Arthritogenic T cells drive the recovery of autoantibody-producing B cell homeostasis and the adoptive transfer of arthritis in SCID mice

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Abstract

T cells orchestrate joint inflammation in rheumatoid arthritis (RA), but B cells/B cell-derived factors are also involved in disease pathogenesis. The goal of this study was to understand the role of antigen-specific T and B cells in the pathological events of arthritis, which is impossible to study in humans due to the small number of antigen-specific cells. To determine the significance of antigen-specific lymphocytes and antibodies in the development of an autoimmune mouse model of RA, we generated TCR transgenic (TCR-Tg) mice specific for the dominant arthritogenic epitope of cartilage proteoglycan (PG) and performed a series of combined transfers of T cells, B cells and autoantibodies into BALB/c.Scid mice. The adoptive transfer of highly purified T cells from naive TCR-Tg, arthritic TCR-Tg or arthritic wild-type mice induced arthritis in SCID recipients, but the onset and severity of the disease were dependent on the sequential events of the T cell-supported reconstitution of PG-specific B cells and autoantibodies. The presence of activated PG-specific T cells was critical for disease induction, establishing a unique milieu for the selective homeostasis of autoantibody-producing B cells. In this permissive environment, anti-PG autoantibodies bound to cartilage and induced activation of the complement cascade, leading to irreversible cartilage destruction in affected joints. These findings may lead to a better understanding of the complex molecular and cellular mechanisms of RA.

Keywords: adoptive transfer, arthritis, lymphocyte homeostasis

Introduction

Rheumatoid arthritis (RA) is a systemic and chronic autoimmune disease characterized by cartilage destruction and bone erosion. Although a number of observations indicate that T cells govern the cellular invasion and inflammation of joints in RA (1, 2), B cells and B cell-derived factors (antibodies and cytokines) are also involved in disease pathogenesis (3–6). Correspondingly, (auto)antibodies against cartilage matrix components, citrullinated proteins or Igs are frequently detected in the sera or synovial fluids of RA patients. Recently, a critical role for B cells has become more evident in clinical trials using B cell-depleting anti-CD20 (Rituximab) immunotherapy, especially in anti-tumor necrosis factor (TNF) therapy-resistant cases (3, 4, 7). However, the small number of antigen-specific lymphocytes, the complexity and timing of T and B cell cooperation, which results in the expression of various receptors and the secretion of a variety of factors, and the unique structural and functional properties of synovial joints make it difficult to understand the immunoregulatory dysfunction in human RA.

In systemic autoimmune animal models of RA [collagen-induced arthritis (CIA), proteoglycan (PG)-induced arthritis (PGIA) and K/BxN spontaneous arthritis (8–10)], the critical function of T cells has been implicated in arthritis induction. In a previous study, we showed selectively recovered subsets of T and B cells after adoptive transfer of healthy and arthritic BALB/c spleen cells into BALB/c.Scid (SCID) mice.
(11). We also found that the transfer of purified T cells from arthritic BALB/c mice required a second transfer of T cells or a second antigen (PG) challenge to generate sufficient amounts of B cells and antibodies for a subsequent induction of arthritis. In the case of arthritogenic T cell transfer in a lymphopenic milieu, appropriate B cell recovery takes a relatively long period of time, a process that is accelerated by transferring T and B cells simultaneously (11, 12).

Therefore, we hypothesized that antigen-specific T cell-mediated B cell expansion and function may be a critical factor in arthritis development (13–15). To confirm this hypothesis, we generated TCR transgenic (TCR-Tg) mice (16, 17) specific for the dominant arthritogenic 5/4E8 epitope of the G1 domain of cartilage PG aggrecan (18) (core sequence underlined) (18, 19). TCR-5/4E8-Tg mice (henceforth: TCR-Tg) were backcrossed into the BALB/c background, and PG-activated spleen cells were used to transfer arthritis into either Rag2−/− or SCID mice (16, 17).

Based on these studies, our goal was to gain insight into the mechanisms and timing of the homeostatic recovery of T and B cells in vivo that lead to autoimmune arthritis and to understand the role of pathogenic autoantibodies in disease development. We used highly purified T and B cells and/or IgGs in different combinations for the adoptive transfer of arthritis into syngeneic SCID recipients. The reconstitution of T and B cell homeostasis was monitored in time-course experiments that measured the existence and activation status of lymphocytes using cell surface markers as well as the functions of antigen-specific T and B cells by cytokine and serum antibody levels. Homeostatic T cell proliferation was fast in a lymphopenic milieu, particularly if the syngeneic T cell population was activated at the time of adoptive transfer (20, 21). As a result, the antigen-specific T cell population selectively supported the recovery of the autoimmune (pathogenic) B cell population and of autoantibody production, which then led to an immediate flare-up of arthritis and cartilage damage.

Materials and methods

Antigens, animals and immunization

Human articular cartilage was collected from patients who had undergone knee joint replacement surgery. The collection of cartilage from consenting patients was approved by the Institutional Review Board of Rush University Medical Center (Chicago). Cartilage PG (aggrecan) was extracted and partially depleted of glycosaminoglycan (GAG) side chains, as previously described (22, 23). A synthetic peptide carrying the 5/4E8 epitope was used as a positive antigen control.

Female wild-type (WT) BALB/c mice aged 16–20 weeks and sex- and age-matched SCID mice in a BALB/c background (NCl/NCr.C.B-17-scid/scid, henceforth: SCID) were purchased from the National Cancer Institute (NCI; Fredrick, MD, USA). TCR-Tg mice were generated and backcrossed into the BALB/c background as previously described (16, 17). WT and TCR-Tg mice were immunized intra-peritoneally with an emulsion of cartilage PG (100 μg) and dimethyldioctyldecyl-ammonium bromide adjuvant (2 mg; Sigma–Aldrich, St Louis, MO, USA) on days 0 and 21 (22). All animal procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Rush University Medical Center, Chicago.

Isolation and transfer of T and B cells and serum IgG to SCID mice

Single-cell suspensions were prepared from the spleens of arthritic and non-arthritic (naive) WT and TCR-Tg mice. To maintain the uniformity of cell transfer and subsequent arthritis development, donor cells were isolated from arthritic mice 2–3 weeks after the onset of primary arthritis in mice, with a cumulative arthritic score of 5–8 (22). Spleen cells from naive or arthritic donor mice were subjected to immunomagnetic purification using the Pan T Cell Isolation Kit and B Cell Isolation Kit for negative selection (Miltenyi Biotec, Auburn, CA, USA). The purity and viability of CD3+ T cells and CD19+/B220+ B cells used for transfer were at least 98%, as determined by flow cytometry and trypan blue exclusion assays. More than 95% of TCR-Tg T cells were CD4+ Vβ4+ double positive.

The optimal number of purified donor T and B cells, the interval between transfers and the effective dose of anti-PG antibodies were determined during preliminary experiments (data not shown). The following transfer system was used as a standard protocol: for the first cell transfer, 5 × 10^6 purified T cells (from naive or arthritic WT mice or from naive or arthritic TCR-Tg mice) in 200 μl sterile PBS were injected intra-peritoneally into SCID mice along with 100 μg of human PG; for the second transfer, the same SCID mice that received one of the four different T cell populations during the first cell transfer were injected intra-peritoneally with 5 × 10^6 T cells (same T cells as those that were used for the first transfer), 5 × 10^6 B cells (from naive or arthritic WT mice) or IgG (isolated from sera from naive or arthritic mice). The second transfers were applied 7 days after the first transfer without PG antigen. For each transfer, two to five mice in each group were simultaneously injected, and the same experimental setup was repeated three to six times. The transfer strategies are summarized in Table 1.

<table>
<thead>
<tr>
<th>First transfer</th>
<th>Second transfer</th>
<th>Recovery (%)</th>
<th>Supernatant cytokines (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive B cells</td>
<td>5/4E8</td>
<td>33</td>
<td>Anti-PG IgG 2.5 mg 64 795</td>
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<tr>
<td>Arthritic WT</td>
<td>Same T cells</td>
<td>41</td>
<td>Anti-PG IgG 2.5 mg 16 160</td>
</tr>
<tr>
<td>Normal IgG</td>
<td></td>
<td>31</td>
<td>Anti-PG IgG 2.5 mg 345 122</td>
</tr>
<tr>
<td>Anti-PG IgG</td>
<td></td>
<td>50</td>
<td>Anti-PG IgG 2.5 mg 166 112</td>
</tr>
</tbody>
</table>

The expression of cell surface molecules was measured in the first and second cell transfers, 3 and 7 days after the first cell transfer. The cumulative results of three to six independent experiments are summarized (n.d., not detectable).

Clinical assessment of arthritis

Recipient SCID mice were examined three times a week for clinical symptoms of arthritis. The time of onset and incidence of arthritis were recorded, and the severity was scored based upon swelling and the redness of each paw, with scores for...
each paw ranging from 0 to 4 (yielding a maximum severity score of 16) (9, 22, 24). For histopathological examination, the limbs of arthritic and non-arthritic mice were dissected, fixed, decalcified and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. As described earlier (11, 17), the inflamed joints of SCID mice with adoptively transferred arthritis were histologically similar to those of WT BALB/c mice with primary PGIA.

Flow cytometry

Peripheral blood, bone marrow, joint-draining lymph node, thymus and spleen cell suspensions were harvested prior to the first and second cell transfers, 3 and 7 days after the second transfer (n = 3–4 SCID mice per group) and at the end of experiments (all animals at 26 days after the second transfer). The expression of cell surface molecules was measured using a FACS Calibur flow cytometer and analyzed by CellQuest software (BD Biosciences, San Jose, CA, USA). The following fluorochrome-labeled or biotinylated mAbs were used: CD3, CD4, CD8, CD19, CD69, CD25, CD80, CD86, B220 and TCR-Vβ4 (BD Biosciences). Appropriate isotype controls were used to determine the background staining.

Measurement of antigen (PG)-specific antibodies and T cell responses

Sera and spleen cells were collected from SCID mice prior to cell transfer, 3 and 7 days after the second transfer and at the end of experiments. IL-2 production was measured by CTLL assay as previously described (11). Antigen-specific T cell responses were measured in quadruplicate samples of spleen cells that were cultured in the presence of human PG protein (50 μg ml⁻¹) or a synthetic peptide (1 μg ml⁻¹) containing the 5/4E8 epitope (18). Spontaneous and antigen-specific production of IL-4, IL-6, TNF-α and IFN-γ was measured in the cell culture supernatants on day 4 using capture ELISA (BD Biosciences), and the results were expressed as delta (Δ) picogram cytokine/1 × 10⁶ cells. PG-specific antibodies were measured using ELISA (11, 22). Sera were applied at a dilution range of 1:10 000–1:320 000 and PG-specific autoantibodies and heteroantibodies were detected with peroxidase-conjugated rabbit anti-mouse IgG (Zymed Laboratories/Invitrogen, Camarillo, CA, USA).

Histology and immunohistochemistry

Femoral heads were used to show the entire articular cartilage surface without fixation and decalcification. Using anatomical features, the articular cartilage was removed in toto from the femoral heads of naïve or acutely arthritic BALB/c mice (n = 4 each) as well as from SCID mice with adoptive transfer-induced arthritis (n = 5 with acute and n = 5 with subacute arthritis). Cryostat sections were incubated with FITC-labeled goat anti-mouse complement C3 (Cappel; MP Biomedicals, Aurora, OH, USA) and Texas Red (TR)-labeled horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA). FITC-labeled non-immune goat IgG (BD Biosciences) and TR-labeled normal horse IgG (Vector Laboratories) served as negative controls. Fluorescence was examined and images were analyzed using a Nikon confocal microscope and MetaMorph image processing program (Meta Imaging Series, version 10.0; Universal Imaging Corporation, Downingtown,

Table 1. T and B cell recovery and cytokine production in cell cultures derived from the spleens of SCID mice with transferred cells or Igs

<table>
<thead>
<tr>
<th>First transfer</th>
<th>Second transfer</th>
<th>Recovery (%)</th>
<th>Supernatant cytokines (Δpg per million cells)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>T cell</td>
<td>B cell</td>
<td>TNF-α</td>
</tr>
<tr>
<td>T cells from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive WT</td>
<td>Same T cells</td>
<td>47 ± 16</td>
<td>5 ± 3</td>
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<tr>
<td></td>
<td>Arthritic B cells</td>
<td>36 ± 13</td>
<td>16 ± 6</td>
</tr>
<tr>
<td></td>
<td>Naive B cells</td>
<td>33 ± 16</td>
<td>15 ± 4</td>
</tr>
<tr>
<td></td>
<td>Anti-PG IgG</td>
<td>39 ± 18</td>
<td>6 ± 3</td>
</tr>
<tr>
<td></td>
<td>Normal IgG</td>
<td>22 ± 12</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Naive TCR</td>
<td>Same T cells</td>
<td>47 ± 18</td>
<td>10 ± 4</td>
</tr>
<tr>
<td></td>
<td>Arthritic B cells</td>
<td>27 ± 9</td>
<td>18 ± 6</td>
</tr>
<tr>
<td></td>
<td>Naive B cells</td>
<td>23 ± 9</td>
<td>12 ± 3</td>
</tr>
<tr>
<td></td>
<td>Anti-PG IgG</td>
<td>30 ± 14</td>
<td>8 ± 3</td>
</tr>
<tr>
<td></td>
<td>Normal IgG</td>
<td>31 ± 14</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>Arthritic WT</td>
<td>Same T cells</td>
<td>41 ± 19</td>
<td>15 ± 7</td>
</tr>
<tr>
<td></td>
<td>Arthritic B cells</td>
<td>45 ± 16</td>
<td>16 ± 5</td>
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<tr>
<td></td>
<td>Naive B cells</td>
<td>59 ± 21</td>
<td>17 ± 5</td>
</tr>
<tr>
<td></td>
<td>Anti-PG IgG</td>
<td>50 ± 18</td>
<td>8 ± 3</td>
</tr>
<tr>
<td></td>
<td>Normal IgG</td>
<td>52 ± 18</td>
<td>7 ± 3</td>
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<tr>
<td>Arthritic TCR</td>
<td>Same T cells</td>
<td>38 ± 13</td>
<td>21 ± 8</td>
</tr>
<tr>
<td></td>
<td>Arthritic B cells</td>
<td>25 ± 9</td>
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<tr>
<td></td>
<td>Naive B cells</td>
<td>19 ± 9</td>
<td>15 ± 6</td>
</tr>
<tr>
<td></td>
<td>Anti-PG IgG</td>
<td>17 ± 5</td>
<td>9 ± 2</td>
</tr>
<tr>
<td></td>
<td>Normal IgG</td>
<td>18 ± 8</td>
<td>10 ± 4</td>
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</table>

T and B cell recovery and cytokines in the supernatants of 5/4E8 peptide-stimulated cell cultures from SCID spleens were measured at the end of transfer experiments (4 weeks after second transfer). Cytokine levels in response to the 5/4E8 peptide (mean ± SEM) were normalized to the medium control. Data in Table 1 are organized according to the sequence of transfers, as shown in Fig. 1. The cumulative results of three to six independent experiments are summarized (n = 6–22 in each experiment); n.d., not detectable.
T cell-driven B cell homeostasis in arthritis

PA, USA). The contralateral hip joints were fixed and embedded for conventional histology. To detect the loss of cartilage PG, serial sections of cartilage from the femoral heads were stained with safranin-O and fast green.

Complement-mediated cytotoxicity of mouse chondrocytes
Chondrocytes were isolated from the femoral head cartilage of adult naïve WT BALB/c mice using pronase/collagenase digestion as previously described (25). High-density chondrocytes were cultured overnight in DMEM:F12 (50%-50%) medium containing 10% foetal bovine serum; live cells attached to the surface were washed with serum-free medium, treated with collagenase D (Roche Diagnostics GmbH, Indianapolis, IN, USA) for 30 min at 37°C and suspended in enzyme-free Cell Dissociation Buffer (Gibco/Invitrogen). After repeated washing in serum-free DMEM, 1 × 10⁴ chondrocytes were treated with undiluted sera or serial dilutions (1:10, 1:50, 1:100 and 1:200) of naïve or pooled sera from arthritic mice together with a 1:10 dilution of Low-Tox™-M rabbit complement (Cedarlane Inc., Burlington, Ontario, Canada) at 37°C for 1 h. All mouse sera were complement-inactivated for 30 min at 56°C. Additional negative control sera from methylated BSA-immunized mice were used. Viable cells were stained with 1 μM chloromethyl fluorescein diacetate (CMFDA) CellTracker (Molecular Probes/Invitrogen) for 45 min, washed and counted by flow cytometry. This cell tracker requires active cell metabolism thus only live cells are stained. The same cell suspensions were smeared on microscope slides and were mounted with 1.5 μl/ml DAPI-containing Vectashield (Vector Laboratories). CMFDA- and DAPI-stained cells were analyzed in epifluorescence mode by a Nikon FX fluorescent microscope. This CellTracker labeling allowed us to visualize and quantify dead and viable cells simultaneously.

Statistical analysis
Descriptive statistics were used to determine group means and standard errors of the means (mean ± SEM) unless otherwise stated. Pillai’s trace criterion was used to detect multivariate significance and Mann-Whitney U-tests were used for intergroup comparisons (SPSS, Chicago, IL, USA).

Results
T cells from arthritic TCR-Tg mice could effectively induce disease in SCID mice
PGIA can be induced by transferring spleen cells from either arthritic WT (11) or PG-specific TCR-Tg mice (17) into SCID recipients. To gain insight into the T cell-controlled mechanisms of this autoimmune arthritis model, purified T cells from naïve or arthritic WT and naïve or arthritic TCR-Tg mice were transferred into SCID mice, followed by a second transfer using T and B cells of different origins and/or antibodies (see transfer strategies in Table 1). A single highly purified (>98%) CD3+ T cell transfer from arthritic TCR-Tg mice injected simultaneously with 100 μg PG induced arthritis in ~30% of recipient SCID mice within 7–10 days, and the incidence was 100% with high severity scores when a second T cell transfer from arthritic TCR-Tg mice was performed (without additional PG injection) (Fig. 1D). In contrast, highly purified T cells from either naïve TCR-Tg or arthritic WT mice induced arthritis only when the first T cell transfer was repeated (Fig. 1C), or the first T cell transfer was followed by the transfer of B cells from arthritic mice or anti-PG antibodies (Fig. 1B and C). T cells transferred from naïve WT mice failed to induce arthritis followed by any combination of second transfer (Fig. 1A). These observations suggested that activated T cells from arthritic TCR-Tg animals could generate an optimal milieu for PGIA development, but T cells from arthritic WT or naïve TCR-Tg mice required the additional help of B cells from arthritic mice, which might be replaced with high-dose auto-antibodies to achieve a successful induction of arthritis.

Recovery of antigen-specific T and B cell homeostasis in recipient SCID mice
While the adoptive transfer of PGIA with total spleen cells (T and B cells together) from either arthritic WT or arthritic TCR-Tg animals were 95–100% successful at inducing arthritis in recipient SCID mice, purified T cells from naïve TCR-Tg (Fig. 1B) or arthritic WT (Fig. 1C) mice induced arthritis only in combination with a second transfer of B cells or antibodies from arthritic animals. This observation indicated the crucial role of PG-specific B cells (or antibodies) in the mechanisms of arthritis development. When we analyzed the peripheral blood of SCID recipients 7 days after the first T cell transfer, we found that 5–10% of the peripheral blood cells were B cells, despite the fact that these mice had only received highly purified CD3+ T cells from either naïve TCR-Tg, arthritic WT or arthritic TCR-Tg mice. CD4+ T cells expressed high levels of early activation markers (CD25 and CD69), and B220+CD69+ B cells were present in the bone marrow and spleen 7 days after the first transfer (data not shown). Based on these findings, we concluded that the trace amount of B cells present in the contaminating non-T-cell population (~2%) proliferated in the immunodeficient SCID milieu. This B cell colonization was more pronounced if the T cells were derived from arthritic TCR-Tg mice and was slightly less obvious when donor T cells were isolated from arthritic WT or naïve TCR-Tg BALB/c mice (data not shown). Four weeks after the second transfer, T cell homeostasis was fully reconstituted in all SCID recipients (34.95 ± 10.42%, same as in WT mice), which was independent of the origin of transferred T cells, whereas B cell recovery was limited to 5–23% in SCID mice (compared with 55–60% B cells present in donor spleens) (Table 1).

These data suggested that a high dose of exogenous anti-PG autoantibodies or B cells from PG-immunized mice could supplement and support the relative antigen-specific B cell deficiency in these SCID mice, which would cause a milder form of transferred arthritis (Fig. 1B and C) compared with mice that had received T cells from arthritic TCR-Tg donors (Fig. 1D). However, these high-dose anti-PG autoantibodies or B cells from PG-immunized mice were not sufficient to induce minimal signs of synovitis when recipient mice did not receive antigen-specific T cells (Fig. 1A). Based on these findings, we concluded that antigen specificity and activation status rather than the number of recovered T cells could orchestrate an optimal milieu for the recovery of arthritogenic B cells in recipient SCID mice.

Arthritis onset, incidence and severity in adoptively transferred SCID recipient mice. Left panels show the time of onset/incidence of arthritis (n=10 mice per group).
Variations in antigen-specific T and B cell responses controlled the success of adoptive transfer

To investigate the pathogenic effect of these colonizing and proliferating B cells, we measured anti-PG antibody levels in the sera of mice after adoptive transfer. There were significantly higher levels of serum anti-PG antibodies in the SCID mice that had received T cells from arthritic TCR-Tg or arthritic WT mice relative to those mice that had received the same number of T cells from naive TCR-Tg mice (Fig. 2A), suggesting a critical role for pre-activated (arthritic) T cells in autoantibody production by pre-activated and colonizing B cells. Notably, a much greater antigen-specific T cell response (as measured by IL-2 production) was found in spleen cell cultures from SCID mice that had received T cells from arthritic mice relative to those who had received T cells from naive TCR-Tg mice, as well as when T cells from arthritic
only T cells from arthritic TCR-Tg donors, but not from arthritic WT donors, could induce arthritis effectively (Fig. 3A and B). SCID mice that had received naive B cells and naive T cells from WT mice did not develop arthritis and were negative for all measured parameters (data not shown). Significant IL-2 (Fig. 3C) and anti-PG antibody (Fig. 3D) production was measured in 5/4E8 peptide-activated spleen cell cultures and in sera of mice received TCR-Tg T cells for the second transfer. These reciprocal transfer experiments further emphasize the role of activated and antigen-specific T cells in the pathology of autoimmune processes.

Anti-PG autoantibodies accelerated disease after adoptive transfer with arthritic T cells

In contrast to B cells, pre-treatment of SCID mice with PG-specific antibodies accelerated the onset and increased the incidence of arthritis when followed by the adoptive transfer of T cells from arthritic donors (Fig. 4A and B). SCID mice that had received T cells from naive TCR-Tg mice for the second transfer developed milder arthritis than arthritic littermates under the same conditions (Fig. 4B). These naive TCR-Tg T cells recovered in SCID mice 24 days after their transfer and responded well to in vitro 5/4E8 peptide stimulation, as indicated by IL-2 production (Fig. 4C). Anti-PG antibodies were measured after the second transfer and were at especially high levels when arthritic T cells from WT animals were used for the second transfer (Fig. 4D). Note that the use of T cells from arthritic TCR-Tg mice was irrelevant in this experimental setup because a single transfer of these T cells could transfer arthritis without the additional transfer of B cells or anti-PG antibodies (see Fig. 1D). There was no PGIA development (Fig. 4A) or detectable serum anti-PG antibody (24 days after the first transfer, data not shown) when SCID mice had received a total of 12.5 mg anti-PG (2.5 mg anti-mouse PG) IgG. Therefore, the serum antibodies in each of the four groups of mice (Fig. 4D) were most likely newly produced by colonizing B cells activated by T cells.

Antibodies and complement C3 bound to the surface of articular cartilage and chondrocytes in inflamed joints

To test the potential involvement of these PG-specific antibodies in situ in cartilage damage, femoral head cartilages from SCID mice with or without adaptively transferred PGIA were removed and cryosectioned. Frozen serial sections were stained with safranin-O (a marker of negatively charged PG molecules) or immunostained for mouse IgG and complement C3. Although safranin-O staining showed only a minimal loss of surface PG from femoral head cartilage (Fig. 5A), which corresponded to little or no synovitis (data not shown), sufficient amounts of mouse IgG and complement C3 were bound to seemingly intact cartilage surfaces in arthritic SCID mice (Fig. 5A1–A3) but never in non-arthritic or naive animals (not shown). Whenever arthritis was more pronounced (Fig. 5B), even in the presence of relatively mild cartilage damage (Fig. 5B0), immunostaining for IgG and C3 was extensive. IgGs typically localized to large areas (including the extracellular matrix) in the superficial layer of articular cartilage (Fig. 5B1), whereas complement C3 staining localized on the surface of chondrocytes (Fig. 5B2). The co-localization
of cartilage surface-bound IgG and chondrocyte-bound C3 suggested that the antibodies produced by activated antigen-specific B cells can actively contribute to irreversible cartilage destruction by the complement system in PGIA.

Antibodies in the sera of arthritic animals were cytotoxic toward syngeneic chondrocytes

Because we detected a sufficient amount of cartilage-bound IgG and complement in the superficial layer of articular cartilage, we were interested in whether these antibodies are cytotoxic toward mouse chondrocytes. Indeed, in a complement-mediated cytotoxic assay using fluorescent CellTracker, we measured massive cytotoxic effect of sera from arthritic mice using an in vitro fluorescent viability test. Isolated chondrocytes were exposed to sera from normal or arthritic mice at various concentrations in the presence of a 1:10 dilution of rabbit complement, and cell death was monitored by flow cytometry and fluorescence microscopy (Fig. 6). Complement alone, IgG isolated from BSA-immunized mice (data not shown) or normal mouse IgG had no cytotoxic effect on chondrocytes (Fig. 6A), whereas sera from arthritic mice killed isolated chondrocytes in a concentration-dependent manner (Fig. 6B and C). These observations, together with the immunohistochemistry results (Fig. 5), indicate a critical involvement of antibody (immune complex)-induced complement activation in the irreversible cartilage damage found in PGIA.

Discussion

The systemic autoimmune rodent models of RA (CIA, PGIA and KxB/N) are T cell-dependent, controlled by genetic (both MHC-dependent and MHC-independent) factors and exhibit a progressive pathology, leading to massive cartilage and bone erosion. In contrast to the systemic models, the serum/antibody transfer (26–28) and the antigen-induced arthritis models (29, 30) can be induced in any strain of mice and are transient in nature. The induced inflammatory events (synovitis) disappear within a few days or a couple of weeks without irreversible joint damage. However, a common denominator in all these chronic and transient arthritis models is an antibody-induced synovitis, which disappears in the absence, yet becomes a chronic and progressive form in the presence, of (auto)antigen-specific T and B cells. Among the above listed animal models of RA, PGIA is unique in terms of its female prevalence (31), particularly in aging females (32). It is a progressive disease and, in contrast to CIA, has a recessive inheritance pattern and does not require oil and/or mycobacterial components of Freund’s adjuvant to induce disease. This disease can be induced by transferring antigen (PG)-activated spleen cells into syngeneic mice, but neither B cells nor anti-PG antibodies alone are sufficient for arthritis induction in the absence of PG-specific T cells. Serum anti-PG antibody titers are the best biomarkers for arthritis development and disease progression. Therefore, we concluded that PGIA represents a T cell-dependent and antibody/B
T cell responses to citrullinated 5/4E8-containing peptide (38), suggesting that B cells and/or antibodies were critical in the development of arthritis, as seen in a longitudinal study where B cell recovery along with PG-specific antibodies in the sera of recipient SCID mice (19), the presence of the 70ATEGRVRVNSAYQDK sequence, designated as the 5/4E8 epitope, appears to be the most pathogenic and arthritogenic epitope (19). While any of these three epitopes, alone or in combination, can induce arthritis in genetically susceptible BALB/c mice (19), the presence of the 70ATEGRVRVNSAYQDK sequence, designated as the 5/4E8 epitope, appears to be the most pathogenic and arthritogenic epitope (19). Importantly, this peptide sequence, either as a whole or in part, has been identified as a T cell epitope in a subgroup of patients with RA or ankylosing spondylitis and in DR4/DQ8-humanized transgenic mice (34–37), and ~68% of RA patients exhibited T cell responses to citrullinated 5/4E8-containing peptide (38).

Because purified CD4+ T cells (>98%) from transgenic mice bearing TCR specific for the 5/4E8 peptide sequence could induce arthritis upon transfer into SCID mice (17), we first thought that there would be no requirement for additional B cell and/or anti-PG antibody assistance for the disease to develop. However, we surprisingly found incomplete B cell recovery along with PG-specific antibodies in the sera of recipient SCID mice with arthritis. In a longitudinal study, the appearance of anti-PG antibodies correlated with the onset of arthritis, suggesting that B cells and/or antibodies were required for disease development. The increasing amounts of serum anti-PG (auto)antibodies could not only predict arthritis onset but also correlate with the severity of arthritis symptoms. As non-T cell contamination was very low in the purified T cell population used for transfer (<2%, <1 × 10^5 non-T cells per transfer) and this contamination could not be reduced with additional purifications, we hypothesized that the number of antigen-specific activated T cells was sufficient to induce the clonal expansion of a small group of contaminating B cells, which resulted in an explosive increase in anti-PG antibody production. As we have previously demonstrated, CD4+ T cells were capable of providing sufficient help in vivo to PG-specific B cells (11, 39). These data suggested that antigen-activated TCR-Tg CD4+ T cells had the capacity to form a supportive milieu for selective expansion of a small population of antigen-specific B cells, which then produced detectable amounts of antigen (PG)-specific auto-antibodies.

Although homeostatic T cell proliferation has been studied extensively (20, 21, 40–42), far less information is available for homeostatic B cell proliferation (12, 13, 43, 44), particularly in a lymphopenic environment. The pathogenic role of autoantibodies and B cell homeostasis in autoimmunity is of critical importance (5, 6, 12–14) and these transfer experiments explored the importance of an activated antigen-specific T cell-dependent and highly specific B cell recovery in autoimmune arthritis. T cells from arthritic WT and naive TCR-Tg mice (34–37), and RA or ankylosing spondylitis and in DR4/DQ8-humanized transgenic mice (34–37), and ~68% of RA patients exhibited T cell responses to citrullinated 5/4E8-containing peptide (38).

Fig. 4. Arthritis onset/incidence, severity and immune responses in SCID recipient mice transferred first with naive or arthritic serum IgG. (A) Time of onset/incidence and (B) severity of arthritis in SCID recipients transferred with IgGs followed by secondary transfers as indicated by the symbols at the left corner of Panel A. (C) T cell responses were measured by IL-2 production and are shown as stimulation indices that are normalized to medium controls. (D) Serum anti-PG antibody levels measured by ELISA in SCID mice that received IgG followed by T cell and PG antigen transfer. Symbols below columns C and D correspond to groups (symbols) used in Panels A and B. The results are shown as the mean values (A and B) or the means ± SEM (C and D), n = 6–12 animals per group. Significant differences are indicated: *P < 0.05, **P < 0.01.
mice induced arthritis only when the recipient SCID mice received T cells with PG antigen, which first supported a homeostatic T cell proliferation and seemingly also triggered the recovery of a highly specific B cell population via an antigen-specific/controlled T and B cell interaction. It is important to note that 100 µg PG antigen alone, even in highly susceptible BALB/c mice, cannot induce a detectable immune response, particularly not arthritis. The second transfer, which included T cells, B cells or anti-PG antibodies from arthritic mice, was an additional challenge for provoking arthritis. Similar results were found in reciprocal experiments in which the injection of anti-PG antibody was followed by a transfer of T cells from arthritic animals. These observations suggest that neither activated T cells (from arthritic WT mice) nor the antigen specificity of T cells (naïve TCR-Tg) alone were sufficient to support the clonal expansion of antigen-specific B cells and their subsequent antibody production, which could then lead to arthritis induction. Thus, a threshold level of antigen-specific T cell number and activation is required to trigger antigen-specific B cell proliferation and subsequent antibody production.

The injection of anti-PG antibodies or B cells from arthritic mice had a similar effect on this adoptive transfer system, suggesting that the important role for B cells (at least in this case) was limited to anti-PG antibody production; other B cell functions (such as antigen presentation and cytokine production) might be critical but not exclusively required. Anti-PG antibodies enhanced the efficacy of PGIA development following transfer, even when administered before the T cells, indicating that the presence of antigen-specific IgG was necessary for disease induction. When anti-PG antibodies were injected before T cell transfer (Fig. 4) or simultaneously with T cell transfer (data not shown), arthritis developed within a few days in SCID recipients, whereas arthritis onset was delayed a few days when B cells from arthritic mice were injected prior to T cell transfer (Fig. 3). Thus, the presence of both antigen-specific T cells, which are joint/cartilage-specific in this case, and antibodies seems to be required for arthritis induction. To translate these observations into the natural situation that occurs in RA, antibodies produced by B cells that are receiving assistance from activated antigen-specific T cells within the proper milieu...
can cause synovitis, which becomes a chronic disease and leads to irreversible joint deformities in patients.

Anti-PG antibodies bind to the surface of cartilage, primarily recognizing the GAG side chains of PG molecules in the extracellular matrix and on the chondrocyte surface (Fig. 5), where newly synthesized PG molecules are entrapped by hyaluronan, as shown by immunoelectronmicroscopy (25, 45). More than 95% of anti-PG antibodies are IgG1 and IgG2a isotypes at the time of arthritis onset and can thus form immune complexes with PG molecules (33) on the cartilage surface or in the joint cavity and may subsequently activate the complement system. The intra-articular activation of the complement cascade might result in complement-dependent leukocyte recruitment (46) and chondrocytolysis (47), as shown in Fig. 6. Metalloproteinases released either by leukocytes in response to activation by pro-inflammatory cytokines or from damaged/dead chondrocytes and joint-invading neutrophils can degrade the extracellular matrix, making the cartilage surface more accessible to PG-specific antibody binding, which can thus amplify tissue damage.

The results from our experimental transfer system have demonstrated that neither T cells nor antibodies (or B cells) alone are sufficient to induce destructive joint inflammation. Rather, a well-controlled and sequential activated antigen-specific T cell-driven B cell selection and activation as well as subsequent autoantibody production can lead to the cartilage destruction executed by the complement system. Studies explaining how the PG-specific CD4+ T cells can control the highly selective proliferation of PG-specific B cells are currently ongoing. Although a uniform TCR repertoire (as present in TCR-Tg mice) does not exist in humans, an oligoclonal cumulative effect over many years may lead to the development of a similar condition. Overall, we believe that this ‘accelerated’ arthritis model may advance our understanding of the mechanisms that control RA.

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