Eosinophils facilitate antigen-specific T-cell proliferation and aggravate antigen-induced arthritis.

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ABSTRACT In the pathophysiology of rheumatoid arthritis (RA), various types of cells, including macrophages, lymphocytes, neutrophils, mast cells, osteoclasts, chondrocytes, and fibroblast-like synoviocytes, contribute to the destructive process. Involvement of eosinophils in RA was first suggested by the presence of eosinophilic cationic protein in the synovial fluid. Later, infiltration of eosinophils in the synovium of RA and the increase of eotaxin in the sera of early RA were reported. Despite these indications, the precise role of eosinophils in RA remains unclear.

In this study, we analyzed whether eosinophils are involved in antigen-induced arthritis (AIA). AIA utilizes Complete Freund's adjuvant (CFA) as an inducer of the Th1 immune response and heat-killed *Bordetella pertussis* as a Th2 immune response inducer. We then used flow cytometry to examine whether eosinophils were present in the knee synovium of animals subjected to AIA. The results revealed that the average total cell number in synovia injected with methylated BSA (mBSA) was 4.8-fold of that in control synovium injected with saline. The numbers of eosinophils in the synovium increased 10.8-fold compared with those in the controls. We also analyzed the gene expression of pro-inflammatory cytokines (*IL-1β, IL-6, TNFα*, and *IL-17*), Th1/Th2 cytokine (*IFNγ* and *IL-13*), and mediators that increase eosinophils (*IL-5, IL-33, GM-CSF, CCL11, CCL24, CCL26, and RANTES*) in the synovium of the knee joints. The result showed that the levels of *IL-17* in the synovium injected with mBSA were significantly higher than those in the controls. In some antigen-challenged synovia, increased gene expression of *IL-6* was observed. By contrast, expression of the other genes showed no consistent changes between synovia from knees with or without antigen challenge. To determine a definitive role for eosinophils in AIA, we used eosinophil-deficient ΔdblGATA mice for the antigen challenge. We found that the severity scores of arthritis in ΔdblGATA mice were milder than those in WT mice. To investigate whether eosinophils are involved in the adaptive immunity associated with AIA, we analyzed serum IgG and lymph node cell proliferation from both WT and ΔdblGATA mice. This revealed that the anti-mBSA IgG levels were similar in the two strains and that mBSA-specific lymph node cell proliferation in ΔdblGATA mice was impaired. Together, these data

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indicate that eosinophils play roles in the support of antigen-specific T cell growth responses, and promotion of arthritis.

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INTRODUCTION

Eosinophils are leukocytes involved in innate immunity that are found in relatively low numbers within the blood (1-4% of total peripheral blood leukocytes). Mature eosinophils have a bilobed nucleus and eosinophil-specific granules in cytoplasm. The granules contain major basic protein, eosinophil peroxidase, eosinophilic cationic protein and eosinophil-derived neurotoxin. Eosinophils function as cytotoxic effector cells, are involved in the immune response to helminth infections, and play a role in allergic responses. However, it is increasingly clear that eosinophils have additional roles. For example, human eosinophils constitutively contain preformed Th1/Th2 cytokines (IFN γ /IL-4 and IL-13) and immunoregulatory cytokines (IL-6, TNF α , IL-10, and IL-12) and can secrete these mediators very rapidly during the immediate innate immune response. In doing so, the eosinophils are able to regulate the microenvironment, albeit by secreting lower amounts of inflammatory mediators when compared to T cells [1]. Furthermore, eosinophils regulate inflammation and tissue-remodeling via both by releasing cytotoxic eosinophil-specific granules and by secreting a large amount of cytokines and growth factors such as TGF β , bFGF, and Th2 cytokines [2].

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by hyperplasia of the synovium, with progressive chronic inflammation leading to joint destruction. The incidence of RA is approximately 1% worldwide, but its etiology and pathophysiology is not completely understood [3]. RA is a polygenic disease and is caused by immunological disorders that develop from the synergistic actions of genetic and environmental factors [4]. Clinical and experimental studies have implicated inflammatory cytokines such as tumor necrosis factor (TNF) α , IL-1 β , and IL-6 in the pathophysiology of RA [2]. In RA, $CD4^+$ T cells, B cells and macrophages infiltrate the synovium; this leads to hyperplasia of the intimal lining due to a marked increase in macrophage-like and fibroblast-like synoviocytes. Locally expressed degradative enzymes, including metalloproteinases, serine proteases, and aggrecanases, digest the extracellular matrix and destroy the articular structures [3, 5-10].

Involvement of eosinophils in RA was first suggested by the presence of eosinophilic cationic protein in the synovial fluid [11]. Subsequently, eosinophils were detected in 3 of 19 rheumatoid synovial specimens [12], and another prospective clinical study showed that 7.6% (15/197) of patients with RA had eosinophilia [13]. Proteomic analysis of secreted proteins in the sera revealed an increase of eotaxin in early RA [14]. Thus, despite these indicators, the actual role of eosinophils in RA remains unclear.

Among the murine arthritis model systems for RA, even in the type II collagen-induce arthritis (CIA) model, which is most popular, there is almost no paper reporting the involvement of eosinophils. Recently, involvement of eosinophils in an arthritis model related to CIA was reported. In the transgenic mouse with the TCR gene specifically recognizing type II collagen, intraperitoneal injection of type II collagen induced chronic arthritis, which was associated with eosinophil infiltration dependent on IL-5 [15]. Although this special system provided the

concept that eosinophils could induce destructive arthritis, conventional CIA is generally not suitable for the analysis of eosinophils because its immune response is strongly skewed to Th1 by using Complete Freund's adjuvant (CFA). Thus, we took advantage of another murine arthritis model, antigen-induced arthritis (AIA) that utilizes CFA as an inducer of the Th1 immune response and heat-killed *Bordetella pertussis* as a Th2 immune response inducer. In AIA, roles for cytokines have been intensively examined. For example, IL-6 plays a key role in the development of arthritis, particularly with regard to bone destruction [16], whereas IFN γ shows anti-inflammatory properties during the initial phase of AIA by inhibition of IL-17 [17]. However, there are no published reports of investigations into whether eosinophils contribute to pathogenesis of AIA.

In this study, we examined the involvement of eosinophils in AIA by performing detailed analyses of the synovium in both WT and eosinophil-deficient (ΔdblGATA) mice.

MATERIALS AND METHODS

Animals

The animal experiments in this study were approved by the committees in the Kawasaki Medical School for the safety of recombinant DNA experiments (No.11-11), and for the animal experiments (No.12-025, 13-061). The eosinophil-deficient ΔdblGATA mice in a C57BL/6 background were kindly provided by Dr. S. H. Orkin [18]. C57BL/6 mice were purchased from Nihon SLC (Sizuoka, Japan). ΔdblGATA mice and C57BL/6 were bred and housed in a temperature-controlled room and given free access to water and food.

Induction of arthritis

AIA was performed by following a previously described protocol [19]. Briefly, 8-12 week-old mice were immunized on day 0 and 7 with 100 μg methylated BSA (Sigma, Tokyo, Japan) emulsified in 0.1 ml CFA containing 200 μg mycobacterial strain H37RA (Difco, Tokyo, Japan) by intradermal injection at the four footpads; 2×10^9 heat-killed *Bordetella pertussis* (Cosmo Bio Co., Tokyo, Japan) were also injected intraperitoneally. On day 21, 100 μg of mBSA in 10 μl of saline was injected into the left knee joint. As a control, the same volume of saline was injected into the right knee joint and mice were sacrificed for analysis on day 35 after priming.

Histological examination

The knees were dissected and fixed in 10% buffered formalin for 1 d. Fixed tissues were decalcified with 10% formic acid for 1 week, dehydrated, and embedded in paraffin. Sections of the whole knee joint were stained with hematoxylin-eosin. The slides were evaluated histologically by two independent observers, and the grade of arthritis was scored from 0 to 4 according to the intensity of lining layer hyperplasia, mononuclear cell infiltration, and pannus formation as described previously [19]. The scores were defined as follows: 0, normal knee joint; 1, normal synovium with occasional mononuclear cells; 2, definite arthritis, a few layers of flat to rounded synovial lining cells and scattered mononuclear infiltrates; 3, clear hyperplasia of the synovium with three or more layers of loosely arranged lining cells and dense infiltration with mononuclear cells; and 4, severe synovitis with pannus and erosion of articular cartilage and subchondral bone.

Cell preparation

On day 35 after priming, mice were sacrificed and the brachial lymph nodes were removed and teased with slide glass. The synovia were removed from the knee joints, minced, and digested in RPMI1640 containing 1 mg/ml collagenase D (Roche, Tokyo, Japan) at 37 ℃ for 3 h. Single-cell suspension was prepared by gentle pipetting and filtration through a

nylon mesh.

Flow cytometry analysis

Multi-color staining for flow cytometry analysis was performed as described previously [20], and cells were analyzed using a FACS Canto Ⅱ flow cytometer (Becton Dickinson, Tokyo, Japan) and BD FACS Diva software. Monoclonal antibodies used were as follows: FITC-conjugated anti-Ly-6G and Ly-6C mAb (Gr-1, clone; RB6-8C5), PE-conjugated anti-siglec F mAb (BD Pharmingen, Tokyo, Japan); PerCP-Cy5.5-conjugated anti-CD3ε (145-2C11) mAb, PerCP-Cy5.5-conjugated anti-CD19 (6D5), PerCP-Cy5.5-conjugated anti-NK1.1 mAb (PK136) (eBioscience, Tokyo, Japan), APC-conjugated anti-F4/80 mAb (BM8) (eBioscience); APC-Cy7-conjugated anti-CD19 mAb (BioLegend, Tokyo, Japan); eFluor-450-conjugated anti-CD11b mAb (M1/70) (eBioscience). Non-specific binding to FcγR was blocked with anti-CD16/32 mAb (2.4G2) and 5% heat inactivated rat serum.

Quantitative PCR

Total RNA was isolated from the synovium of the knee joints using TRIzol (Invitrogen, Tokyo, Japan). RNA samples were treated with DNase I (Sigma). cDNA was synthesized with ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) following the manufacturer's instructions. cDNA samples were amplified with specific primers in the Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan) and run on a ABI 7300 or 7500 cycler (Applied Biosystems). The specific PCR primers were designed using Universal Primer Design Tools available on the web site of Roche. The sequences for the primers (sense, anti-sense) were as follows: *IL-1β*, 5'-TTGACGGACCCCAAAAGAT-3' and 5'-GAAGCTGGATGCTCTCATCTG-3'; *IL-5*, 5'-ACATTGACCGCCAAAAAGAG-3' and 5'-ATCCAGGAACTGCCTCGTC-3'; *IL-6*, 5'-GCTACCAAACTGGATATAATCAGGA-3' and 5'-CCAGGTAGCTATGGTACTCCAGAA-3'; *IL-13*, 5'-CCTCTGACCCTTAAGGAGCTTAT-3' and 5'-CGTTGCACAGGGGAGTCT-3'; *IL-33*, 5'-GGTGAACATGAGTCCCATCA-3' and 5'-CGTCACCCCTTTGAAGCTC-3'; *IFNγ*, 5'-ATCTGGAGGAACTGGCAAAA-3' and 5'-TTCAAGACTTCAAAGAGTCTGAGGTA-3'; *TNFα*, 5'-TGCTGGGAAGCCTAAAAGG-3' and 5'-CGAATTTTGAGAAGATGATCCTG-3'; *RANTES*, 5'-CTACTCCCACTCCGGTCCT-3' and 5'-GATTTCTTGGGTTTCGTGGTC-3'; *CCL11*, 5'-AGAGCTCCACAGCGCTTCT-3' and 5'-GCAGGAAGTTGGGATGGA-3'; *CCL24*, 5'-GCAGCATCTGTCCCAAGG-3' and 5'-GCAGCTTGGGGTCAGTACA-3'; *CCL26*, 5'-GCACCAGTGACGGTGTGATA-3' and 5'-TGAATCTCTGCACCCATTTG-3'; *β-actin*, 5'-CTAAGGCCAACCGTGAAAAG-3' and 5'-ACCAGAGGCATACAGGGACA-3'. The expression levels of target cDNAs were normalized to the endogenous transcription levels of the *β-actin*.

Measurement of serum anti-mBSA IgG

Sera were collected on days 0 (before immunization), 7, and 14. Anti-mBSA IgG levels were measured by ELISA as described previously [16]. Further, 96-well plates were coated with 100 µl of 5 µg/ml mBSA (Sigma) in coating buffer (Carbonate-bicarbonate buffer pH9.6) for 1 h at RT and blocked by incubation with 0.1% goat serum in PBS for 1 h at RT. After washing 3 times with PBS containing 0.1% Tween 20 (washing buffer), a 100-µl aliquot of mouse serum diluted 40-fold in PBS was applied and incubated for 30 min at RT. After another wash, 100 µl of goat anti-mouse IgG conjugated with alkaline phosphatase (SouthernBiotech, Birmingham, AL) (1:1000 dilution) was added and incubated for 30 min at RT. The plates were washed 3 times and p-nitrophenylphosphate (0.1

μg/ml in substrate buffer; 0.05 M $NaHCO_2$, 10 mM $MgCl_2$, pH 9.8) was applied in each well. Color development was monitored by absorbance at 405 nm with Varioscan (Thermo Electron Corporation, Yokohama, Japan).

Proliferative response of the lymph node cells

Mice were immunized with mBSA twice on days 0 and 7 by the same protocol with the induction of AIA. On day 14, the brachial lymph nodes were removed and single lymph node cell suspensions were prepared. Lymph node cells (5×10^5 cells/well) were cultured in 96-well plates with 0.1 ml of DMEM supplemented with 1% mouse serum and antibiotics in the absence or presence of either mBSA (100 μg/ml) or hamster anti-mouse CD3ε mAb (145-2C11; 1.0 μg/ml) for 72 h. Cell proliferation was measured using the MTT assay (Roche) [21].

Statistical analysis

Statistical significance for all experiments was analyzed using the Mann-Whitney U-test run in StatMate 4.01 software (ATMS Co. Ltd., Tokyo, Japan).

RESULTS

Marked increases of various hematopoietic cells in synovia after intra-articular injection of mBSA

To investigate whether eosinophils are associated with development of arthritis, we analyzed cells in the synovium of mice subjected to AIA. Flow cytometrical definitions for myeloid cells are as reported by Chu *et al.* [22]. Briefly, lymphoid cells expressing either CD3ε, CD19 or NK1.1 were gated out to enrich for myeloid cells. Among the $CD3\varepsilon^-CD19^-NK1.1^-$ myeloid fraction, the $Gr\text{-}1^{hi}CD11b^+$ fraction contains neutrophils, whereas $Gr\text{-}1^{lo}F4/80^+$ fraction is further divided into 3 fractions:

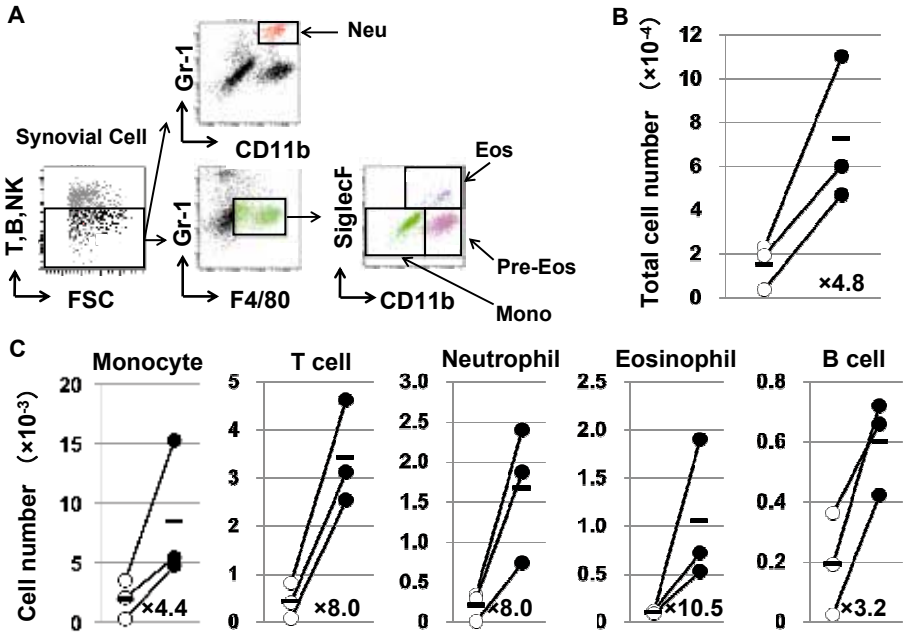

Fig. 1. Marked increases in the number of eosinophils in the synovia of mice subjected to AIA. Identification of monocytes, neutrophils, eosinophils, and eosinophil precursors by multicolor flow cytometry analysis (A). Total cell numbers in the synovium (B) and the individual cell populations in the synovium (C). Numbers of cells prepared from the synovium injected with saline (white circle) and mBSA (black circle) are shown. The data of each individual mouse are connected with a line. The circles and horizontal bars indicate the values for each mouse and the average, respectively.

$CD11b^{int}SiglecF^{+}$ (eosinophils), $CD11b^{int}SiglecF^{-}$ (eosinophil precursors), and $CD11^{lo}SiglecF^{-}$ (monocytes) (Fig. 1A).

The average total cell number in synovia injected with mBSA increased 4.8-fold from that of the synovia injected with saline as control (Fig. 1B). The majority of these cells were monocytes, and others include, T cells ($CD3\varepsilon^{+}$), neutrophils, eosinophils, and B cells ($CD19^{+}$) were also present. The number of eosinophils in the Ag-challenged synovia increased 10.5-fold compared with the control (Fig. 1C). The increase of eosinophils was the highest among the mature hematopoietic cells in the synovia. Although the eosinophils accounted for only a minor fraction of the total cells, their frequency in the arthritic synovium was almost equivalent to that of neutrophils at day 35. Taken together, these data suggest that eosinophils may play a role in the pathophysiology of AIA.

Marked increase of IL-17 gene expression in the synovium after intra-articular injection of mBSA

To investigate which cytokines are associated with the inflammatory response and the increase of eosinophils in the AIA synovium, we quantitatively analyzed the expression of a panel of genes. This included pro-inflammatory cytokines (*IL-1β*, *IL-6*, *TNFα*, and *IL-17*), Th1/Th2 cytokines (*IFNγ* and *IL-13*), and mediators that induce a local increase of eosinophils (*GM-CSF, IL-5, IL-33, CCL11, CCL24, CCL26,* and *RANTES*). This revealed that levels of *IL-17* were significantly higher in the synovia injected with mBSA than in controls. Although there was no overall significant difference in *IL-6* levels between groups with or without Ag challenge, 2 of 5 mice showed more than 10-fold increase in this cytokine from the levels in the controls. The expression of mediators affecting eosinophils showed no consistent changes between the right and left synovium of the knee joint (Fig. 2). These data suggest that IL-17 and/or IL-6 likely play pivotal roles in the inflammatory component of AIA.

Arthritis of eosinophil-deficient mice was milder than WT mice

The joints of WT mice injected with mBSA developed severe arthritis with marked synovial hyperplasia, mononuclear cell infiltration, and erosions of the articular cartilage and bone (Fig. 3B) unlike the control joints injected with saline (Fig. 3A). To determine the importance of eosinophils in AIA, we utilized eosinophil-deficient mice,

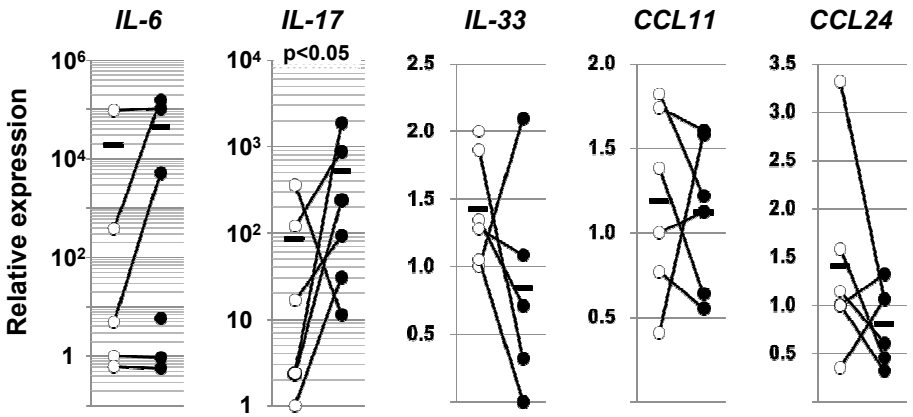

Fig. 2. Expression of the *IL-17* gene increased in the synovium after intra-articular injection of mBSA. The relative amount of each gene from samples of synovium injected with saline (white circle) and mBSA (black circle) are shown. The expression level of each gene was normalized to the amount of *β-actin* in the synovium from one control joint injected with saline alone.

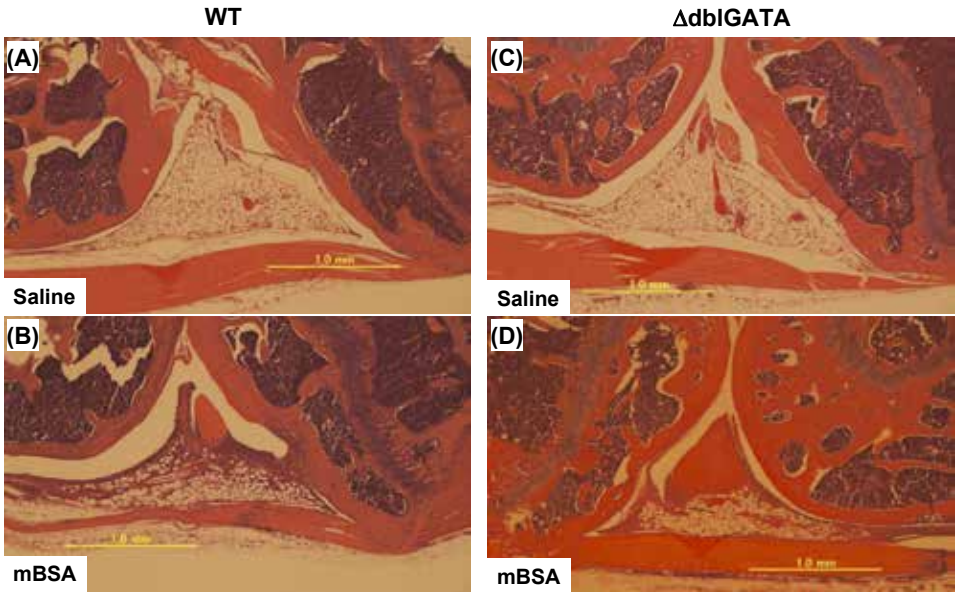

Fig. 3. Arthritis in eosinophil-deficient mice was milder than WT mice. WT mice developed severe arthritis with marked synovial hyperplasia, mononuclear cell infiltration (B) compared with the control (A). Arthritis of ΔdblGATA mice had a less pronounced infiltration of inflammatory cells in the synovial tissue (D). The knee joint of ΔdblGATA mice injected with saline (C) is almost same as that of WT mice (B). Bars =1mm.

Table 1. Histological scores for AIA in the eosinophil-deficient and WT mice.

The numbers of mice in each histological grade of AIA			
Grade	Histological characteristics	WT	ΔdblGATA
0	normal knee joint.	0	0
1	normal synovium with occasional mononuclear cells.	0	0
2	definite arthritis, a few layers of flat to rounded synovial lining cells and scattered mononuclear infiltrates.	0	1
3	clear hyperplasia of the synovium with three or more layers of loosely arranged lining cells and dense infiltration with mononuclear cells.	1	3
4	severe synovitis with pannus and erosions of articular cartilage and subchondral bone.	3	1

Both groups were immunized in the standard manner and the mice were sacrificed 35 days after priming (14 days after injection of mBSA to the joint space).
The histopathological characteristics of the knee joints scored from 0 to 4 are indicated.

ΔdblGATA, in the AIA model. The arthritis in ΔdblGATA mice was milder than in WT mice, especially with regard to the degree of cellular infiltration into synovial tissue (Fig. 3-D). As shown in Table 1, the majority of arthritis grades for WT was 4, whereas that of ΔdblGATA was 3, clearly indicating that eosinophils play roles in promoting arthritis in AIA.

Antigen-specific response of T cells but not B cells is impaired in ΔdblGATA mice

A previous study showed that alum treatment induced eosinophil recruitment to the spleen and that lack of eosinophils led to reduced antibody production during the thymus-dependent antigen response [23]. To determine the locations at which eosinophil activity affects the development of arthritis, we analyzed the acquired humoral immune

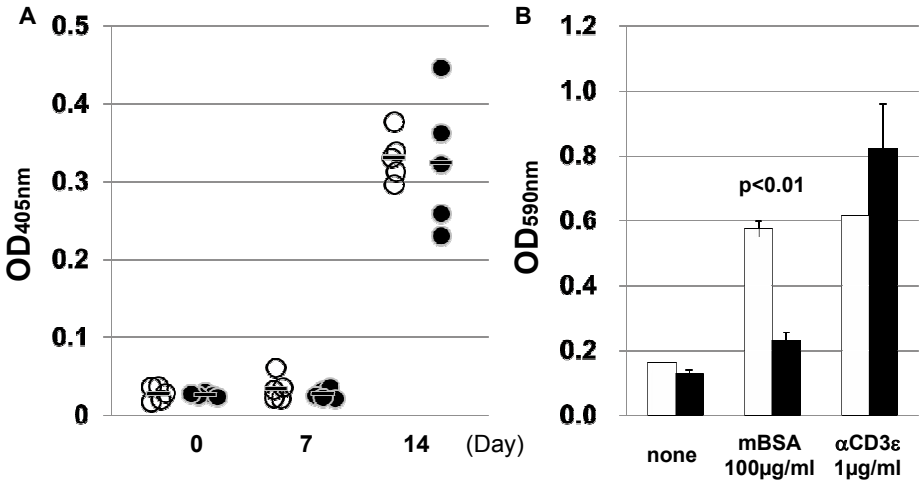

Fig. 4. Antigen-specific responses of T cells but not B cells were impaired in eosinophil-deficient mice. Mice were immunized with mBSA twice on days 0 and 7 according to the same protocol used for induction of AIA. (A) Sera were collected before and 7 or 14 d after first immunization. The titers of antibodies (IgG class) against mBSA were measured by ELISA. White and black circles indicate the values of WT mice and ΔdblGATA mice, respectively. (B) 7 d after the second immunization (Day 14 after priming) with mBSA, the brachial lymph node cells were prepared and cultured at 5×10^5 cells/well in the absence or presence of either mBSA (100 μg/ml) or anti-CD3ε mAb (1.0 μg/ml) for 72 h. The proliferative response was measured using the MTT assay. Data are representative of 2 independent experiments. White (WT mice) and black (ΔdblGATA mice) bars indicate the average values of triplicate and standard deviations are shown.

response by measuring serum mBSA-specific IgG as well as the cellular immune response by quantifying mBSA-specific proliferation of lymph node cells. As shown in Fig. 4, measurement of serum anti-mBSA IgG levels revealed no difference between ΔdblGATA and WT mice on days 7 and 14 after immunization. On the other hand, mBSA-specific proliferative responses of lymph node cells from ΔdblGATA mice were reduced to less than half of those from WT mice. No difference was observed in proliferation induced by anti-CD3ε antibody between ΔdblGATA and WT mice, which indicates that the TCR-mediated T cell response itself is normal in the eosinophil-deficient mice. FACS analysis revealed no difference in the ratio of either CD3/CD19 or CD4/CD8 in the lymph node cells between the two groups. Thus, eosinophils appear to have a functional role in the antigen-specific T cell responses during AIA.

DISCUSSION

In this study, we report that the number of eosinophils increases in the synovium of mice subjected to AIA. Additionally, our use of eosinophil-deficient mice underscores a critical role for these cells in AIA pathology. Furthermore, we showed that the antigen-specific T-cell growth responses, but not the B-cell humoral immune responses, were impaired in the eosinophil-deficient mice. Owing to the selective increase in *IL-17* expression in the synovium at day 35, we speculate that eosinophils may also regulate the Th17 cell development. A marked increase of *IL-6* expression in some of the antigen-challenged synovia suggests that the axis of IL-6/gp130/STAT3/Th17 might contribute to the late phase of AIA. Additionally, eosinophils secrete various cytokines [2] and some of them (IL-1β, IL-2, and IL-6) could facilitate T cell proliferation. Thus, it will be interesting to examine the profile of cytokines in the

supernatant of T cells stimulated *in vitro*.

T cell proliferation and differentiation are regulated by signals delivered not only through the TCR recognizing the antigen peptide, but also by cytokines and co-stimulatory molecules of antigen presenting cells (APCs) such as dendritic cells [24, 25]. Our results suggest that eosinophils positively regulate antigen-specific T cell proliferation. A previous study by others showed that eosinophils act as APCs in an ovalbumin-induced bronchial asthma model [26]. Similar to this, we speculate that eosinophils may also play the role of APCs in AIA.

To find the functional interaction between eosinophils and T cells, future studies will focus in the kinetics of eosinophil and T cell in the synovium during priming or after antigen challenge. Since eosinophils contain cytotoxic granule proteins and various secretory mediators, including cytokines, chemokines, growth factors and lipid, analyses of the production and release of such mediators by eosinophils in the synovium or the lymph nodes in AIA will also be informative.

In this study, the numbers of specimens for histological analyses were limited, but they showed clear but not significant difference in the severity of arthritis due to eosinophil deficiency. We are currently extending this study to a larger, comprehensive one to clarify the relationship between the gene expression in the synovia, the cellular localization especially of T cells and eosinophils, and the histological grades of arthritis.

Although it has been difficult to understand the roles of eosinphils in the RA synovia, our results could provide an intriguing possibility that eosinophils involve in the pathophysiology of RA by facilitating T cell proliferation to certain antigens including self-antigens.

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