritis, using both B cell-deficient mice and Ig-deficient mice (mIgM) mice that express an H chain transgene encoding for membrane-bound, but not secreted, IgM. The IgH transgene, when paired with endogenous λ L chain, recognizes the hapten 4-hydroxy-3-nitro-phenyl acetyl and is expressed on 1–4% of B cells. B cell-deficient and mIgM mice do not develop arthritis after immunization with PG. In adoptive transfer of PG-induced arthritis into SCID mice, T cells from mIgM mice immunized with PG were unable to transfer disease even when B cells from PG-immunized wild-type mice were provided, suggesting that the T cells were not adequately primed and that Ag-specific B cells may be required. In fact, when PG was directly targeted to the B cell Ig receptor through a conjugate of 4-hydroxy-3-nitrophenyl acetyl-PG, T cells in mIgM mice were activated and competent to transfer arthritis. Such T cells caused mild arthritis in the absence of autoantibody, demonstrating a direct pathogenic role for T cells activated by Ag-specific B cells. Transfer of arthritic serum alone induced only mild and transient arthritis. However, both autoreactive T cells and autoantibody are required to cause severe arthritis, indicating that both B cell-mediated effector pathways contribute synergistically to autoimmune disease.** ***The Journal of Immunology,*** **2005, 174: 3781–3788.**

B cells are critically required for the development of several spontaneous and induced murine models of autoimmune disease (1–14). One way that B cells contribute to autoimmune disease is through the production of autoantibodies. These autoantibodies induce disease directly and indirectly in the form of immune complexes (1, 2, 12, 14, 15). The pathogenic consequences of activated autoreactive B cells are not restricted to autoantibody production, because B cells have the capacity to promote T cell activation (16–19). The relative importance of B cells as APCs for priming naive $CD4^+$ T cells is somewhat controversial, although it has been demonstrated to be important in some experimental models (20–24). Furthermore, recent data indicate that B cells function as critical APCs for memory $CD4^+$ T cells (25). Ag-specific B cells regulate Ag processing and presentation by two separate mechanisms. One mechanism involves direct binding and internalization of Ag through the BCR (26–32) whereas a second mechanism involves the formation of immune complexes (IC)[3] and their internalization through Fc$\gamma$R expressed on dendritic cells (DC) or macrophages (33–39).

The relevance of B cells in disease pathogenesis has been shown for several experimental models of autoimmune disease, including systemic lupus erythematosus (3, 9, 16) and diabetes mellitus (5, 6, 40). Chan et al. (17) recently demonstrated that mice deficient in the secretion of circulating Ig develop lupus nephritis. These data exclude the simple explanation that B cells influence this autoimmune disease solely through the production of autoantibody, and instead suggest a potential function for B cells as APCs for T cell activation. Although these results clearly defined a critical role for B cells independent of circulating IC, they did not determine whether the Ag specificity of the B cell contributes to their APC function. In a separate model of autoimmune disease, several investigators have examined the role of B cells in the NOD mouse, and their results suggest a role for Ag-specific B cells as APCs (7, 18, 41–43). However, because B cells were still competent to secrete Ab, IC-mediated Ag presentation by DCs or macrophages could have contributed to the level and type of T cell priming necessary for disease.

To clarify the role of Ag-specific B cells as APCs independent of circulating ICs in autoimmune disease, we examined the requirement for Ag-specific B cells in proteoglycan (PG)-induced arthritis (PGIA) using Ig-deficient (mIgM) mice. PGIA is induced in BALB/c mice after immunization with the human PG aggrecan, resulting in clinical manifestations and histology of the diarthrodial joints with many similarities to rheumatoid arthritis (RA) (44–47). $CD4^+$ T cells are necessary for the development of PGIA (48), and the higher ratio of IFN-$\gamma$ to IL-4 indicates that these are Th1 cells

*Department of Immunology/Microbiology, †Departments of Biochemistry and of Orthopedic Surgery, Section of Biochemistry and Molecular Biology, and ‡Department of Internal Medicine, Section of Rheumatology, Rush University Medical Center, Cohn Research Building, Chicago, IL 60612; and §Department of Laboratory Medicine, Section of Immunobiology, Yale University School of Medicine, New Haven, CT 06510

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[2] Address correspondence and reprint requests to Dr. Alison Finnegan, Rush University Medical Center, 1735 West Harrison Street, MC 109, Chicago, IL 60612. E-mail address: alison_finnegan@rush.edu

[3] Abbreviations used in this paper: IC, immune complex; DC, dendritic cell; mIgM, Ig-deficient mice; NMS, normal mouse serum; NP, 4-hydroxy-3-nitro-phenyl acetyl; NP-OSu, NP succinimide ester; NP-PG, PG conjugated to NP; PG, proteoglycan; PGIA, proteoglycan-induced arthritis; RA, rheumatoid arthritis; WT, wild type; NIP, 4-hydroxy-3-iodo-5-nitrophynyl acetyl.



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(46, 49, 50). Adoptive transfer experiments demonstrate that both T cells and APCs cooperatively participate in the development of disease (51). Also, autoantibody to mouse PG arise before disease onset (52). Although arthritic serum alone did not induce disease, earlier onset of PGIA occurred when arthritic serum was concurrently transferred with lymphocytes into recipient mice (47, 51), suggesting that autoantibody contributed to arthritis inception.

In this study we demonstrate that Ag-specific B cells are indispensable as APCs for the activation of autoreactive T cells. Only when Ag-specific B cells activate T cells are they competent to induce arthritis. Importantly, these studies also reveal that autoreactive T cells and autoantibodies are required to cause severe arthritis, indicating that both aspects of B cell function contribute synergistically to autoimmune disease.

## Materials and Methods

### *Mice*

Female BALB/c and SCID mice were purchased from the National Cancer Institute. The mIgM, (m+s)IgM transgenic mice and B cell-deficient mice were bred and maintained in our animal care facility (Rush University Medical Center). B cell-deficient mice contain a homozygous deletion of the joining H chain region of Ig DNA ($J_HD$) due to a neo insertion in the $J_H$ locus and are incapable of developing mature B cells (53). B cell deficiency was validated by confirming the absence of surface $IgM^+$ and low levels of $B220^+$ spleen cells by flow cytometry; additionally, neither IgM nor IgG was detectable in the serum of B cell-deficient mice by radial immunodiffusion assays (The Binding Site).

Variable H chain ($V_H$186.2) Ig transgenic mice contain a transgene with a rearranged VDJ region ligated to an $IgM^a$ (BALB/c-derived) H chain. B cells from $V_H$186.2 transgenic mice express an H chain that can pair with any endogenous Ig L chain; however, pairing with a λ L chain confers Ig specificity for the hapten, 4-hydroxy-3-nitrophenyl acetyl (NP), in 2–4% of B cells (54). The mIgM transgenic mice were created using the $V_H$186.2 transgene containing a deletion of the DNA encoding secretion ($\mu$s) and polyadenylation sequence ($pA_s$) of the IgM H chain (17). Additionally, a transgenic control strain, referred to as (m+s)IgM, contains an intact transgene encoding for both membrane-bound and secreted IgM. The transgenic mice ($IgM^a$ transgene expression on a CB-17 background) were backcrossed to the B cell-deficient ($J_HD$/CB-17) strain to fix homozygosity of the $J_HD$ mutation, which eliminates the expression of endogenously rearranged IgH chains as previously described (54). CB-17 mice normally express $IgM^b$ on the BALB/c genetic background; thus, $IgM^a$ transgenic mice more closely resemble BALB/c animals, which were used as the control group. The mIgM mice and (m+s)IgM mice were assessed by flow cytometry and were found to contain equivalent percentages of splenic $IgM^+$ and $B220^+$ cells compared with wild-type (WT) BALB/c animals (54) (our unpublished observation). Total serum IgM levels in the (m+s)IgM mice (0.08 ± 0 mg/ml) were less than those in WT mice (0.31 ± 0.01 mg/ml), whereas mIgM mice had no detectable serum IgM by radial immunodiffusion assay (The Binding Site). Furthermore, transgenic mice, (m+s)IgM or mIgM, had significantly diminished serum IgG (0.01 ± 0.01 mg/ml) compared with nonimmune WT mice (8.4 ± 2.2 mg/ml). The $V_H$186.2 transgene was detected by PCR using the $V_H$186.2–5′ primer (5′-TGCTCTTCTTGGCAGCAAC-3′) and the $V_H$186.2–3′ primer (5′-TGAGGAGACTGTGAGAGTG-3′), as previously described (17).

### *Ag preparation*

Human cartilage was obtained after joint replacement surgery, and PG was subsequently isolated as previously described (46). Briefly, liquid nitrogen-frozen cartilage was cut into 20-$\mu$m sections and extracted with 4 M guanidium chloride in 50 mM sodium acetate, pH 5.8, containing protease inhibitors at 4°C. High buoyant density PG monomers (aggrecan) were isolated by dissociative cesium chloride gradient centrifugation, followed by sequential digestion with endo-$\beta$-galactosidase and chondroitinase ABC (Seikagaku America) overnight at 37°C. Additional purification was performed using a Sephacryl S-200 column (Pharmacia Biotech). Murine PG was isolated after obtaining cartilage from newborn mice and was prepared in a similar method to human cartilage PG, without deglycosylation.

PG was haptenated with NP succinimide ester (NP-OSu; Biosearch Technologies) by dissolving PG (measured as protein) in 0.1 M borate buffer to 5 mg/ml, pH 9.2. NP-OSu was dissolved in dimethylformamide to 10 mg/ml. To achieve a substitution ratio of ~1 PG:20 NP molecules, 11.6 $\mu$l of 10 mg/ml NP-OSu/dimethylformamide was added to 1 ml of PG (5 mg/ml) dissolved in borate buffer at 4°C and stirred overnight. The remaining free hapten was separated from the hapten-protein conjugate by dialysis against TS buffer (50 mM Tris and 150 mM NaCl).

### *Induction and assessment of arthritis*

Female mice, ≥3 mo of age, were immunized i.p. with either 150 $\mu$g of human PG or PG conjugated to NP (NP-PG) measured as protein, emulsified in CFA. Mice received booster immunizations at wk 3 and 6 with 100 $\mu$g of PG or NP-PG in IFA. Mice were monitored biweekly for signs of arthritis subsequent to the final immunization, and scoring was blinded. Paws were scored for clinical signs of arthritis on a scale from 1 to 4 as follows: 0, normal; 1, mild swelling affecting more than one digit; 2, moderate erythema and swelling of the paw; 3, more intense erythema, swelling, and redness affecting a greater proportion of the paw; and 4, severe erythema, swelling, and redness affecting the entire paw. Each animal received a cumulative score ranging from 0 to 16, based on individual paw scores of 0–4.

### *Detection of in vitro cytokine secretion by ELISA*

T cells were purified from spleens of PG- or NP-PG-immunized mice by passage through nylon wool. T cells ($2.5 \times 10^5$ cells/well) from PG- or NP-PG-immunized mice plus irradiated (2500 rad) spleen cells from naive WT mice were cultured in triplicate in 96-well Falcon plates (Fisher Scientific) in serum-free HL-1 medium, 100 $\mu$g/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM L-glutamine in the presence of PG (25 $\mu$g/ml). IL-2 was measured from the supernatants collected on day 1 by ELISA using an OPT enzyme immunoassay mouse IL-2 kit (BD Biosciences). The samples were run in duplicate on 96-well Maxisorp plates (Nalge Nunc International). Data represent the mean ± SEM of three or four individual mice.

### *Detection of serum Ab titers by ELISA*

Serum from immunized mice were evaluated for Abs that recognize mouse PG or human PG, using a PG-specific ELISA. Enzyme immunoassay tissue culture 96 "half-area" plates (Costar Corning) were coated overnight at 4°C with 0.5 $\mu$g of chondroitinase avidin-biotin peroxidase complex-digested human PG or 0.75 $\mu$g of native mouse PG in carbonate buffer. Serum was serially diluted in buffer (PBS/0.5% Tween 20). To detect IgG1 or IgG2a Abs in WT mice, serum dilutions of 1/500, 1/2,500, and 1/12,500 were used to determine the concentration of anti-mouse PG Abs, whereas dilutions of 1/2,500, 1/12,500, and 1/62,500 were used to detect anti-human PG Abs. Samples were run in duplicate. When testing for the presence of PG-specific Abs in the serum of mIgM mice, (m+s)IgM transgenic mice, or B cell-deficient mice, serum was diluted at 1/10, 1/50, and 1/100 for plates coated with either human PG or mouse PG. Ab isotypes were detected with HRP-labeled rabbit anti-mouse IgG1, IgG2a, or IgM (Zymed Laboratories), which was then detected with the substrate *o*-phenylenediamine. Colorimetric change in each sample was measured with a spectrophotometer at 490 nm and were compared with a standard curve of known concentrations of unlabeled murine IgG1, IgG2a, or IgM Abs (Southern Biotechnology Associates). Data represent the mean ± SEM of three to five individual mice.

### *Adoptive transfer of PGIA into SCID mice*

Nylon wool-purified T cells ($1 \times 10^7$) from PG- or NP-PG-immunized WT or mIgM mice or naive WT mice plus Thy1.2 (CD90)-microbead (Miltenyi Biotec)-depleted spleen cells ($2 \times 10^7$) from arthritic WT mice, and 100 $\mu$g of Ag (PG or NP-PG) were mixed in saline and adoptively transferred i.p. into female SCID mice. SCID mice were examined for arthritis onset and severity every 3 days after cell transfer. Enriched T cells were >90% $CD3^+$, and CD90-depleted cells were <2% $CD3^+$ by flow cytometry. In separate experiments, SCID mice were injected i.p. with splenocytes ($3 \times 10^7$/mouse) from NP-PG-immunized mIgM mice or WT mice on days 0 and 16. SCID mice that received spleen cells from NP-PG-immunized mIgM mice were injected i.p. with 500 $\mu$l of pooled sera from arthritic WT mice or normal mouse serum (NMS) every 3 days for 4 wk after the initial cell transfer. SCID mice were administered arthritic serum alone (500 $\mu$l) according to the same transfer schedule.

### *Flow cytometry*

Spleen cells from WT and mIgM mice were depleted of RBCs, washed, and stained for 20 min on ice with an optimal dilution of the relevant Ab. Cells were analyzed by flow cytometry (FACSCalibur and CellQuest software; BD Biosciences). $V_H$186.2 IgH chain transgenic B cells from mIgM

mice recognize 4-hydroxy-3-iodo-5-nitrophenyl acetyl (NIP) with high affinity when paired with a λ L chain (54). PE (Molecular Probes) was haptenated with NIP-hydroxysuccinimide ester (Biosearch Technologies), and the NIP-PE conjugate was used to identify $V_H186.2^+Ig\lambda^+$ B cells. Naive WT and mIgM mice spleen cells were additionally stained with rat anti-mouse λ FITC (Southern Biotechnology Associates). Day 12 spleen cells from PG- or NP-PG-immunized mIgM mice were stained with a combination of NIP-PE and biotin rat anti-mouse CD86 (B7-2)-biotin, then washed and stained with streptavidin-allophycocyanin (BD Biosciences).

### *Statistical analysis*

The Mann-Whitney *U* test was used to compare nonparametric data for statistical significance. Significance is based on a value of $p < 0.05$.

## Results

### *B cells are required for the development of arthritis*

To evaluate whether B cells are necessary for the development of PGIA, we used B cell-deficient ($J_HD$) mice. WT and B cell-deficient mice were immunized with PG and assessed for clinical signs of arthritis. Arthritis developed in a few WT mice before the third injection with PG and progressed until 100% ($n = 14$) of the animals were arthritic with a maximum mean arthritic score of 6.70 ± 1.13 (Fig. 1, *A* and *B*). B cell-deficient mice (0 of 26) were completely resistant to disease. B cell-deficient animals remained resistant to arthritis even 100 days after the final immunization (data not shown), confirming an absolute requirement for B cells in the initiation of PGIA.

### *The mIgM mice are resistant to arthritis*

B cells may function as autoantibody-producing cells and as APCs in the development of arthritis. To address whether B cells contribute to the development of PGIA independent of autoantibody, we used mIgM mice. B cells in the mIgM mice express a membrane-bound Ig H chain transgene in association with an endogenously generated L chain, but do not secrete Ig (17). The $V_H186.2$ transgene contains a deletion of the DNA encoding secretion ($\mu s$) and polyadenylation sequence ($pA_s$) of the IgM H chain (17). Control animals, termed (m+s)IgM mice, similarly express a transgenic IgM H chain that is both expressed on the B cell surface and secreted. WT, mIgM, and (m+s)IgM mice were immunized with PG and examined for disease progression. None of the mIgM ($n = 15$) or (m+s)IgM ($n = 21$) mice developed arthritis compared with 100% of WT mice that developed severe disease (Fig. 1).

To determine whether B cells specific for PG are present in mIgM mice, we used the (m+s)IgM mice that secrete Ig and assayed serum from the PG-immunized mice. PG-specific IgM levels in (m+s)IgM mice to human PG (0.55 ± 0.16 μg/ml) or mouse PG (0.98 ± 0.19 μg/ml) were not statistically different from those in naive WT animals (human PG, 1.33 ± 0.57 μg/ml; mouse PG, 1.13 ± 0.46 μg/ml), in contrast to significantly increased Ab levels in PG-immunized WT mice (human PG, 11.89 ± 3.14 μg/ml; mouse PG, 7.51 ± 0.44 μg/ml). These data demonstrate that PG-specific Abs are undetectable and suggest that (m+s)IgM and mIgM mice are deficient in PG-specific B cells.

Our results show that simply restoring B cells is not sufficient to induce arthritis. There are two potential explanations for why B cells in the mIgM and (m+s)IgM mice were not capable of inducing arthritis. First, a deficiency in PG-specific autoantibodies in mIgM and (m+s)IgM mice may have prevented the development of arthritis, because we have previously shown that autoantibodies enhance arthritis onset (47, 51). The second possibility is that a lack of Ag-specific B cells that function as APCs in the mIgM and (m+s)IgM mice prevented adequate priming of autoreactive T cells that are competent to induce arthritis.

### *PGIA is reduced in the absence of Ag-specific B cells in mIgM mice*

Because T cells are required for the development of PGIA, it is possible that autoreactive T cells are not effectively primed in mIgM mice immunized with PG. To assess whether the T cells from PG-immunized mIgM mice were capable of inducing arthritis, we performed a SCID adoptive transfer experiment to test the function of the T cells in an environment where B cells were capable of producing PG-specific autoantibody. Purified T cells from PG-immunized WT or mIgM mice and Thy1.2 (CD90)-depleted spleen cells (B cells plus APCs) from arthritic WT mice were mixed with 100 μg of PG and transferred into SCID recipients. SCID mice that received T cells from PG-immunized WT mice developed persistent severe arthritis in 100% of cases; in contrast, T cells from PG-immunized mIgM mice developed very mild and transient disease in only 40% of mice (Fig. 2*A*). These results indicate that T cells in the PG-immunized mIgM mice were not adequately primed to induce severe arthritis, suggesting that Ag-specific B cells may be required for sufficient T cell priming.

### *Ag-specific B cells restore autoreactive T cell activation*

Specific recognition of Ag by B cells through the BCR leads to cell activation and efficient endocytosis of Ag, which substantially enhances Ag presentation to $CD4^+$ T cells. To address whether Ag-specific B cells were essential for the activation of autoreactive T cells, we targeted PG to NP-specific B cells in mIgM mice by conjugating PG to the hapten NP. Because ~2–4% of B cells from mIgM mice are NP specific (54), immunization with NP-PG allows B cells from mIgM mice to specifically recognize PG through

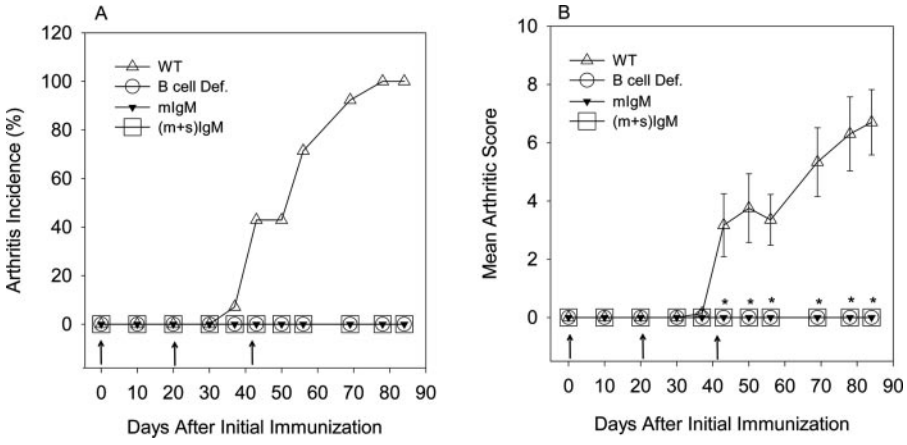


**FIGURE 1.** B cell-deficient mice and mIgM mice are resistant to PGIA. WT, B cell-deficient (B cell Def.), mIgM, and membrane-bound and secreted IgM (m+s)IgM mice were immunized with PG on wk 0, 3, and 6, as indicated by the arrows. Arthritis onset (*A*) and severity (*B*) were monitored. One hundred percent of WT mice ($n = 14$) became arthritic, whereas none of the B cell-deficient ($n = 26$), mIgM ($n = 15$), or (m+s)IgM mice ($n = 21$) developed arthritis. Arthritis scores are the mean ± SEM for two separate experiments. *, Data are statistically significantly different ($p < 0.05$) between WT and B cell-deficient mice, mIgM mice, or (m+s)IgM mice.

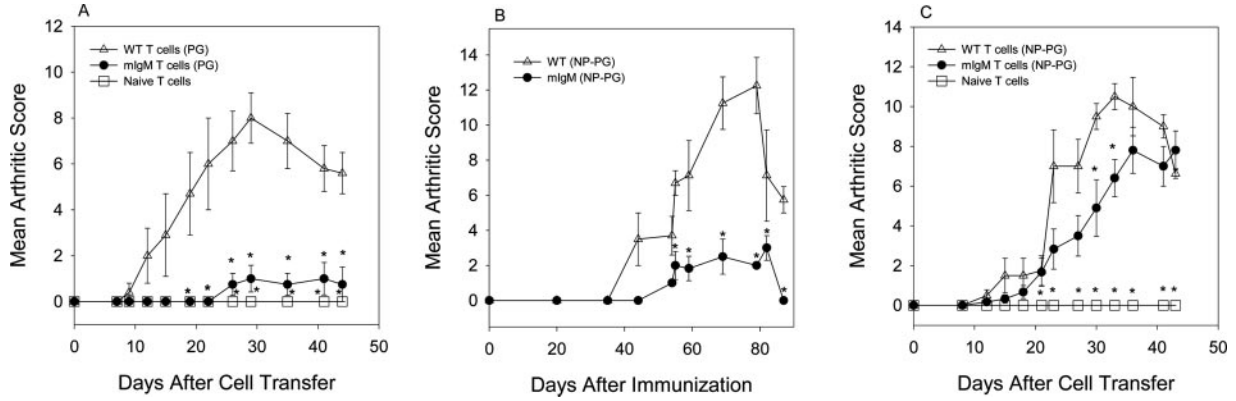


**FIGURE 2.** Ag-specific B cells are essential APCs for the activation of autoreactive T cells that induce arthritis. *A*, Purified T cells ($1 \times 10^7$) from PG-immunized WT mice, PG-immunized mIgM mice, or naive WT mice were transferred with CD90-depleted spleen cells (B cells plus APCs; $2 \times 10^7$) from WT arthritic mice and 100 $\mu$g of PG i.p. into SCID recipients. Arthritis onset and severity were monitored. *B*, WT and mIgM mice ($n = 5$) were immunized with NP-PG and monitored for arthritis incidence and severity. Arthritis scores are the mean $\pm$ SEM of arthritic mice. The incidence of arthritis was 100% in WT and 60% in mIgM mice. $*$, Data are significantly different ($p < 0.05$) between WT and mIgM mice. *C*, Purified T cells ($1 \times 10^7$) from NP-PG-immunized WT, NP-PG-immunized mIgM, or naive WT mice were transferred in conjunction with CD90-depleted spleen cells ($2 \times 10^7$) from WT arthritic mice and 100 $\mu$g of NP-PG i.p. into SCID recipient animals. SCID mice were examined for arthritis severity after cell transfer. Arthritis scores are the mean $\pm$ SEM for four or five SCID mice (*A* and *C*) and are representative of one of two experiments. $*$, Data are statistically significantly different ($p < 0.05$) between SCID mice that received T cells from WT mice immunized with PG or NP-PG vs T cells from PG- or NP-PG-immunized mIgM mice or naive WT mice.

the NP-specific BCR. Although NP-PG-immunized WT mice developed severe disease as anticipated (maximum arthritis score, 12.3 $\pm$ 1.7), we observed unexpectedly that mIgM mice immunized with NP-PG developed mild, transient arthritis (maximum arthritic score, 3.0 $\pm$ 0.7) in 60% of the animals (Fig. 2*B*). These data suggest that even in the absence of Ab, autoreactive T cells activated by Ag-specific B cells could induce some degree of arthritis. We next investigated whether T cells from NP-PG-immunized mIgM mice were competent to induce severe arthritis in the presence of WT B cells that are capable of secreting autoantibody. Purified T cells from WT or mIgM mice were mixed with CD90-depleted spleens (B cells plus APCs) from NP-PG-immune WT mice, along with 100 $\mu$g of NP-PG, and transferred into SCID mice. T cells efficiently induced disease when derived from either WT or mIgM mice, but not from naive WT mice (Fig. 2*C*). These data suggest that targeting PG to the BCR (in mIgM mice) using NP-PG led to priming of arthritis-inducing T cells. Activation of the T cells in mIgM mice occurred in the absence of circulating Ig, indicting that presentation of Ag by ICs was not necessary to prime T cells for arthritis.

### *B cells contribute to arthritis by two distinct, but synergistic, mechanisms: autoantibody production and APC function*

We have shown that PG-specific autoreactive T cells were activated in mIgM mice using NP-PG as the immunizing Ag, although these mice only developed mild and transient arthritis. However, when the T cells from the NP-PG-immune mIgM mice were transferred to SCID mice with B cells from PG-immune WT mice severe arthritis developed, suggesting that autoantibodies were needed for severe disease (Fig. 2*C*). Therefore, we investigated whether PG-specific autoantibodies were essential for the development of severe arthritis. We first assessed whether autoantibody alone mediates severe arthritis. SCID mice were injected with 500 $\mu$l of pooled serum from arthritic WT mice every third day for 4 wk (Fig. 3). Transfer of arthritic serum alone induced only mild and transient arthritis in 40% of SCID mice, indicating that autoantibody partially induced disease.

To determine whether there was synergy between the autoreactive T cells and autoantibody in generating arthritis, spleen cells from NP-PG-immunized mIgM mice, which provided a source of primed T cells, were adoptively transferred into SCID mice in conjunction with either serum from arthritic WT mice or normal mouse serum. SCID mice that received arthritic serum and spleen cells from NP-PG-immune mIgM mice developed severe arthritis, resembling mice that received NP-PG WT arthritic splenocytes alone (Fig. 3). In contrast, no disease was apparent in those mice

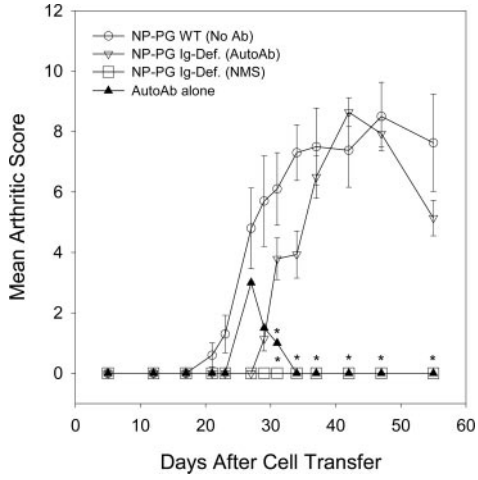


**FIGURE 3.** AutoAbs and autoreactive T cells are collectively required to transfer severe arthritis. SCID mice received spleen cells ($3 \times 10^7$) from NP-PG-immunized mIgM mice or WT mice on days 0 and 16. SCID mice that received mIgM mouse spleen cells were administered either 500 $\mu$l of NMS or pooled WT arthritic serum (AutoAb) i.p. every 3 days starting 1 day after the initial cell transfer and continuing for 4 wk. Another set of SCID mice was administered only WT arthritic serum using the same serum transfer schedule (AutoAb alone). SCID mice were monitored for onset and severity of arthritis after cell transfer. Arthritis developed in SCID mice that received NP-PG WT cells without Ab (No Ab; $n = 5/5$), NP-PG mIgM cells plus arthritic serum (AutoAb; $n = 7/7$), NP-PG mIgM cells plus NMS ($n = 0/5$), or arthritic serum (AutoAb alone; $n = 2/5$). Data represent the mean $\pm$ SEM of arthritic scores. $*$, Data are statistically significantly different ($p < 0.05$) between SCID mice that received arthritic serum and those that received NMS.

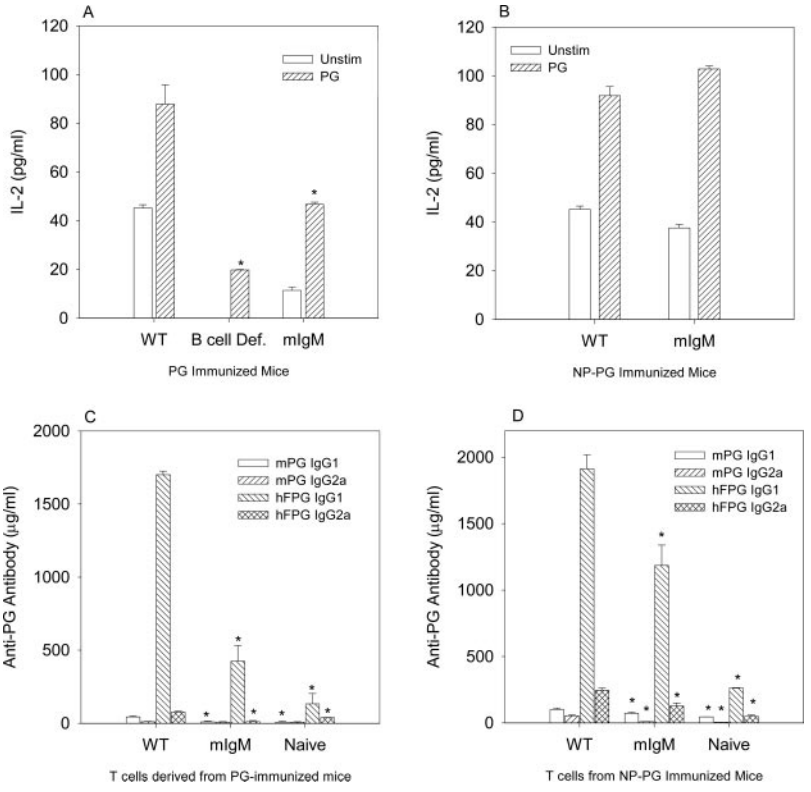


**FIGURE 4.** T cell activation is more efficient in the presence of Ag-specific B cells. *A* and *B*, Purified T cells ($2.5 \times 10^5$) from the spleens of PG-immunized (*A*) WT, B cell-deficient, or mIgM mice ($n = 4$) or NP-PG immunized (*B*) WT or mIgM mice ($n = 4$) were incubated with irradiated spleen cells ($2.5 \times 10^5$) and PG or medium alone. Supernatants were collected at 24 h and examined for Ag-specific T cell production of IL-2 by ELISA. Data are the mean ± SEM of four mice per group and are representative of one of three experiments. *, Data are statistically significantly different ($p < 0.05$) between WT and B cell-deficient mice or mIgM mice. *C* and *D*, Purified T cells ($1 \times 10^7$) from PG-immunized (*C*) or NP-PG-immunized (*D*) WT, mIgM, or naive WT mice were transferred in conjunction with CD90-depleted spleen cells ($2 \times 10^7$) from WT mice plus 100 μg of NP-PG into SCID recipients ($n = 5$ SCID mice/group). Serum obtained from the SCID mice was examined for IgG1 and IgG2a Abs that recognize human PG (hFPG) or native mouse PG (mPG) by ELISA. Ab production is the mean ± SEM of five SCID mice and represents one of two experiments. *, Data are statistically significantly different ($p < 0.05$) between SCID mice that received T cells from PG- or NP-PG-immunized WT mice and SCID mice that received T cells from PG- or NP-PG-immunized mIgM or naive WT mice.

that received spleen cells from the NP-PG-immunized mIgM mice treated with normal mouse serum (Fig. 3). Thus, generation of severe arthritis required a source of PG-specific autoreactive T cells in addition to PG-specific autoantibody. From these data, it is evident that Ag-specific B cells contribute to arthritis by two discrete mechanisms, autoantibody production and APC function, each of which is necessary for severe and persistent arthritis.

### *T cells primed in the presence of Ag-specific B cells provide efficient B cell help for Ab production*

Because the above experiments suggested that there is a difference in T cell priming, we directly examined T cell responses in vitro. Purified T cells were isolated from B cell-deficient mice and mIgM mice immunized with either PG or NP-PG, and IL-2 production was assessed. T cell priming in mice lacking B cells was first evaluated. T cells from B cell-deficient animals produced significantly less IL-2 in response to PG stimulation compared with WT T cells (Fig. 4*A*). These results demonstrate that PG-specific T cell activation was substantially reduced in B cell-deficient animals and could in part account for the lack of arthritis in these mice.

To determine whether the presence of B cells leads to T cell activation, T cell responses in mIgM mice were evaluated. T cells from mIgM mice produced significantly less IL-2 than WT T cells; however, IL-2 levels were significantly higher than those in T cells from B cell-deficient mice (Fig. 4*A*). These data demonstrate that B cells partially activate T cells, independently of their Ag specificity or secreted Ig. Thus, non-Ag-specific B cells contribute to T cell priming to some degree, although this level or type of activation is not sufficient to transfer arthritis.

Next, T cell responses were examined in NP-PG-immunized WT and mIgM mice to determine whether the presence of Ag-specific B cells reconstitutes T cell responses. T cells from NP-PG-immunized WT and mIgM mice produced similar levels of IL-2 in response to PG, indicating that T cells were efficiently primed in the presence of Ag-specific B cells (Fig. 4*B*). These in vitro results correlate with the SCID adoptive T cell transfer experiments in which T cells from NP-PG-immunized mIgM mice were competent to transfer arthritis (Fig. 2*B*).

To determine whether Th activity was affected by the priming of T cells, we assessed autoantibody production. T cells from PG- or NP-PG-immunized WT or mIgM mice and B cells plus APCs from WT arthritic mice were transferred into SCID mice. Serum from the SCID recipients was examined for IgG1 and IgG2a Abs to human PG or native mouse PG. Ab levels were severely reduced in SCID mice that received T cells from PG-immunized mIgM mice compared with T cells from PG-immunized WT mice (Fig. 4*C*), indicating that T cells primed in the presence of non-Ag-specific B cells were ineffective at providing B cell help. Conversely, T cells primed in mIgM mice in the presence of Ag-specific B cells were markedly more efficient at providing T cell help for Ab production than T cells primed by nonspecific APCs (compare Fig. 4, *C* and *D*). These data suggest that Ag-specific B cells are critical for the activation of PG-specific T cells that can provide Th activity for B cell autoantibody production. Thus, a reciprocal relationship exists by which Ag-specific B cells provide activating signals to PG-specific T cells, which are, in turn, required to provide T cell help for autoantibody production that is essential for severe autoimmune arthritis.

### *Ag-specific B cells proliferate and are markedly activated in mIgM mice immunized with NP-PG, but not PG*

Activation of B cells and the expression of costimulatory molecules enhance the Ag-presenting function of B cells. To determine whether NP-PG activates proliferation and costimulatory molecule expression, we assessed the NP-specific population of B cells in mIgM mice. As previously shown, ~1.5% of $V_H186.2^+$ B cells that bind NP were identified by FACS analysis in naive mIgM mice (Fig. 5*B*), whereas this population of cells was undetectable in naive WT mice (Fig. 5*A*). To assess whether NP-specific B cells expand in mIgM mice immunized with NP-PG, we compared the

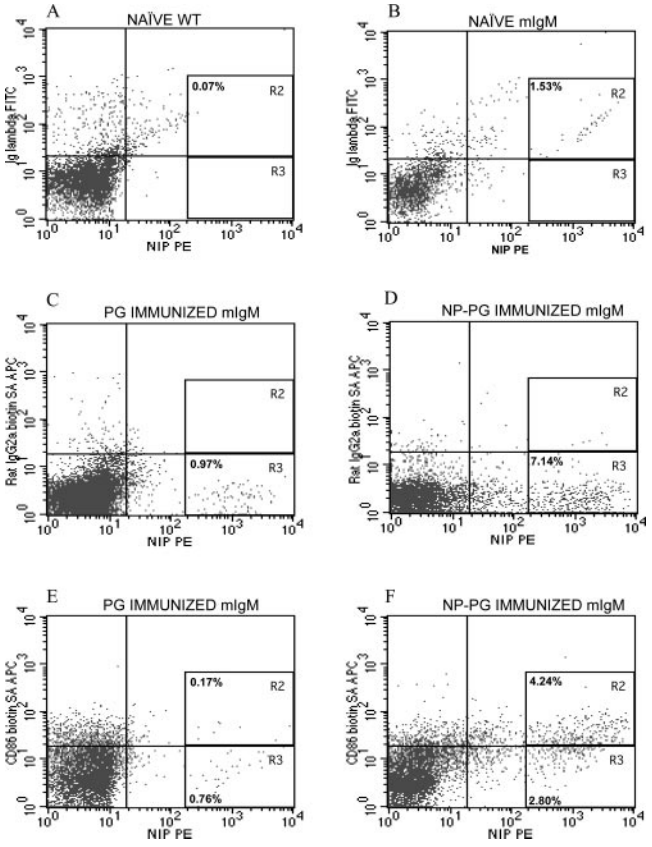


**FIGURE 5.** NIP-specific B cells expand in vivo and increase CD86 surface expression in mIgM mice immunized with NP-PG. *A* and *B*, Naive WT and mIgM mouse spleen cells were analyzed by FACS for the expression of $NIP^+Ig\text{-}\lambda^+$ double-positive cells. A distinct population of $NIP^+Ig\text{-}\lambda^+$ double-positive cells is evident in transgenic mIgM mice and is undetectable in WT mice ($n = 3$). *C–F*, mIgM mice ($n = 3$ mice/group) were immunized with either PG (*C* and *E*) or NP-PG (*D* and *F*) and examined on day 12 for the percentage of $NIP^+$ cells (*C* and *D*) and the percentage of $NIP^+CD86^{high}$ cells (*E* and *F*; R2 gate) by FACS.

percentage of NIP-binding B cells from spleens of PG- (Fig. 5*C*) or NP-PG- (Fig. 5*D*) immunized mIgM mice. In mIgM mice immunized with NP-PG, the NIP-binding B cells comprised 6.60 ± 0.65% of the spleen, in contrast to the PG-immunized mice where the NIP-binding B cells failed to expand (0.84 ± 0.18%).

To prove that Ag-specific B cells that have expanded in NP-PG-immunized mIgM mice also become activated, we examined the percentage of NIP-binding cells that express high levels of the activation marker CD86 (B7-2) on day 12 after PG (Fig. 5*E*) or NP-PG (Fig. 5*F*) immunization. The percentage of $B7\text{-}2^{high}$ $NIP^+$ cells in mIgM mice ($n = 3$) immunized with NP-PG was 4.39 ± 0.17%, which is significantly greater than that in the PG-immunized mice, 0.26 ± 0.08% ($p < 0.05$). These results clearly demonstrate that Ag-specific B cells from mIgM mice substantially proliferated and became activated only after NP-PG immunization.

## Discussion

Autoreactive B cells are activated in PGIA, but our understanding of how they contribute to disease susceptibility is incomplete. The present work provides direct evidence that B cells are essential for the development of arthritis and provides several different functions by which they contribute to disease. One of the major findings of our studies is that Ag presentation by B cells is critical for the activation of autoreactive T cells. Moreover, the APC function of B cells requires the direct recognition of Ag through the BCR to activate autoreactive T cells. Once activated, the autoreactive T cells can induce a very mild form of arthritis, indicating that T cells subsequent to activation by Ag-specific B cells have a direct pathogenic role in disease. Although DCs and macrophages are present in mIgM mice, they are not sufficient to function as APCs for autoreactive T cell activation. Our second major finding is that B cell production of autoantibody is critical and that autoantibodies alone induce mild and transient disease. However, both autoreactive T cells and autoantibody are required to cause severe arthritis, indicating that both B cell effector pathways contribute synergistically to autoimmune disease. We also show that autoantibody in the form of ICs are not required to activate autoreactive T cells, because T cells competent to induce arthritis are activated independent of circulating Ab.

There is some controversy over whether B cells can prime naive T cells; however, activated and memory T cells are critically dependent on B cells (25). Our results similarly demonstrate that T cell activation is reduced, and arthritis is prevented in B cell-deficient mice. However, in mIgM mice, PG-specific T cell recall responses are restored to some degree. We found that T cells primed to PG in mIgM mice secrete reduced IL-2 compared with WT T cells. However, these T cells are not sufficient to transfer arthritis. The inability to transfer arthritis correlates with a reduction in B cell helper activity for PG-specific autoantibody production. These findings indicate that the presence of B cells allows some degree of T cell activation that is independent of the B cell specificity; however, this level of activation is not sufficient to transfer arthritis.

The inability of B cells in mIgM mice to fully prime autoreactive T cells may be due to the low frequency of B cells specific for PG. In support of this idea, targeting PG to the high frequency, NP-specific B cells by haptenating PG with NP led to the activation of T cells that induced mild arthritis, and upon transfer with autoantibody caused severe arthritis. Development of T cells in mIgM mice that are competent to induce arthritis correlates with an increase in IL-2 production and the ability to provide Th activity for autoantibody-producing B cells.

Several potential mechanisms could explain why Ag-specific B cells are required for the efficient activation of autoreactive T cells. Ag processing and presentation by Ag-specific B cells can stimulate T cells nearly 1,000–10,000-fold more efficiently than nonspecific B cells. Furthermore, engagement of the BCR by Ag leads to B cell proliferation and up-regulation of costimulatory molecules (54). We demonstrate that Ag-specific B cells are expanded to nearly 7% of the spleen population in NP-PG-immunized mIgM mice, and 60% of these B cells are $B7\text{-}2^{high}$. Activation of Ag-specific B cells could also promote the expansion of PG-specific T cells that induce arthritis. It is possible that internalization of Ag through the BCR could generate a different set of epitopes that are presented to T cells (21, 29, 55, 56), thereby activating a unique subset of Ag-specific T cells. Studies of autoantigens have shown that B cells elicited with foreign cross-reactive Ag can present self-peptides, resulting in priming of autoreactive T cells (57, 58). With regard to PGIA, immunization with human PG activates B cells that cross-react with mouse PG (45, 47). Based on this scenario, PG-specific T cells may be initially activated by DCs and macrophages, but as clones of autoreactive B cells expand, they may increasingly become important APCs for autoreactive T cell activation.

In PGIA, severe and persistent arthritis cannot be induced through arthritic serum transfer; nevertheless, we demonstrate that autoantibodies are critical for the induction of severe arthritis once autoreactive T cells are primed. SCID mice develop severe disease

only after receiving both serum from arthritic mice and autoreactive T cells from NP-PG-immunized mIgM mice (Fig. 3). We have shown that autoantibody are important for initiating inflammation in the joint by binding to the cartilage surface and initiating chemokine and cytokine responses in the joint (59). Development of joint inflammation is at least in part an Fc$\gamma$R-dependent mechanism (59). However, the activation of autoreactive T cells is independent of Fc$\gamma$R expression. In this study we also show that autoreactive T cells are primed in the absence of circulating Ig. Taken together, these data demonstrate that ICs are not necessary for the priming of autoreactive T cell in PGIA.

This study demonstrates that Ag-specific B cells regulate the initiation of autoimmune murine arthritis by functioning as APCs and producing autoantibody. Neither mechanism alone orchestrates robust disease; however, autoreactive T cells and autoantibody contribute synergistically to severe arthritis. The implication of our study is that autoreactive Ag-specific B cells are crucial at multiple stages of PGIA and, by extension, may be regulating human RA by several different mechanisms. This, in turn, could explain the potent therapeutic effects of B cell depletion in RA (60–66).

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## Disclosures

The authors have no financial conflict of interest.

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