

T_H1/T_H17 polarization and acquisition of an arthritogenic phenotype in arthritis-susceptible BALB/c, but not in MHC-matched, arthritis-resistant DBA/2 mice

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Abstract

Proteoglycan (PG) aggrecan-induced arthritis (PGIA) is a murine model of rheumatoid arthritis (RA). Although BALB/c and DBA/2 mice share the same MHC (H-2d) haplotype, the BALB/c strain is susceptible to PGIA, while DBA/2 mice are resistant. Therefore, these two inbred mouse strains provide an opportunity to study arthritis susceptibility factors excluding the effects of MHC-associated genetic components. The goal of this study was to monitor changes in the cellular composition and activation state following intra-peritoneal (i.p.) immunization to induce PGIA; additionally, we sought to identify new susceptibility factors by comparing PG-induced immune responses in BALB/c and DBA/2 mice. Upon i.p. PG injection, resident naive B1 cells are replaced by both T cells and conventional B cells in the peritoneum of BALB/c mice. These peritoneal T cells produce IFN γ and IL-17, cytokines shown to be important in RA and corresponding arthritis models. Moreover, peritoneal cells can adoptively transfer PGIA to SCID mice, demonstrating their arthritogenic properties. Our results indicate that repeatedly injected antigen leads to the recruitment and activation of immune cells in the peritoneum; these cells then trigger the effector phase of the disease. The migration and activation of T_H1/T_H17 cells in the peritoneal cavity in response to PG immunization, which did not occur in the arthritis-resistant DBA/2 strain, may be critical factors of arthritis susceptibility in BALB/c mice.

Introduction

Proteoglycan (PG) aggrecan-induced arthritis (PGIA) is one of the murine models of rheumatoid arthritis (RA). Repeated intra-peritoneal (i.p.) immunization with human cartilage PG (aggrecan) leads to joint inflammation, progressive cartilage destruction, bone erosion and ankylosis in diarthrodial joints of genetically susceptible BALB/c mice (1). Autoimmune features of PGIA, such as autoreactivity, T and B cell involvement and the cytokine profile in serum and synovial fluid closely resemble RA (2). Additional features common to RA are that this model includes early involvement of small synovial joints, similar X-ray and histopathologic abnormalities of peripheral joints, greater susceptibility of aging females, dominant, but not exclusive, effect of MHC, the polygenic character and the recessive inheritance of the disease [reviewed in (2)]. Immunization with human cartilage PG aggrecan leads to the breakdown of self-tolerance, and it

triggers an autoimmune response to murine cartilage PG due to the presence of cross-reactive epitopes in human and murine PGs (3–5).

The i.p. route of immunization appears to be critical for disease induction in the PGIA model. Although intra-dermal, subcutaneous or intra-muscular administration of the same PG antigen induces massive T and B cell responses, the severity of arthritis is weaker. Therefore, it is a logical approach to study immunological events in the peritoneum that may determine the development and progression of this autoimmune disease.

The peritoneal cavity is a unique microenvironment, lined by a serous membrane that consists of a mesothelial layer and sub-mesothelial connective tissue, containing blood and lymphatic vessels, and lymphoid tissue (6). 'Milky spots' on the human omentum, containing mostly macrophages

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and lymphocytes, may be considered secondary lymphoid foci within the peritoneal cavity (7). The peritoneal fluid contains different types of immune cells, such as macrophages, lymphocytes and dendritic cells (6). B1 lymphocytes represent a major cell population in the peritoneum; however, this cell type is rare in other tissues (8). A major function of these B1 cells is thought to be the production of IgM (9), although effective antigen presentation is another significant feature of these B1 cells (10, 11). Furthermore, two functionally different B1 cell sub-populations (B1a/b) can be defined based on the expression of the CD5 molecule, a cell surface receptor that is otherwise expressed by T cells (12).

Besides the site of immunization (antigen entry), the direction of the immune response is largely determined by the adjuvant. For example, CFA and dimethyldioctadecyl-ammonium bromide (DDA) are known to induce T_H1 type immune responses, while alum adjuvant favors T_H2 polarization (13–15). Adjuvants exert these effects mainly by activating the cells of innate immunity (14, 16). DDA enhances both cellular and humoral immune responses when injected i.p. but primarily supports cellular immunity when administered subcutaneously (17).

PGIA can be induced in genetically susceptible BALB/c mice, however, most other strains are resistant, for example the MHC-matched DBA/2 is a resistant strain (1, 18, 19). Although the difference in arthritis susceptibility between these two strains has been known for >20 years (18) and the fundamental cellular and immunological mechanisms leading to the development of PGIA have been described [see for review, ref. (2)], the most critical susceptibility factors have not been elucidated. PGIA is a T cell-dependent, B cell- and/or antibody-mediated disease (2, 18). The disease cannot be adoptively transferred by T cells, B cells or antibodies alone to naive BALB/c or SCID mice; successful transfer requires both T cells and either B cells or antibodies from arthritic animals (5, 20). A shift toward a T_H1 -dominant cytokine profile is necessary for disease development (21, 22), and IL-4 has been shown to act as a negative regulator, or a protective cytokine, in PGIA (23).

The induction phase of this autoimmune disease begins with the activation of PG-specific T and B cells, eventually overcoming self-tolerance; this takes place during the immunization regime (18). Therefore, we chose to characterize the early events following i.p. immunization, when joint inflammation was not yet present. We then extended our study by analyzing changes in cellular composition, activation markers, cytokine production, T cell response and antibody production during the entire immunization period.

Here we report that the earliest events in the peritoneum following i.p. PG immunization are the disappearance of resident naive B1 cells and their replacement by T cells and conventional B cells in BALB/c mice. This re-organization of the cellular composition following repeated injections of antigen suggests that the generation of activated effector cells, beginning in the peritoneum, triggers the effector phase of the disease. Moreover, the arthritogenic potential of these activated peritoneal cells is confirmed by their ability to adoptively transfer PGIA to SCID mice. Adjuvant DDA plays a crucial role in initiating the change of cellular composition in the peritoneal cavity, but DDA itself is not capable of disease induction. Changes in the cellular composition in re-

sponse to PG injection, which do not occur in the MHC-matched arthritis-resistant DBA/2 strain, appear to determine the arthritis susceptibility of BALB/c mice to PGIA.

Materials and methods

Chemicals

All chemicals, unless indicated otherwise, were purchased from Sigma Chemical Co. (St Louis, MO, USA) or Fisher Scientific (Chicago, IL, USA). Mouse recombinant cytokines and ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA) or BD Biosciences (San Jose, CA, USA). PBS (pH 7.4) was used for washing and short-term storage of cells until use. Cell surface labeling with mAbs was carried out in flow cytometry wash buffer (PBS containing 0.1% NaN_3 and 0.1% BSA). Intracellular FoxP3 labeling was performed in permeabilization buffer after fixation of surface-stained cells (eBioscience, San Diego, CA, USA).

Antigen, animals and immunization

Articular cartilage was collected from patients undergoing knee joint replacement surgery. The collection of cartilage from consenting patients was approved by the Institutional Review Board of Rush University Medical Center. Cartilage PG (aggrecan) was extracted and depleted of glycosaminoglycan side chains as described (24).

All animal procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Rush University Medical Center. Female BALB/c and DBA/2 mice of 16–20 weeks of age and sex- and age-matched SCID mice of BALB/c background (NCI/NCrC-B-17-*scid/scid*; henceforth SCID) were purchased from the National Cancer Institute (Frederick, MD, USA). Mice were immunized via i.p. injection with an emulsion of cartilage PG (100 μg protein) and 2 mg DDA adjuvant on days 0, 21 and 42 (24). Groups of animals were sacrificed 5, 10 and 20 days after the first, second and third immunizations, respectively. Adoptive transfer of PGIA from BALB/c to SCID mice was performed as described (20). Briefly, $1-3 \times 10^6$ peritoneal lavage fluid (PLF) cells, harvested 10 days after the first, second and third immunization, were injected i.p. with 100 μg PG into SCID mice, twice 10–14 days apart. In preliminary experiments, PLF cells of PG/DDA-immunized DBA/2 mice (after four immunizations) were also used for adoptive transfer, and cell compositions of the PLF were determined in CD44- and CD62L-deficient BALB/c mice after PG/DDA injections.

Clinical assessment of arthritis

PG-immunized BALB/c, DBA/2 and recipient SCID mice were examined three to four times a week for clinical symptoms of arthritis. The time of onset and incidence of arthritis were recorded, and severity was scored visually based upon the swelling and redness of each paw, ranging from zero to four, yielding a maximum severity score of 16 per mouse (1, 18, 24).

mAbs

The following fluorochrome-labeled or biotinylated mAbs were purchased from BD Biosciences: FITC-conjugated Armenian

hamster anti-mouse CD69 (clone H1.2F3), PE-conjugated rat anti-mouse CD8 (clone 53-6.7), PerCP-Cy5.5-conjugated rat anti-mouse CD4 (clone RM4-5), PE-Cy7-conjugated rat anti-mouse B220 (clone RA3-6B2), Allophycocyanin (APC)-conjugated rat anti-mouse CD25 (clone PC61), APC-Cy7-conjugated Armenian hamster anti-mouse CD3 (clone 145-2C11), FITC-conjugated rat anti-mouse IgD (clone 11-26c.2a), PE-conjugated rat anti-mouse CD43 (clone S7), PerCP-Cy5.5-conjugated rat anti-mouse IgM (clone R6-60.2), PE-Cy7-conjugated rat anti-mouse CD19 (clone 1D3), APC-conjugated rat anti-mouse CD5 (clone 53-7.3), biotin-conjugated rat anti-mouse CD23 (clone B3B4), Alexa Fluor 488-conjugated rat anti-mouse CD44 (clone IM7) and PE-conjugated rat anti-mouse CD62L (clone MEL-14). PE-conjugated rat anti-mouse FoxP3 (clone FJK-16s) was purchased from eBioscience. Biotinylated mAbs were detected by the use of APC-Cy7-conjugated streptavidin.

Fluorescent cell surface labeling

We used a three- to six-color labeling technique for the simultaneous detection of cell surface and intracellular molecules in cells harvested from the PLF and spleen of mice. Briefly, 1×10^6 cells were seeded in 96-well U-bottom assay plates (BD Falcon). Fc receptors were blocked with anti-CD16/32 (FcBlock, BD Biosciences) at 4°C for 15 min, and cells were then incubated with mAb cocktails in 100 μ l flow cytometry staining buffer at 4°C in the dark for 30 min. Cells were washed twice and finally re-suspended in 200 μ l of 0.1% formaldehyde in PBS. If biotinylated mAb was used, fluorochrome-labeled streptavidin was added to the samples after washing, and cells were then incubated at 4°C in the dark for another 30 min. Intracellular FoxP3 was stained after fixation and permeabilization of cells. Isotype-matched control IgG (rat or hamster) for each color was used for background staining.

Flow cytometric measurement and phenotypic analysis, definition of cell populations

Samples were measured and analyzed using a FACS Canto II flow cytometer and FACS DIVA software (BD Biosciences). Acquisition was performed by gating on lymphoid cells within forward/side-scatter dot plots. Cell surface marker-defined populations were as follows—CD3+: total T cells; CD3+ CD4+: CD4+ T cells; CD3+ CD8+: CD8+ T cells; CD4+ CD25+ FoxP3+: regulatory T cells (Tregs); B220+: total B cells; IgD^{low} IgM^{high} CD19+ CD23– CD5+/-: B1 cells (B1a/b) and IgD^{high} IgM^{low} CD19+ CD23+: conventional B2 cells (25). Specific cell proportions were expressed as percentage of total cells unless otherwise stated. Data were collected from 10 000 cells within the lymphoid gate in each sample. We used fluorescent histogram plots to compare mean fluorescence intensities of different samples and to calculate the proportions of positively stained cells.

Measurement of PG-specific antibodies and T cell response

Serum samples, PLF and spleen cells were harvested from BALB/c and DBA/2 mice before and at different time points after each immunization and also from adoptively transferred SCID mice 40–50 days after the second cell transfer. PG-

specific antibodies were measured using ELISA as described (20, 24). In brief, sera were diluted at the range of 1:100–1:102400, and PG-bound antibodies were detected with peroxidase-conjugated rabbit anti-mouse IgG1 or IgG2a (Zymed Laboratories, San Francisco, CA, USA).

Antigen-specific T cell responses were measured in quadruplicate samples of spleen cells (3×10^5 cells per well in 200 μ l on 96-well plates) cultured in the absence or presence of 50 μ g human PG protein per milliliter. T cell proliferation was assessed using [³H]thymidine incorporation on day 5 of culture (20, 26). Spontaneous and antigen-specific production of IL-4 (detection level: 10 pg ml⁻¹), IL-6 (detection level: 20 pg ml⁻¹), IL-12/23 p40 (detection level: 30 pg ml⁻¹), IL-17 (detection level: 20 pg ml⁻¹), tumor necrosis factor α (TNF α) (detection level: 50 pg ml⁻¹) and IFN γ (detection level: 80 pg ml⁻¹) was measured in PLF and spleen cell culture supernatants (1.8×10^6 cells per well in 600 μ l on 48-well plates) harvested on day 5 using capture ELISAs (from BD Biosciences or R&D Systems), and the results were expressed as cytokine (picograms) secreted by 10^6 cells (26).

Statistical analysis

Descriptive statistics were used to determine group means and standard error of the mean (SEM) (mean \pm SEM) unless otherwise stated. The difference between two groups was tested for statistical significance using Student's *t*-test, and the difference among three or more groups was tested using analysis of variance. A *P* \leq 0.05 value was considered to be statistically significant.

Results

Immunophenotypic characterization of naive BALB/c and DBA/2 mice

Since basal immunologic traits of mice may affect the immune response to antigen, in this case PG, which causes arthritis, we first assessed the cellular composition of the PLF, the first site of antigen entry after i.p. injection, and the spleen, which is involved later in the systemic response, in naive BALB/c mice, focusing on lymphoid cell types.

Table 1 compares the distribution and phenotypic properties of T and B cells in these two compartments in naive BALB/c mice. The CD4+/CD8+ T cell ratio was higher in the PLF than the spleen, but the CD62L^{high}/CD44^{high} CD4+ ratio, which denotes resting and activated CD4+ T cells, respectively, was similar in the two compartments (Table 1). A more detailed cell surface marker analysis of the B cell pool in naive BALB/c mice showed that in the PLF, a majority of B cells belonged to the B1 cell sub-population, while the spleen contained more conventional B (B2) cells (Table 1). In the PLF, the CD5+/CD5– B1 cell ratio was 0.145 (Table 1), indicating the dominance of CD5– B1 cells in the naive peritoneal milieu.

In MHC-matched, but PGIA-resistant, naive DBA/2 mice, we found significantly fewer T cells but similar numbers of B cells as in naive BALB/c mice, both in the PLF and the spleen (Tables 1 and 2). The CD4+:CD8+ T cell ratio was higher in DBA/2 than in BALB/c mice (Tables 1 and 2). There were significantly fewer CD62L^{high} CD4+ T cells, CD44^{high} CD4+ T cells and Tregs in the spleen of naive DBA/2 mice

Table 1. Immunophenotypic composition of lymphoid cells in PLF and spleen of naive BALB/c mice

	PLF	Spleen
T cells (CD3+)	0.172 ± 0.039*	19.690 ± 1.858*
CD4+ T cells	0.100 ± 0.029*	13.820 ± 1.337*
CD8+ T cells	0.029 ± 0.011*	5.201 ± 0.594*
CD4/CD8	3.500	2.657
CD62L ^{high} CD4+	0.058 ± 0.012*	7.286 ± 0.883*
CD44 ^{high} CD4+	0.038 ± 0.012	5.410 ± 0.690*
CD62L ^{high} /CD44 ^{high}	1.530	1.347
Tregs	0.013 ± 0.002*	1.797 ± 0.166*
B cells (B220+)	1.779 ± 0.272	29.200 ± 1.635
B1 cells ^a	1.228 ± 0.315	3.135 ± 0.743
CD5+ B1 cells	0.154 ± 0.047*	0.149 ± 0.074
CD5- B1 cells	1.063 ± 0.036	2.526 ± 0.743
CD5+/CD5-	0.145	0.059
Conventional B (B2) cells	0.444 ± 0.093*	23.330 ± 3.715
B1/B2	2.766	0.134

^aIn spleen, this population is comprised of transitional B cells, marginal zone B cells and B1 cells. Numbers represent mean ± SD of cell numbers ($\times 10^6$) of five independent measurements. Bold-faced numbers indicate significant ($P < 0.05$) differences when values from BALB/c and DBA/2 mice (Table 2) are compared (*BALB/c value > DBA/2 value).

when compared with BALB/c (Tables 1 and 2). In the PLF, the B1:B2 (conventional) cell ratio was higher, but the CD5+/CD5- B1 cell ratio was lower in DBA/2 mice than in BALB/c mice (Tables 1 and 2).

Changes in cellular composition of the PLF upon i.p. immunization with PG

The most efficient way of immunization leading to PGIA is the i.p. injection of PG with either CFA (1) or DDA (14). The use of DDA, instead of CFA, however, became a routine process lately to avoid the side effects of CFA including the complicating effects of heat shock proteins, bacterial cell wall components etc. present in mycobacteria preparations (14). The peritoneal cavity is the entry site of the antigen, where the cells of the immune system first encounter PG and adjuvant. Therefore, we monitored the changes in cellular composition of the PLF at different time points during immunization with PG and DDA, as well as when DDA adjuvant was injected without antigen in both BALB/c and DBA/2 mice.

The number of T cells in BALB/c PLF increased significantly compared with naive animals by 10–20 days after the first PG injection (Fig. 1A and G). Most of these T cells were CD4+, and 26.4 ± 5.4% of the CD4+ cells expressed high levels of CD25 already at day 5 after the first i.p. injection of PG in DDA (data not shown). There was a 2-fold increase (up to 79.3 ± 2.4%) in the CD44^{high} CD4+ cell percentage, compared with that measured in naive animals (data not shown). After a transient decrease, detected between the second and third i.p. PG/DDA injections, the number of T cells increased again after the third immunization in the PLF of BALB/c mice (Fig. 1A and G). In the PLF of DBA/2 mice, the T cell number remained lower than in BALB/c through the whole immunization period. However, there were transient but

Table 2. Immunophenotypic composition of PLF and spleen of naive DBA/2 mice

	PLF	Spleen
T cells (CD3+)	0.052 ± 0.010	8.430 ± 1.686
CD4+ T cells	0.032 ± 0.010	6.575 ± 1.349
CD8+ T cells	0.005 ± 0.005	1.630 ± 0.337
CD4/CD8	6.500	4.034
CD62L ^{high} CD4+	0.006 ± 0.003	1.966 ± 0.309
CD44 ^{high} CD4+	0.024 ± 0.003	3.498 ± 0.447
CD62L ^{high} /CD44 ^{high}	0.260	0.562
Tregs	0.005 ± 0.001	1.203 ± 0.184
B cells (B220+)	1.295 ± 0.184	28.887 ± 1.068
B1 cells ^a	0.875 ± 0.370	3.372 ± 0.899
CD5+ B1 cells	0.030 ± 0.007	0.112 ± 0.011
CD5- B1 cells	0.841 ± 0.367	3.035 ± 0.899
CD5+/CD5-	0.035	0.037
Conventional B (B2) cells	0.221 ± 0.112	24.335 ± 1.686
B1/B2	3.966	0.139

^aIn spleen, this population is comprised of transitional B cells, marginal zone B cells and B1 cells. Numbers represent mean ± SD of cell numbers ($\times 10^6$) of five independent measurements. Statistical differences of different cell populations and/or ratios in different lymphoid compartments are not indicated because the presence of these cells represents the normal (naive) cell distribution in the PLF and spleen of naive (non-immunized) DBA/2 mice. Significant differences between BALB/c and DBA/2 are indicated in Table 1.

significant increases in the T cell number after the second and third i.p. PG + DDA injections compared with the naive DBA/2 PLF (Fig. 1A).

In contrast to the significant expansion of T cells in the peritoneal cavity of BALB/c mice in response to i.p. immunization, the number of B1 cells (IgD^{low} IgM^{high} CD19+ CD23-) measured in the PLF of naive mice declined from $>10^6$ cells to $<10^5$ cells within 5 days and remained at a significantly lower level throughout the immunization period (Fig. 1B and G). The number of conventional B cells (IgD^{high}, IgM^{low}, CD19+ and CD23+) dropped after each i.p. injection but always returned close to the levels measured before immunization (Fig. 1C and G). Changes in the B1 cell and conventional B cell numbers showed similar kinetics after i.p. PG injections during the entire immunization period in both BALB/c and DBA/2 mice (Fig. 1B and C).

To further dissect whether the changes in cellular composition described above could be attributed to the antigen (PG) or the adjuvant (DDA), we repeated the same immunization protocol with PBS/DDA and monitored the same parameters. We found that DDA alone caused very similar changes in cellular composition (increased T cell and conventional B cell numbers and decreased B1 cell numbers) in the peritoneal cavity of BALB/c mice to what was observed when the antigen (PG) was also present (Fig. 1D–F). However, the kinetics of the T cell number changes were different when we injected mice with DDA alone: the T cell number did not increase after the first DDA injection. On the contrary, especially after the second injection of adjuvant alone, T cell and conventional B cell number changes were more pronounced than after the second injection of PG in adjuvant (Fig. 1D and F). Importantly, in the peritoneal cavity of DBA/2 mice, the DDA injections affected only the B1 cell

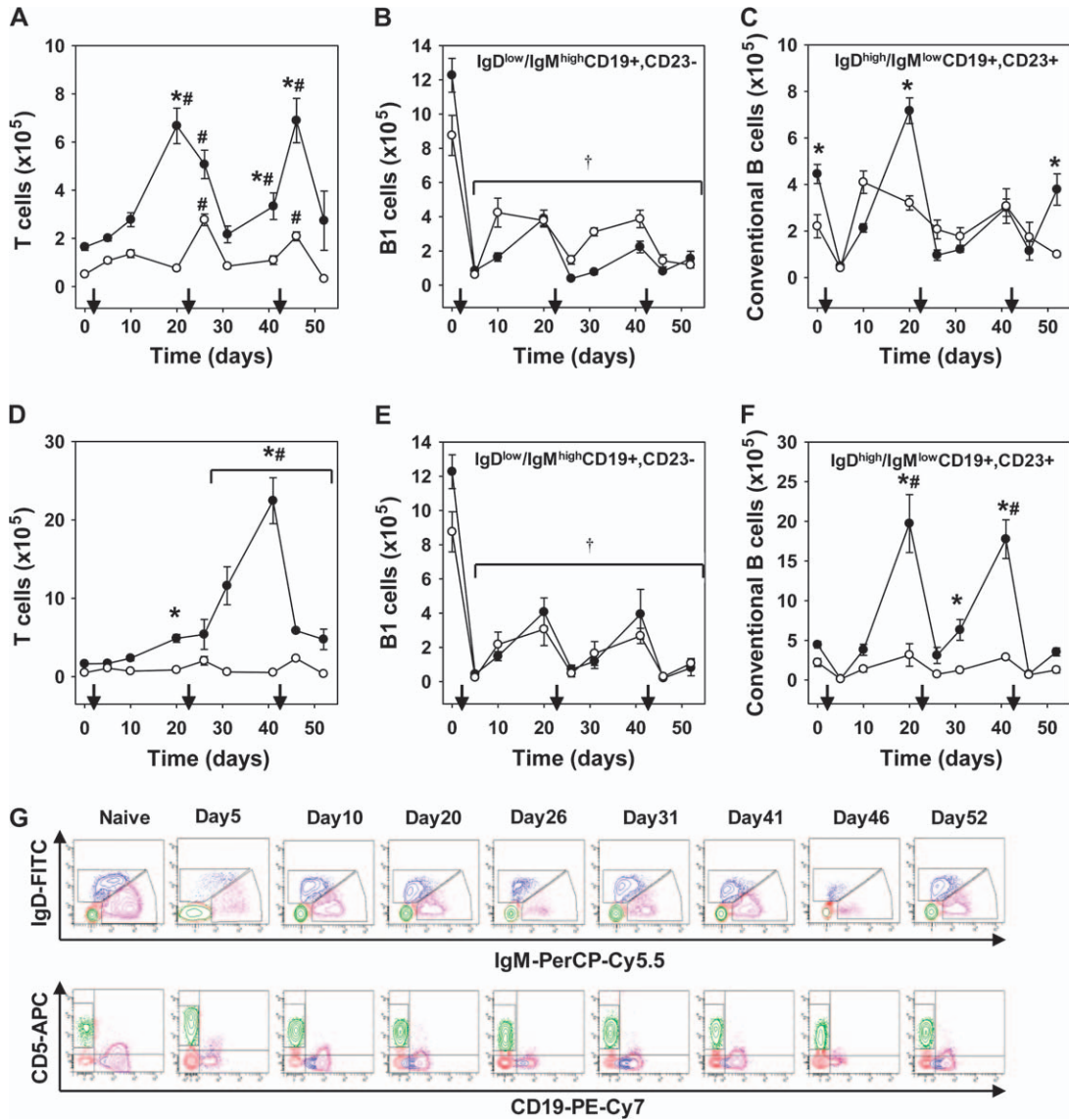


Fig. 1. Cellular composition changes in the peritoneum of BALB/c (black circles) and DBA/2 (open circles) mice in response to PG immunization (A, B and C) or repeated DDA adjuvant injections (D, E and F) as assessed by flow cytometry. Time-dependent changes in the number of T (A and D), B1 (B and E) and conventional B (C and F) cells in the PLF of BALB/c and DBA/2 mice. Values significantly higher ($*P < 0.05$) in BALB/c than DBA/2, or values that were significantly higher ($\#P < 0.05$) or lower ($\dagger P < 0.05$; in both strains) than in naive animals, are indicated. Data present mean \pm SEM of three to five animals at each time point. Arrows on the x-axis (days 0, 21 and 42) indicate the injections. (G) Flow cytometric contour plots show T cells (green), B1 cells (magenta) and conventional B cells (blue) based on their IgM, IgD, CD19 and CD5 staining in BALB/c mice.

population (Fig. 1E) and did not cause the T cell or conventional B cell number to increase as seen in BALB/c mice (Fig. 1D and F).

In vitro cytokine production by BALB/c PLF cells

Because a significant T cell influx was detected in the peritoneum of BALB/c mice as early as day 10 after the first i.p. injection of PG/DDA and since the number of T cells in the peritoneum (Fig. 1A) and their activation profile (as measured by CD25 and CD44) also showed dynamic changes during the immunization period, we measured cytokines that are known to be important regulators of the immune

response in PGIA in supernatants of cultured PLF cells (Fig. 2A).

After the first PG injection, on days 5, 10 and 20, there were measurable amounts of spontaneous IL-12/23 p40, IL-17 and IFN γ in BALB/c PLF cultures (Fig. 2A); the level of IFN γ was elevated after the second PG injection. In response to *in vitro* PG stimulation, PLF cells secreted large amounts of IFN γ , IL-17 and IL-6 and lesser amounts of TNF α and IL-4 (Fig. 2A). In general, the *in vitro* cytokine-producing capacity of PLF cells decreased after the third PG injection, with TNF α and IL-4 in PG-stimulated PLF cell cultures being the only exceptions (Fig. 2A).

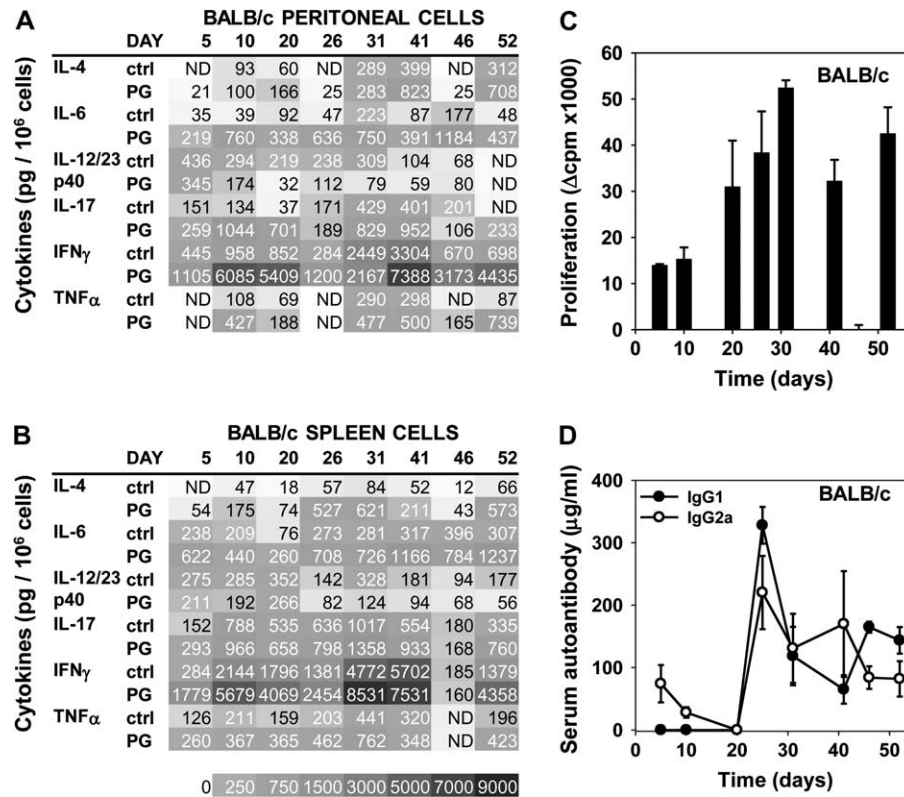


Fig. 2. Kinetics of the immune response to PG in BALB/c mice. Spontaneous (ctrl) and PG-induced (PG) *in vitro* cytokine production of PLF (A) and spleen (B) cell cultures from BALB/c mice ($n = 3$ animals at each time point). Numbers show the mean cytokine concentrations of experimental groups (pg 10^{-6} cells) (mean \pm SEM values are listed in Supplementary table 1, available at *International Immunology Online*). Grayscale intensity coding of cytokine concentrations is shown below panel B. (C) *In vitro* proliferation response measured in the presence of PG antigen. Values represent Δ counts per minute (c.p.m.) (PG-stimulated c.p.m. – ctrl c.p.m.) (mean \pm SEM) of spleen cells ($n = 3$ animals were sacrificed at each time point). (D) Serum IgG1 (black symbols) and IgG2a (white symbols) concentrations against mouse PG ($n = 3$ animals at each time point).

Since we noticed earlier that DDA alone also triggered T cell influx into the peritoneal cavity, we measured the spontaneous *in vitro* cytokine-producing capacity of PLF cells isolated from BALB/c mice injected once with PBS/DDA. We detected IL-12/23 p40, IL-17, IFN γ and TNF α , although in lesser amounts than in the supernatants of PLF cells of mice immunized with PG in DDA (data not shown).

In conclusion, BALB/c PLF cells can produce considerable amounts of IFN γ , IL-17 and IL-12/23 p40, *in vitro* spontaneously, and the levels of these cytokines further increase upon antigen challenge, except for the IL-12/23 (Fig. 2A). A similar spontaneous cytokine secretion pattern, with the exception of IL-4 and IL-6, was found when cells were harvested from PBS/DDA-injected mice (data not shown).

Cytokines measured in BALB/c spleen cultures

After i.p. injection, antigen is first processed by local cells; however, the systemic response is controlled by the spleen. Since the direction of the immune response, as determined by the T_H1/T_H2 balance, is mainly regulated by pro- and anti-inflammatory cytokines in different lymphatic compartments (27), we compared cytokine production in non-stimulated and PG-stimulated spleen cell cultures (Fig. 2B–C; Supplementary table 1, available at *International Immunology Online*).

Spleen cells spontaneously secreted relatively large amounts of IL-6, IL-12/23 p40, IL-17 and IFN γ during the entire immunization period (Fig. 2B; Supplementary table 1, available at *International Immunology Online*). Increased production of IL-17 and IFN γ was detected as early as 10 days after the first PG injection, and IL-17 and IFN γ levels reached a maximum in spleen cell cultures after the second PG injection (Fig. 2B; Supplementary table 1, available at *International Immunology Online*). Spleen cells from mice that received only DDA secreted measurable amounts of IL-12/23 p40, IL-17, IFN γ and TNF α 5 and 10 days after a single DDA injection (data not shown).

Splenic T cell response to PG and serum antibody levels in BALB/c mice

Next, we monitored the *in vitro* proliferation responses of spleen cells to PG (Fig. 2C). BALB/c splenocytes showed significant proliferation upon PG stimulation as early as 5 and 10 days after the first PG injection (Fig. 2C), and spleen cell proliferation further increased after the second and third PG injections (Fig. 2C). The results of the proliferation assays correlated with the ratio of activated CD25^{high} CD4⁺ T cells in the spleen as assessed by flow cytometry at the same time points (data not shown).

It has been previously shown that in the development of PGIA, in addition to T cells, antibodies produced by B cells in response to PG immunization also play an indispensable role (2, 18). Since cartilage destruction in PGIA is most likely amplified by auto-antibody production against mouse cartilage PG, we assessed circulating mouse PG-specific auto-antibody levels (Fig. 2D). BALB/c mice produced significant amounts of IgG1 and IgG2a to mouse PG after the second and third PG immunizations (Fig. 2D). As expected, increasing concentrations of IgG1 and IgG2a to human PG were also detectable in the serum of mice after the second immunization (data not shown).

Comparison of PG-specific immune responses in BALB/c and DBA/2 mice

The significant difference in the T cell numbers in PLF between the two strains (Fig. 1A) already indicated that there could be substantial differences between their immune responses to PG. Beyond exploring the differences in cellular composition between the two strains (Tables 1 and 2), we monitored cytokine and antibody production following repeated PG immunization that may have contributed to the activation of T and B cells and their differentiation into an arthritogenic phenotype in BALB/c mice (Fig. 2). To decipher which components of these 'BALB/c-specific' features play

crucial roles in PGIA susceptibility, we made a systematic comparison with arthritis-resistant DBA/2 mice using approximately the same number of animals and exactly the same immunization protocol. Although the DBA/2 strain carries the same MHC (H-2d) as the BALB/c strain, DBA/2 mice are resistant to PGIA (2, 18, 24).

The *in vitro* cytokine production profile of PLF cells harvested from DBA/2 mice (Fig. 3A) was fundamentally different from that of BALB/c (Fig. 2A). IL-6 was the most dominant cytokine at all time points of the immunization protocol in DBA/2 PLF cell cultures, whereas minimal or no IL-4, IL-12/23 p40, IL-17 or IFN γ production was detected (Fig. 3A). DBA/2 spleen cells produced significantly less IL-17 and IFN γ at almost all time points after immunization than BALB/c splenocytes (Figs 3B and 2B; Supplementary table 1, available at *International Immunology Online*).

DBA/2 spleen cultures exhibited higher *in vitro* proliferation responses to PG when compared with identical BALB/c cell cultures between the second and third immunizations (Fig. 3C). However, after the third immunization, this spleen cell proliferation capacity declined (Fig. 3C). The auto-antibody levels against mouse PG in the sera of DBA/2 mice were only 10–30% of those measured in BALB/c mice when compared at the same time points of immunization (compare Fig. 3D with Fig. 2D).

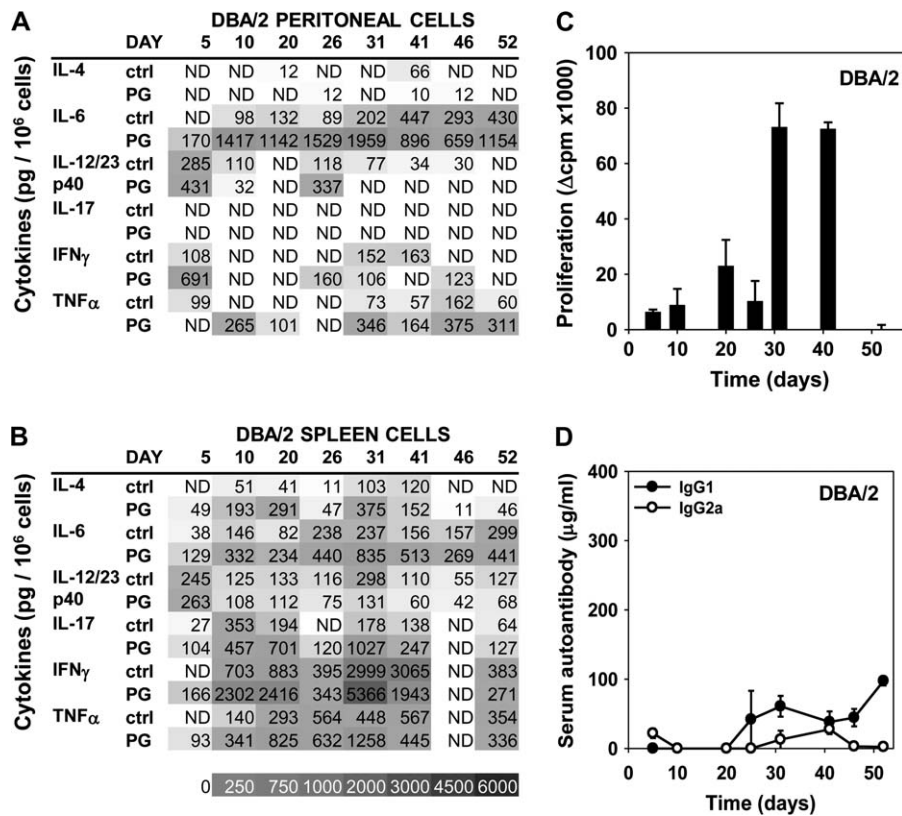


Fig. 3. Kinetics of the immune response to PG in DBA/2 mice. *In vitro* cytokine production of PLF (A) and spleen (B) cells. Numbers show the mean cytokine concentrations of experimental groups (pg 10⁻⁶ cells). Grayscale intensity coding is shown below the spleen panel. (C) *In vitro* T cell proliferation in response to PG stimulation. Bars represent Δ counts per minute (c.p.m.) (PG-stimulated c.p.m. – ctrl c.p.m.) (mean \pm SEM) of spleen cells ($n = 3$ animals at each time point). (D) Serum IgG1 (black symbols) and IgG2a (white symbols) concentrations against mouse PG ($n = 3$ animals at each time point) in DBA/2 mice. Note, the y-axis scale is the same as in Fig. 2(D).

Transfer of PGIA to SCID mice with PLF cells from BALB/c mice

PGIA can be adoptively transferred into syngeneic SCID mice using spleen cells from arthritic BALB/c donors and PG antigen (20). Side-by-side comparison of BALB/c and DBA/2 immune responses to PG revealed the most pronounced difference in T cells and their cytokine production in the peritoneal cavity. Since the arthritogenic potential of peritoneal cells from PG-immunized BALB/c mice has never been tested, we injected PLF cells, comprised mostly of T cells and conventional B cells and some B1 cells (Fig. 1).

The results of the transfer experiments are summarized in Table 3. PGIA was transferred most efficiently (100% incidence, highest arthritis score) with PLF cells from arthritic BALB/c mice (Table 3, Group C). Importantly, transfer of PGIA was also possible with PLF cells harvested 10 days after the second i.p. injection from not yet arthritic BALB/c mice (Table 3, Group B). There was no arthritis at all in the SCID recipients when PLF cells harvested from mice immunized only once with PG and DDA were transferred (Table 3, Group A). PLF cells from DBA/2 mice induced mild arthritis (using the same conditions) only after repeated (four times) PG/DDA injections (data not shown).

Table 3. Summary of the results of peritoneal cell transfer to SCID mice

Recipient (SCID) group	A	B	C
Donor (BALB/c) status	Non-arthritic	Non-arthritic	Arthritic
Donor immunization (times)	1	2	3
Incidence (arthritic/total)	0/3	2/3	4/4
Onset ^a	NA	19–31	19–22
Maximum arthritic score	NA	5.5 ± 1.5	6.8 ± 1.5
<i>In vitro</i> spleen cell proliferation ^b	1.5 ± 0.2	0.8 ± 0.02	2.3 ± 0.6
<i>In vitro</i> spleen cell cytokine production (pg million cells ⁻¹)			
IL-4	214 ± 33	143 ± 96	457 ± 26*
IL-6	111 ± 17	182 ± 103	532 ± 63*
IL-17	ND	ND	532 ± 64
IFN γ	ND	ND	1343 ± 73
TNF α	42 ± 27	200 ± 180	157 ± 109
Serum antibodies to			
hu PG IgG (mg ml ⁻¹)	ND	2.6 ± 1.1	6.2 ± 1.1*
hu PG IgG2a (mg ml ⁻¹)	ND	ND	0.21 ± 0.11
mPG IgG1 (lg ml ⁻¹)	8.9 ± 8.7	12.7 ± 12.5	70 ± 11.3*
mPG IgG2a (lg ml ⁻¹)	46.3 ± 45.5	ND	ND

PLF cells from BALB/c donors after one, two or three PG immunizations were injected with PG into SCID recipients. Transfers were repeated after 14 days. Table shows the incidence, onset and arthritis score of transferred SCID mice, the *in vitro* measured immunologic parameters (PG-specific T cell proliferation and cytokine production) and serum antibody levels in recipients at the end of transfer experiments (45 days after the second transfer). All values are shown as mean ± SEM. Numbers in bold face (Group C) indicate significant (**P* < 0.05) differences when compared with either Group A or B. NA, not applicable; ND, not detected; hu PG, human PG; mPG, mouse PG.

^aDays after the second transfer.

^bSI, stimulation index.

SCID mice were sacrificed 45 days after the second cell transfer, and cells from PLF and spleen were harvested. Flow cytometric analysis revealed that these compartments in all SCID recipients were efficiently repopulated with donor BALB/c PLF lymphocytes, mainly T cells (most of which were CD44^{high} CD4+), conventional B cells and B1 cells (Fig. 4A and B; Supplementary table 2, available at *International Immunology Online*). Moreover, large proportions of both T and B cells expressed CD69 and CD25 activation markers at high levels (Fig. 4C and D; Supplementary table 2, available at *International Immunology Online*).

PG-induced *in vitro* T cell proliferation was highest in Group C SCID mice (Table 3). *In vitro* production of IL-4 and IL-6 by spleen cells upon PG stimulation was significantly higher in Group C than in Group A or B (Table 3). IFN γ and IL-17 productions were only detectable in spleen cultures of Group C (Table 3). Serum IgG1 to both human and mouse PG was highest in Group C, while IgG2a to human PG was only detectable in Group C (Table 3).

In summary, we found that BALB/c PLF cells co-injected with antigen were able to transfer PGIA to SCID hosts even if the cells were harvested from twice-immunized, but not yet arthritic, donor mice. Importantly, these PLF cells were already committed to be highly arthritogenic, since the transfer of 1–3 × 10⁶ cells was sufficient to induce arthritis, whereas conventional transfer usually required 1–2 × 10⁷ spleen cells from severely arthritic BALB/c mice (20).

Discussion

In this study, we have analyzed and compared the distribution of T and B lymphocytes and their responses to PG immunization in PLF and spleen of arthritis-susceptible BALB/c and arthritis-resistant DBA/2 mice. The early changes induced by PG immunization in these tissue compartments, especially in the peritoneal cavity, most likely led to the differentiation and activation of arthritogenic T and B cells which then triggered synovial joint inflammation in BALB/c mice. The adjuvant (DDA) proved to be a major contributor to these early cellular changes, but DDA alone was insufficient to induce any inflammatory reaction in synovial joints as confirmed by histology.

Relatively, early after the first i.p. immunization, there was a robust T cell influx and/or proliferation in the peritoneal cavity in BALB/c mice, accompanied by the disappearance of the resident naive B1 cell population. Upon repeated immunizations, this skew in the cellular composition became more apparent. The continuously elevated T cell numbers were most likely the result of both the homing of antigen-specific T cells to the peritoneum and the local expansion of resident T cells. This notion is further supported by the fact that the absence of neither CD44 nor CD62L molecules [in knockout (KO) mice] could completely abolish the T cell number increase in the peritoneal cavity (data not shown). The transient decrease in T cell number after the second PG/DDA injection might be the result of the systemic (joint) recirculation of autoreactive T cells and activation-induced cell death locally in the peritoneal cavity (28). The T cell-enriched peritoneal effusion in BALB/c mice, which could not be detected in arthritis-resistant DBA/2 mice, might well

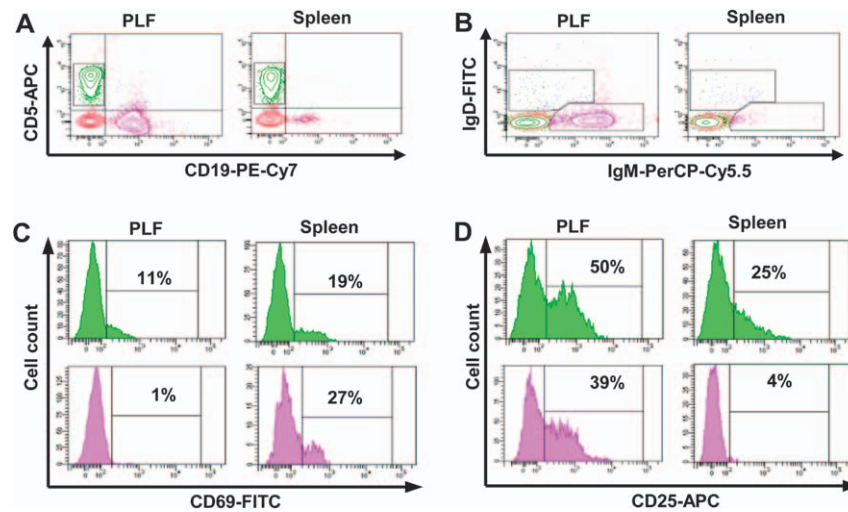


Fig. 4. Flow cytometric evaluation of cellular reconstitution in SCID recipients after transfer of PLF cells from arthritic BALB/c donors (Table 3, Group C). (A and B) Representative flow cytometric contour plots show the cellular composition of PLF and spleen of SCID recipients. (C and D) Activation marker (CD69 and CD25) expression of T and B cells is shown on histograms (T cells: green and B cells: magenta). Corresponding data (PG-induced T cell proliferation, PG-induced *in vitro* cytokine production and serum antibodies to human and mouse PG) of SCID recipients from all groups are summarized in Table 3, and more detailed information for cellular composition of PLF and spleen are summarized in Supplementary table 2 (available at *International Immunology Online*).

be one of the critical upstream contributors that set the stage for the development of PGIA.

In BALB/c mice, a dominant production of pro-inflammatory cytokines (IL-6, IL-12/23 p40, IFN γ , IL-17 and TNF α) was already detected at the early stages of i.p. immunization-induced peritonitis at 5 and 10 days following the first i.p. injection. This was not due to the injection itself, because i.p. injection of 200 μ l PBS did not induce cytokine production and had no evident effect upon peritoneal cell composition (our unpublished data). The massive shift in cellular composition and cytokine production in PLF, which might be due to the combined effects of adjuvant and strain specific factors, may dictate arthritis susceptibility in BALB/c and resistance in DBA/2 mice.

IL-12 and IL-27 have been shown to promote T_h1 differentiation, whereas IL-6, transforming growth factor β and IL-23 (which shares a common p40 chain with IL-12) promote and maintain the T_h17 lineage (29, 30). Both T_h1 and T_h17 cytokines have been associated with arthritis in different mouse models (21, 31). T_h1 dominance was shown to be characteristic of PGIA (21, 22), whereas the absence of IL-17 alone in IL-17a-deficient BALB/c mice did not seem to have a significant effect on PGIA (32). Nonetheless, IL-17 has been shown to be an important mediator in human RA (33, 34), collagen-induced arthritis (35) and spontaneous arthritis in either SKG (36) or IL-1 receptor antagonist-deficient mice (37), as well as in experimental autoimmune encephalomyelitis (38). In contrast to the *in vivo* experiments in which IL-17a deficiency alone seems to play a limited role in PGIA (32, 39), we show that one of the prominent cytokines produced during the PG immunization period is IL-17 in BALB/c mice. This suggests that in addition to IFN γ , IL-17 might play a role in PGIA similar to its implicated role in other autoimmune disease models and RA. Indeed, IFN γ and IL-17 were the cytokines that were produced in significantly lesser amounts at most time points (especially by the PLF cells) in

the resistant DBA/2 strain, suggesting that high levels of IFN γ and IL-17 might contribute to PGIA susceptibility. Results obtained in IFN γ and IL-17 double KO mice (A. Finnegan, personal communication) revealed the role of IL-17 in PGIA. While IFN γ is a key cytokine in PGIA, IFN γ deficiency alone does not abolish completely PGIA. This 'residual arthritis', however, is completely eliminated by additional IL-17 deficiency. Thus, the absence of IL-17 in the IL-17a-deficient mice most likely is compensated by IFN γ . In turn, in wild-type BALB/c mice, both cytokines are present and involved in PGIA.

B cells are excellent APCs (40), and B cells from PG-immunized BALB/c mice are 1000 times more effective APCs than peritoneal macrophages (4, 41). Antigen presentation is known to influence both the magnitude and the direction of the immune response (42). It has been shown recently that B1 and conventional B (B2) cells induce different pathways of T cell differentiation (11). According to Zhong *et al.* (11), when B1 cells were used as APCs under *in vitro* conditions, T_h1/T_h17 cells accumulated, whereas conventional B cells as APCs induced the proliferation of Tregs. In naive (non-immunized) conditions, the peritoneal cavity is rich in B1 cells (Fig. 1 and Table 1), and these B1 cells, through antigen presentation, may initially direct the immune response toward T_h1/T_h17 differentiation. Although the number of B1 cells decreases upon repeated PG/DDA injections, a low number of B1 cells may be sufficient for maintaining the polarization and dominance of T_h1 and T_h17 CD4⁺ cells in the peritoneal cavity of BALB/c mice. In contrast, conventional B2 cells represent the majority of B cells in spleen of DBA/2 mice. This may be one of the reasons for the expansion of the splenic Treg population detected at day 5 after the first PG injection in DBA/2 mice (data not shown). Similar expansion of splenic Tregs was also observed by Zhong *et al.* (11). This early Treg induction in the spleen could also contribute to the PGIA-resistant phenotype of the DBA/2 mouse strain.

Upon the second PG injection, T and B cells are reactivated in the peritoneal cavity, and this event is accompanied by a robust change in the T and B cell composition and activation state (Fig. 1). This change is reflected by the production of IFN γ , IL-17 and auto-antibodies against mouse PG in BALB/c, but not in DBA/2 mice. These PG-specific auto-antibodies, together with activated effector T_h1 and T_h17 cells, may find their targets in the joints (5, 41, 43). Deposition of PG-specific antibodies in the joints and T cell migration to the synovium, also indicated by the transient decrease in the T cell number of the PLF 10 days after the second PG + DDA injection, may trigger the effector phase of autoimmune arthritis, amplifying the local response through pro-inflammatory cytokine (IL-1 β , TNF α , IFN γ , IL-17 and IL-6) production, thereby leading to progressive joint tissue destruction. Since in DBA/2 mice there was significantly less influx of T cells into the peritoneal cavity, T cell help was probably not sufficient for auto-antibody production. Subtle differences in co-stimulation, which might influence the T-B cell interactions, cannot be ruled out between the two strains. Our PLF cell transfer experiments show that boosting peritoneal cells with a second PG injection in donor BALB/c mice enhanced the arthritogenic potential of these cells in SCID recipients. This supports the hypothesis that, although PG-primed T and B cells already appear in the peritoneum after the first immunization, their reactivation via clonal expansion is necessary to reach the fully 'armed effector' state.

A potentially interesting implication of this study is the importance of the peritoneal lymphoid cells in controlling autoimmune joint inflammation. B1 cells, residing in the peritoneal cavity, have been implicated in autoimmunity (44). They produce auto-antibodies spontaneously at low concentrations, and these cells may be considered as physiological regulators of autoimmunity (45). Although there is no direct evidence for their role in RA so far, it might be interesting to speculate that changes in the physiological peritoneal milieu in susceptible humans, similar to what happens in BALB/c mice as a consequence of i.p. PG/DDA injections, could pave the way to autoimmune joint inflammation. Theoretically, repeated peritoneal antigen (endogenous or exogenous) stimuli, together with chronic exposure to chemicals with potential adjuvant activity, might contribute to such cellular changes, eventually leading to impaired immune regulation.

A key observation of this study is that injection of adjuvant (DDA) alone induced very similar changes in peritoneal cell composition as the injection of DDA with PG. However, only BALB/c mice injected with PG/DDA developed strong antigen-specific immune responses and arthritis. DDA has been shown to skew the immune response toward the T_h1 direction (46, 47) in addition to strongly activating innate immune cells (14). Here, we show that DDA induces the recruitment of T cells into the peritoneum, which is in line with earlier observations (17), but the kinetics of T cell influx was different when the antigen was present (Fig. 1). This effect, however, can be attributable to the local induction of inflammatory adhesion molecules and chemokines. The propensity of these T cells to produce IFN γ , TNF α , IL-12 and IL-17 in the early stage of immunization may create an optimal milieu for the differentiation and activation of autoimmune T and B cells

when antigen (PG) is present. Such peritoneal T cell response was lacking in DBA/2 mice, which raises the possibility that the adjuvant itself might not effectively activate innate immune cells in this strain. On the contrary, systemic T cell response and serum antibody levels against the immunizing antigen showed that the immunization was effective, although at different levels, in both strains. This correlates with earlier studies which have shown that DBA/2 mice, similar to BALB/c, are good responders to DDA (48).

As we have shown, PGIA resistance in DBA/2 mice is not the consequence of the lack of the systemic immune response against the immunizing (human) PG. However, the 'autoimmune switch' and the subsequent immune attack on the joints (self-PG) can only be observed in BALB/c mice. Our preliminary transfer experiments with PLF cells from hyper-immunized DBA/2 mice also support the idea that indeed, DBA/2 cells carry some arthritogenic potential, which, otherwise, is suppressed in the original wild-type background. Several candidate molecules can be involved in the development of autoimmunity, including innate immune receptors, like Toll-like receptors (TLRs) (49, 50), and co-stimulatory molecules (51). For example, flow cytometry analysis has shown that higher percentage of peritoneal macrophages express TLR1 in DBA/2 than in BALB/c mice (data not shown).

Therefore, we conclude that the arthritis-susceptible phenotype of BALB/c mice is due to an autoimmune switch during the immunization with human PG. This is potentially determined by differences in the adjuvant response, locally, in the peritoneal cavity and in joint-draining lymph nodes of BALB/c mice with PGIA (4). The profound immunophenotypic differences between the two strains found here, in addition to data from genetic studies (2, 52), may advance our understanding of the critical events leading to autoimmunity in PGIA in the BALB/c strain.

Supplementary data

Supplementary tables are available at *International Immunology Online*.

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Abbreviations

APC	antigen-presenting cell
DDA	dimethyldioctadecyl-ammonium bromide
i.p.	intra-peritoneal

KO	knockout
PG	proteoglycan
PGIA	PG aggrecan-induced arthritis
PLF	peritoneal lavage fluid
RA	rheumatoid arthritis
SEM	standard error of the mean
TNF α	tumor necrosis factor
TLR	Toll-like receptor
Treg	regulatory T cell

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