The interaction of dendritic cells and $\gamma\delta$ T cells promotes the activation of $\gamma\delta$ T cells in experimental autoimmune uveitis

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Uveitis is a severe inflammatory disease that can cause visual impairment. Recently, activated $\gamma\delta$ T cells were proved to play a central role in the development of experimental autoimmune uveitis (EAU). However, the mechanism underlying $\gamma\delta$ T-cell activation in EAU is incompletely known. In this study, we determined the percentage changes in and the phenotypes of $\gamma\delta$ T cells and dendritic cells (DCs) obtained from the spleens of immunized C57BL/6 (B6) mice, an animal model of EAU. We found that the number of $\gamma\delta$ T cells and DCs obviously increased during the inflammation phase of EAU (days 16–20 of our experiment), and that during this time, $\gamma\delta$ T cells expressed high levels of CD69 and the integrin lymphocyte function-associated antigen-1 (LFA-1) and secreted high levels of interleukin (IL)-17A. Moreover, DCs obtained during this phase expressed high levels of CD80, CD83, CD86, and intracellular cell adhesion molecule-1 (ICAM-1). Furthermore, we studied the interaction between DCs and $\gamma\delta$ T cells by using flow cytometry and confocal microscopy in order to determine whether DCs affected $\gamma\delta$ T-cell activation *in vitro*. Co-cultures of the two types of cells showed that DCs induced high levels of CD69, LFA-1, and

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IL-17A in $\gamma\delta$ T cells. Imaging studies revealed contact between the DCs and $\gamma\delta$ T cells. This interaction was mediated by the accumulation of ICAM-1 and LFA-1 at the interface of DCs- $\gamma\delta$ T cells. Thus, the activation of $\gamma\delta$ T cells in EAU was promoted by DCs interacting with $\gamma\delta$ T cells.

Keywords: $\gamma\delta$ T cells; dendritic cells; experimental autoimmune uveitis; integrin lymphocyte function-associated antigen-1 (LFA-1).

1. Introduction

Uveitis is a serious inflammatory disease that can cause visual impairment. This condition usually occurs in young adults and children.¹ A disorder of the immune system is considered to be the key factor in the occurrence of uveitis. However, the exact mechanism underlying the development of uveitis remains unclear. Recently, many reports have shown that $\gamma\delta$ T cells play an important role in the regulation and resolution of inflammatory processes associated with infections, tumors, and autoimmunity diseases.^{2–4} Moreover, $\gamma\delta$ T cells can have either an up-regulation or a down-regulation effect on adaptive immune responses.^{5–9} Studies have shown that $\gamma\delta$ T cells play a central role in the development of experimental autoimmune uveitis (EAU), and the effect of $\gamma\delta$ T cells critically depends on their state of activation.^{10,11} Activated $\gamma\delta$ T cells promote the activation of IL-17⁺ autoreactive T cells to enhance EAU development, whereas nonactivated $\gamma\delta$ T cells have little effect on EAU.^{10,11} However, the mechanism of $\gamma\delta$ T cells activation in EAU is still incompletely understood. Although antigen and cytokines might promote the activation of $\gamma\delta$ T cells, the function of interactions between $\gamma\delta$ T cells and other immune cells on activation of $\gamma\delta$ T cells has not been well defined. Herein, we studied whether $\gamma\delta$ T cells were activated by interacting with dendritic cells (DCs), which were an initiator of adaptive immune responses.

DCs are the most powerful antigen-presenting cells (APCs) and they initiate and regulate a broad repertoire of immune responses.^{12,13} DCs are the link between innate and adaptive immunity. They have an immense capacity to regulate T-cell activation.^{14,15} They also interact with innate lymphocytes such as natural killer (NK) cells, NK T cells, and $\gamma\delta$ T cells to regulate innate immunity. DCs promote the secretion of cytokines and the activation of innate lymphocytes, which in turn regulate the maturation of DCs.^{16,17} Mature DCs highly

express major histocompatibility complex (MHC) and co-stimulatory molecules such as intracellular cell adhesion molecule-1 (ICAM-1), CD40, CD80, and CD86, and secrete interleukin (IL)-12.^{18,19} DCs are believed to be an initiator of adaptive immune responses in some diseases, such as EAU. Some reports have shown that the number, phenotype, and function of DCs are altered in EAU, and thereby participate in the development of EAU.^{20,21} The aim of the present study was to determine whether DCs participate in the development of EAU by regulating the activation of $\gamma\delta$ T cells.

In this study, we constructed an EAU model to detect the number and phenotypes of DCs and $\gamma\delta$ T cells during the “inflammatory phase” of EAU (days 16–20 of our experiment), and determined the effects of DCs on $\gamma\delta$ T cell activation. Furthermore, by using flow cytometry and confocal microscopy, we analyzed the mechanism of DC promoted $\gamma\delta$ T-cell activation. Our results suggested that the interactions between DCs and $\gamma\delta$ T cells play a major role in the regulation of $\gamma\delta$ T-cell activation.

2. Materials and Methods

2.1. Animals and reagents

Pathogen-free female C57BL/6 Mice (B6) (6 to 8-weeks-old) were purchased from Peking Vital River Laboratory Animal Ltd., Beijing, China and were fed and maintained in specific pathogen-free conditions according to the guidelines of Care and Use of Laboratory Animals published by the China National Institute of Health. $\gamma\delta$ TCR- $\delta^{-/-}$ mice (8 weeks old) were purchased from Jackson laboratory (Bar harbor, ME, USA). All experimental procedures adhered to the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and vision research. The human interphotoreceptor retinoid-binding protein peptide (IRBP)_{1–20} was synthesized by ChinaPeptides Co., Ltd., Shanghai, China. Complete Freund's adjuvant (CFA) was obtained from Sigma

(St. Louis, MO, USA). Pertussis toxin (PTX) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Fluorescein antibodies of CD3e, MHC-II, CD11c, IL-17A, CD69 and LFA-1 were purchased from eBioscience (San Diego, CA, USA). PE-anti-mouse TCR $\gamma\delta$ was obtained from BD Biosciences (San Jose, CA, USA). Anti-TCR gamma and TCR delta antibody [UC7-13D5] (Phycoerythrin), anti-CD11c antibody [N418] and goat anti-armerian hamster IgG H&L (Alexa Fluor 647) were purchased from Abcam (Cambridge, MA, USA). Recombinant murine IL-2 and IL-23 were purchased from R&D Systems (Minneapolis, MN, USA). CD11c+ isolated kit, $\gamma\delta$ T cells isolated kit were purchased from Miltenyi Biotec (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.2. Induction and evaluation of EAU

B6 mice were immunized subcutaneously at six spots (on the footpads, tail base, and flank) with 200 μ l of an emulsion containing 300 μ g IRBP_{1–20} emulsified in CFA. Concurrently, a single dose of 500 ng PTX was injected intraperitoneally.

After immunization, the mice were examined for clinical signs of EAU on days 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 by using a Genesis-D camera (Kowa Company Ltd., Hamamatsu City, Japan). The disease was graded using scoring systems based on acknowledged and modified criteria,²² as displayed in Supplementary Table 1.

In addition, inflammation in the eye was confirmed by histopathologic examination. Eyes were obtained from the immunized mice on days 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36, and were fixed for 48 h in 4% buffered glutaraldehyde. The fixed tissues were embedded in paraffin, sectioned (4–6 μ m) through the papillary–optic nerve plane, and stained with hematoxylin and eosin (H&E). They were observed under a microscope (Ti, Nikon, Japan), and the disease was graded on the basis of cellular infiltration and structural changes, which have been described in Supplementary Table 2.

2.3. Purification of DCs and $\gamma\delta$ T cells

DCs were purified from the spleens of normal or IRBP_{1–20}-immunized B6 mice, by using CD11c+ Microbeads and an autoMACS separator (both from Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

Similarly, $\gamma\delta$ T cells were purified from the spleens of IRBP_{1–20}-immunized or normal B6 mice by using a $\gamma\delta$ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, for $\gamma\delta$ T-cell isolation, 1×10^8 lymphocytes were incubated with 50 μ l Non-T-Cell Depletion Cocktail in 450 μ l phosphate-buffered saline (PBS) at 4°C for 15 min. The cells were then washed once with PBS. The cells were resuspended in PBS and loaded on an autoMACS separation column, and 50 μ l Anti-Biotin MicroBeads in 450 μ l PBS were added. The resuspended cells were separated at 4°C for 15 min. The purity of the isolated cells was determined using flow cytometry (Supplementary Fig. 1).

2.4. Analysis of $\gamma\delta$ T cell and DC phenotypes

Splenic cells were obtained from normal mice and EAU mice on days 4, 8, 12, 16, 20, 24, 28, 32, and 36 after immunization. T cells were isolated from the spleen by passage through a nylon wool column. The cells were then collected by Ficoll-Hypaque density gradient centrifugation, and cultured at 37°C in a carbon dioxide incubator for another 12 h. The obtained cells were stained by direct immunofluorescence and analyzed using flow cytometry (FACSVerse; BD Biosciences, USA). To determine the number of $\gamma\delta$ T cells and their activation status, we stained aliquots of 1×10^6 T cells with phycoerythrin (PE)-conjugated $\gamma\delta$ T-cell receptor (TCR) and APC-conjugated CD3e antibodies, or with PE-conjugated lymphocyte function-associated antigen-1 (LFA-1) and fluorescein isothiocyanate (FITC)-conjugated CD69. To determine the number of DCs, we stained splenic cells with PE-conjugated MHC-II and FITC-conjugated CD11c antibodies. Next, to analyze the phenotype of DCs, we stained the DCs with APC-conjugated ICAM-1, CD80, CD83, or CD86. Data collection and analysis were performed using a FACSVerse flow cytometer and CellQuest software.

2.5. Assessment of the interaction between DCs and $\gamma\delta$ T cells *in vitro* by flow cytometry

We purified $\gamma\delta$ T cells and DCs from the spleens of immunized mice by using autoMACS. The $\gamma\delta$ T cells were incubated at 37°C in RPMI-1640

Fig. 1. EAU model creation. (a) B6 mice were immunized subcutaneously with IRBP_{1–20}, and disease symptoms were evaluated by funduscopy. Minimal vasculitis was observed on day 12. Multifocal chorioretinal lesions, severe vasculitis, and linear lesions were observed on days 16–20. Ocular inflammation was gradually resolved on day 28. (b) Histopathology of a representative eye section from control (day 0) and IRBP_{1–20}-immunized mice on days 12, 16, and 28 (hematoxylin and eosin; original magnification, $\times 100$). Image of a representative eye on day 16 after immunization (in the severe inflammation phase) shows heavy cell infiltration and retinal folds (arrows). (c) EAU scores after immunization. The data presented are the average scores of three separate experiments. The results are expressed as mean $\pm$ SD ($n = 6$).

medium in 24-well plates coated with IL-2 (10 ng/ml) and IL-23 (10 ng/ml). DCs were stimulated with IL-4 (20 ng/ml) and granulocyte macrophage colony-stimulating factor (100 ng/ml). Lipopolysaccharide (200 ng/ml) to promote maturation. Mature and immature DCs were washed and incubated for 2 h in the above medium supplemented with IRBP_{1–20} (20 ng/ml). Then, mature and immature DCs were separately co-cultured with $\gamma\delta$ T cells at a 1:1 ratio in 24-well plates, at 37°C. After 24 h, the cells were harvested and stained with PE-conjugated LFA-1, FITC-conjugated CD69, and APC-conjugated IL-17A. Activated $\gamma\delta$ T cells were prepared by incubating the isolated $\gamma\delta$ T cells with IRBP_{1–20} (20 ng/ml), IL-2 (10 ng/ml) and IL-23 (10 ng/ml) for 24 h. With anti-ICAM-1 antibody treatment, anti-ICAM-1 antibody (10 μ g/ml)

pretreated the DCs for 2 h and then pulsed IRBP_{1–20} to DCs for 2 h and $\gamma\delta$ T cells were added to co-culture with DCs.

2.6. Flow cytometric analysis

Aliquots of 1×10^6 cells were stained with different monoclonal antibodies. After being incubated for 30 min and washed twice, cells from each sample were analyzed using FACSVerse and the CellQuest data acquisition and analysis software (BD Biosciences, USA). To assess intracellular cytokine expression, we stimulated the prepared cells for 5 h with leukocyte activation cocktail (BD Biosciences, USA) at 37°C in a 5% CO₂ environment. The cells were then harvested and transferred to tubes, washed once with PBS, and incubated with APC-conjugated IL-17A antibody

after fixation and permeabilization, according to the manufacturer's instructions.

2.7. Confocal microscopy assessment of $\gamma\delta$ T-cell–DC interaction

DCs and $\gamma\delta$ T cells were purified from the spleens of the IRBP_{1–20}-immunized B6 mice. The $\gamma\delta$ T cells were incubated with IRBP_{1–20}-pulsed mature DCs for 30 min, until $\gamma\delta$ T-cell–DC contact had been established. Then, all the cells were fixed in PBS/4% paraformaldehyde for 10 min, followed by incubation with PBS/0.1 M glycine for 3 min and blocking with PBS/2% bovine serum albumin buffer for 20 min. Next, the cells were stained with a 1:10 dilution of PE-conjugated anti- $\gamma\delta$ TCR antibody and APC-conjugated ICAM-1 antibody for 60 min. After being washed three times, the cells were observed using confocal microscopy.

The spleen from the immunized mice was frozen in O.C.T. Compound (VWR scientific Products, So. Plainfield, NJ). Seven-micrometer-thick spleen sections were cut, fixed and then were permeabilized with 0.1% Triton-X-100 for 10 min, and blocked with 5% serum for 1 h at 25°C. Anti-mouse CD11c antibody at a dilution of 1:20 was incubated overnight at 4°C in a humidified chamber, cells were washed with PBS and incubated with secondary antibody (1:200) at room temperature in the dark. Then anti-TCR γ/δ antibody (phycoerythrin) (1:50) was incubated overnight at 4°C. Nuclei were counterstained with Hoechst (1:10000).

Images of the cells were taken with a confocal microscope (LMS 780, Zeiss, Germany) equipped with an APO oil immersion objective lens (63 $\times$, NA=1.40). To quantify the redistribution of molecules at the contact site, $\gamma\delta$ T-cell–DC doublets were chosen from bright-field images, and only $\gamma\delta$ T-cell–DC pairs whose contact orientation was proper for the x – y plane projection were taken into consideration for further analysis. The images were analyzed with the Imaris software (Bitplane AG, Zurich, Switzerland).

2.8. Statistical analysis

The data analysis was performed by SPSS 17.0 software (SPSS, Chicago, IL, USA). Each experiment was carried out in duplicate and repeated three times. Data were represented as the mean $\pm$ standard deviation.

3. Results

3.1. Increase in the number of $\gamma\delta$ T cells and DCs and their activation in the spleen in the inflammatory phase of EAU

We prepared the EAU model in C57BL/6 mice to analyze the station of $\gamma\delta$ T cells in EAU development. EAU was successfully induced by IRBP_{1–20} in C57BL/6 mice (Fig. 1). Compared to the normal eye, the diseased eye showed increased blood vessel growth and inflammatory cell infiltration in the vitreous and retinal disorganization. The inflammatory symptoms in the eyes appeared on day 12 of the experiment. The initial changes were fundal blood vessel thickening and minimal inflammatory cell infiltration. Then, multifocal chorioretinal lesions, severe vasculitis, and linear lesions were observed on days 16–20, along with abundant lymphocyte infiltration in the vitreous and retinal disorganization. The inflammatory symptoms in the eyes began to abate from day 28 onwards. Representative images of the changes are shown in Figs. 1(a) and 1(b). Clinical scores were recorded using a Genesis-D camera, and histological scores were obtained from H&E analysis (Fig. 1(c)). The clinical and histological scores peaked on days 16–20, indicating that the severity of the inflammation was greatest during this period, which we have termed as the “inflammatory phase” in this study. We studied the changes in $\gamma\delta$ T cells and DCs in the inflammatory phase in the immunized mice, and compared these findings with those observed in the normal mice during the same period.

Using flow cytometry, we determined the number of $\gamma\delta$ T cells and DCs obtained from the spleens of the normal and immunized mice. In the normal mice, only 1.58% $\pm$ 0.2% of the splenic T cells were $\gamma\delta$ T cells, but in the immunized mice in the inflammatory phase, 5.62% $\pm$ 1.09% of the splenic T cells were $\gamma\delta$ T cells (Fig. 2(a), left column, each figure shows a representative result of three experiments). In the normal mice, 2.19% $\pm$ 0.13% of the splenic cells were DCs, whereas in the immunized mice in the inflammatory phase, 9.77% $\pm$ 1.91% of splenic cells were DCs (Fig. 2(a), right column, each figure shows a representative result of three experiments). Furthermore, the $\gamma\delta$ T cells harvested from the immunized mice in the inflammatory phase expressed higher levels of the surface

Fig. 2. The status of $\gamma\delta$ T cells and DCs obtained from the spleens of immunized mice in the inflammatory phase, as analyzed using flow cytometry. (a) The changes in the percentage of $\gamma\delta$ T cells among splenic T-cells are shown in the left column. T-cells are stained with PE-conjugated $\gamma\delta$ TCR and APC-conjugated CD3e antibodies. T-cells that coexpressed $\gamma\delta$ TCR and CD3e are $\gamma\delta$ T cells. The changes in the percentage of DCs among lymphocyte cells isolated from the spleens of mice are shown in the right column. (b) The expression of CD69 and LFA-1 in the $\gamma\delta$ T cells obtained from the spleens of normal mice (shadow line) compared with the expression in cells obtained from the spleens of mice with severe inflammatory symptoms (solid line). (c) The level of IL-17A in $\gamma\delta$ T cells obtained from the spleens of normal mice (left panel) and mice with severe inflammatory symptoms (right panel). (d) The expression of ICAM-1, CD80, CD86, and CD83 in DCs obtained from the spleens of normal mice (shadow line) compared with the expression in cells obtained from the spleens of immunized mice with severe inflammatory symptoms (solid line). (e) Changes in the percentages of $\gamma\delta$ T cells and DCs obtained from the spleens of diseased mice on days 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 after immunization.

molecule CD69 and LFA-1, which are markers of T-cell activation (Fig. 2(b)) and secreted greater amounts of IL-17A ($19.09\% \pm 3.86\%$ versus $1.88\% \pm 0.28\%$; Fig. 2(c), each figure shows a

representative result of three experiments) than did the $\gamma\delta$ T cells obtained from the normal mice. This indicated that the number of $\gamma\delta$ T cells was increased in the inflammation phase, and that these

cells were activated. In addition, the DCs from the immunized mice expressed higher levels of ICAM-1, CD80, CD83, and CD86 than did DCs from the normal mice (Fig. 2(d)). This indicated that the DCs had undergone maturation in the immunized mice. We also analyzed the changes in the percentages of $\gamma\delta$ T cells and DCs obtained from the spleens of the immunized mice at different time points (days 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 after immunizations). The results showed that the percentages of both $\gamma\delta$ T cells and DCs increased from day 8, and peaked on days 16–day 20. The above results show that the number of $\gamma\delta$ T cells and DCs increased during the inflammatory phase, and that the $\gamma\delta$ T cells were activated and the DCs matured in the immunized mice. This suggested that both types of cells participated in the development of EAU. Moreover, we found that increase in the number of $\gamma\delta$ T cells and DCs were associated with increased severity of EAU and both types of cells were activated in the inflammatory phase. This further indicated that activated $\gamma\delta$ T cells and mature DCs participated in the progression of EAU. Then, the activated $\gamma\delta$ T cells were transferred into the IRBP_{1–20} immunized mice. The symptom of the mice with $\gamma\delta$ T cells injection was more aggravated than that of mice without $\gamma\delta$ T cells injection (Supplementary Fig. 2(a)). However, the symptom of immunized $\gamma\delta$ TCR- $\delta^{-/-}$ mice was slighter than that in EAU model in the inflammatory phase (Supplementary Fig. 3).

3.2. Co-culture of mature DCs and $\gamma\delta$ T cells promoted $\gamma\delta$ T cell activation

Pervious report showed that there is a crosstalk between DCs and $\gamma\delta$ T cell,^{16,23–26} which could influence the activation of each other. Thus, we speculated the increasing of DCs in the process of EAU might correlate with the activation of $\gamma\delta$ T cells. To analyze the relationship between DCs and $\gamma\delta$ T cells, we isolated DCs from the spleens of immunized mice, induced their maturation, and pulsed them with IRBP_{1–20}. Then, these mature DCs were transferred into the mice which were immunized by IRBP_{1–20} after eight days. The inflammation symptoms in the eyes and the percentage of $\gamma\delta$ T cells in the spleen were analyzed after transfer of DCs for four days. Increasingly severe symptom was found in mice with transfer of mature DCs. Supplementary Fig. 2(a) shows representative examples of histopathology without and with mature DCs injection. The percentage of $\gamma\delta$ T cells from spleen increased and expressed high level CD69 and LFA-1 (Supplementary Figs. 2(b), 4(a) and 4(b)). However, the inflammation symptoms could not become aggravated and the percentage of $\gamma\delta$ T cells was not changed, after immature DCs injection (supplementary Figs. 2(a) and 2(b)).

Furthermore, we isolated DCs from the spleens of immunized mice, induced their maturation, and pulsed them with IRBP_{1–20}, and finally, co-cultured them with $\gamma\delta$ T cells at a ratio of 1:1 in 24-well plates at 37°C. After 24 h, the cells were harvested,

Fig. 3. (a) Activation of $\gamma\delta$ T cells co-cultured with DCs. (b) The $\gamma\delta$ T cells co-cultured with mature DCs expressed higher levels of activation markers (CD69, LFA-1; green line) than did $\gamma\delta$ T cells not cultured with DCs (shadow line), or that on the $\gamma\delta$ T cells co-cultured with immature DCs (red line), or that on the $\gamma\delta$ T cells co-cultured with anti-ICAM-1 antibody pretreated DCs (blue line). (c) IL-17A expression is greater in $\gamma\delta$ T cells co-cultured with mature DCs (green line) than in $\gamma\delta$ T cells cultured without DCs (shadow line), or that on the $\gamma\delta$ T cells co-cultured with immature DCs (red line), or that on the $\gamma\delta$ T cells co-cultured with anti-ICAM-1 antibody pretreated DCs (blue line).

Fig. 4. The interaction between $\gamma\delta$ T cells and DCs. (a) The location of ICAM-1 and $\gamma\delta$ TCR at the interface between the DCs and $\gamma\delta$ T cells. The upper line indicates the interaction between wild-type (WT) DCs and $\gamma\delta$ T cells, and the $\gamma\delta$ T cells were labeled with PE-conjugated $\gamma\delta$ TCR antibody (red), and DCs were labeled with APC-conjugated ICAM-1 antibody (green). The bottom line indicates the interaction between an ICAM-1-EGFP/DC2.4 cell and a $\gamma\delta$ T-cell. The $\gamma\delta$ T cells were labeled with PE-conjugated $\gamma\delta$ TCR antibody (red). (b) The location of LFA-1 at the interface between the DCs and $\gamma\delta$ T cells. A $\gamma\delta$ T-cell interacts with a WT DC labeled with FITC-conjugated LFA-1 antibody (green) and PE-conjugated $\gamma\delta$ TCR antibody (red). A $\gamma\delta$ T-cell interacts with an ICAM-1-EGFP/DC2.4 cell (this cell line is a gift from professor Yiwei chu,⁴⁰ Fudan university, Shanghai, China) labeled with PE-conjugated LFA-1 antibody (red); the ICAM-1 on the DC 2.4 cell line is labeled with EGFP (green). The upper line indicates the interaction between a WT DC and a $\gamma\delta$ T-cell, and the bottom line indicates the interaction between an ICAM-1-EGFP/DC2.4 cell and a $\gamma\delta$ T-cell. (c) The amount of interaction between mature DC2.4 cell and $\gamma\delta$ T cells and between immature DC2.4 cell and $\gamma\delta$ T cells. The amount of interaction between DC2.4 cell and $\gamma\delta$ T cells, without antigen. The amount of interaction between $\gamma\delta$ T cells and DC2.4 cell with anti-ICAM-1 antibody pretreated. (d) Calcium signaling released in $\gamma\delta$ T cells interacting with mature DC2.4 cells, immature DC2.4 cells or pretreated DC2.4 cells. Data are expressed as mean $\pm$ s.e.m.; ** p < 0.01, *** p < 0.001 (two-tailed Student t -test). Scale bar = 2 μ m. (e) The contact of DC- $\gamma\delta$ T cell with or without anti-ICAM-1 antibody treatment. Scale bar = 10 μ m.

and the status of the $\gamma\delta$ T cells was determined using FACSVerse flow cytometry. The data showed that the $\gamma\delta$ T cells co-cultured with mature DCs expressed higher levels of CD69 and LFA-1, and secreted higher levels of IL-17A than did $\gamma\delta$ T cells not incubated with DCs (Figs. 3(a)–3(c)). In addition, immature DCs pulsed with IRBP_{1–20} could not induce the increase in LFA-1 expression and IL-17A secretion by $\gamma\delta$ T cells (Figs. 3(a)–3(c)). Moreover, $\gamma\delta$ T cells co-cultured with mature DCs were induced a slight increase in LFA-1 expression and IL-17A secretion than activated $\gamma\delta$ T cells did, which was stimulated by cytokines *in vitro*, although activated $\gamma\delta$ T cells expressed higher levels of CD69 and LFA-1, and secreted higher levels of IL-17A than did $\gamma\delta$ T cells not incubated

with DCs (Figs. 3(a)–3(c)). These data indicated that DCs might promote the activation of $\gamma\delta$ T cells *in vitro*. The mature DCs possessed a much stronger ability to activate $\gamma\delta$ T cells than did the immature DCs.

Furthermore, DCs-pretreated $\gamma\delta$ T cells or $\gamma\delta$ T cells-pretreated DCs were transferred into the IRBP_{1–20} immunized mice. The symptoms of those mice were more aggravated and the percentage of DCs or $\gamma\delta$ T cells was higher than that of mice without cell injection (Supplementary Figs. 2(a)–2(c), and Supplementary Fig. 4). In addition, a DC was found to contact with two or more $\gamma\delta$ T cells in the spleen of immunized mice (Supplementary Fig. 5). Thus, the interaction of DCs and $\gamma\delta$ T cells might aggravate the symptoms of EAU.

3.3. ICAM-1 and $\gamma\delta$ TCR accumulated at the interface of DCs and $\gamma\delta$ T cells

To further analyze how DCs promoted $\gamma\delta$ T-cell activation, we isolated DCs and $\gamma\delta$ T cells from the spleens of EAU mice at 16 days after immunization. We then pulsed the DCs with IRBP_{1–20} and co-cultured them with $\gamma\delta$ T cells at a 1:1 ratio in 24-well plates at 37°C. A confocal system was used to measure the interaction between the DCs and $\gamma\delta$ T cells. For this analysis, we labeled the $\gamma\delta$ TCR of CD4⁺ T cells with PE-conjugated $\gamma\delta$ TCR antibody in order to mark the locations of TCR clusters in CD4⁺ T cells. Similarly, we labeled DCs with APC-conjugated ICAM-1 antibody to indicate ICAM-1 clusters. Our data showed that ICAM-1 and $\gamma\delta$ TCR accumulated at the interface between the mature DCs and $\gamma\delta$ T cells obtained from the immunized mice (Fig. 4(a), upper line). Furthermore, we found that LFA-1 and $\gamma\delta$ TCR were co-located at the interface of mature DCs and $\gamma\delta$ T cells (Fig. 4(b), upper line). The same results were found in the case of the IRBP_{1–20}-pulsed DC2.4-ICAM-1-GFP cell line and $\gamma\delta$ T cells (Figs. 4(a) and 4(b) bottom line). Both mature and immature DCs could interact with $\gamma\delta$ T cells, but the interaction was more frequently found at mature DCs– $\gamma\delta$ T cells than immature DCs– $\gamma\delta$ T cells ($84.3 \pm 5.36\%$ versus $35 \pm 10.48\%$) (Fig. 4(c)). Moreover, IRBP_{1–20} pulsed immature DCs contacted $\gamma\delta$ T could accumulate ICAM-1 and $\gamma\delta$ TCR at the interface of DC and $\gamma\delta$ T cell (date not shown). Thus, the DCs from the diseased mice could preferentially interact with $\gamma\delta$ T cells. The accumulation of LFA-1 and ICAM-1 at the interface between the DCs and $\gamma\delta$ T cells might be beneficial for DC– $\gamma\delta$ T-cell interaction.

Calcium release is a marker of T-cell activation. To determine whether the DC– $\gamma\delta$ T-cell interaction promoted the activation of $\gamma\delta$ T cells, we measured the calcium release from $\gamma\delta$ T cells interacting with DCs. By imaging analysis, we found that $\gamma\delta$ T cells in contact with mature DCs released higher levels of calcium than did cells that were in contact with immature DCs (Fig. 4(d)). This suggested that the DC– $\gamma\delta$ T cell interaction might be a factor to contribute the activation of $\gamma\delta$ T-cell. Furthermore, mature DCs might play a major role in the interaction of DCs with $\gamma\delta$ T cells to promote the activation of the latter.

3.4. Blocking the interaction of DC– $\gamma\delta$ T-cell, the activation of $\gamma\delta$ T-cell was decreased

To confirm that $\gamma\delta$ T-cell activation was promoted by the DC– $\gamma\delta$ T-cell interaction, we blocked this interaction by using anti-ICAM-1 antibody. We found the expression of LFA-1, CD69, and IL-17A in $\gamma\delta$ T cells in contact with the treated DCs which did not significantly differ from the expression in $\gamma\delta$ T cells not in contact with DCs (Figs. 3(a)–3(c)). The contact between the DCs and $\gamma\delta$ T cells was decreased by the anti-ICAM-1 antibody (Figs. 4(c) and 4(e)). The calcium response was decreased in $\gamma\delta$ T cells contacted treated DCs, as compared to $\gamma\delta$ T cells cultured with mature DCs (Fig. 4(d)).

In addition, we pretreated the mature DCs with anti-ICAM-1 antibody to block the binding of DCs and $\gamma\delta$ T cells and transferred those DCs to immunized mice. We found the symptom of the mice with transfer of ICAM-1 antibody pretreated DCs was slighter and the percentage of activated $\gamma\delta$ T cells decreased, comparing mice with transfer of mature DCs (Supplementary Figs. 2(a) and 2(b), Supplementary Fig. 4). It indicated blocking the contact sites of DCs and $\gamma\delta$ T cells, the activated $\gamma\delta$ T cells decreased.

4. Discussion

It was previously believed that $\gamma\delta$ T cells bridge the gap between innate and adaptive immunity^{27–29} and regulate the intensity of adaptive immune responses.^{30,31} These cells are increasingly being recognized as an important source of IL-17A in several autoimmune diseases.^{32–34} The cells might reside in the target organ, where they incite a rapid immune response upon activation even before naive CD4⁺ T cells detect their cognate antigens in the secondary lymphoid organs and enhance the development of IL-17A-producing $\alpha\beta$ T cells, thereby damaging the target organ and aggravating the autoimmune disease.^{35,36} In a previous report, $\gamma\delta$ T cells were found to represent a critical element of the autoreactive responses involved in IRBP_{1–20}-induced EAU in B6 mice.¹¹ The activation of $\gamma\delta$ T cells is believed to promote the development of EAU.^{10,11,37} Moreover, the proinflammatory effect of $\gamma\delta$ T cells was found to be augmented by their activation.^{10,11} However, the mechanism of $\gamma\delta$

T-cells activation has not been reported. In the present study, we found that the DCs, especially mature DCs activated $\gamma\delta$ T cells, and the interaction of DCs and $\gamma\delta$ T cells might be a factor to promote the activation of $\gamma\delta$ T cells in EAU.

Firstly, we measured the dynamic change of $\gamma\delta$ T cells following with EAU progression, and then transferred the activated $\gamma\delta$ T cells into EAU model, the symptoms of EAU were aggravated. However, the symptoms of immunized $\gamma\delta$ TCR- $\delta^{-/-}$ mice were relieved obviously. Thus, activated $\gamma\delta$ T cells play a pathogenic role in EAU.

Furthermore, mature DCs were transferred into EAU model, the symptom of EAU was aggravated and the percentage of activated $\gamma\delta$ T cells increased. However, the transfer of immature DCs did not aggravate the symptoms of EAU. *In vitro* experiment, we found that mature DCs promoted higher level of CD69 and LFA-1 expression and IL-17A secretion in the $\gamma\delta$ T cells co-cultured with DCs than that in activated $\gamma\delta$ T cells, which activated by cytokines or that in resting $\gamma\delta$ T cells. Thus, mature DCs might promote the activation of $\gamma\delta$ T cells, and the ability of activating $\gamma\delta$ T cells of this process was much stronger than that of antigen and cytokine stimulation *in vitro*.

To further analyze whether mature DCs promote the activation of $\gamma\delta$ T cells through interacting with $\gamma\delta$ T cells, we measure the interaction of DCs and $\gamma\delta$ T cells by confocal and flow cytometry. By imaging study, the contact formed between DCs and $\gamma\delta$ T cell was found *in vitro* and *ex vivo*. Blocking the contact of DCs- $\gamma\delta$ T cells by anti-ICAM-1 antibody, the activation of $\gamma\delta$ T cells was decreased. In addition, the transfer of DCs-pretreated $\gamma\delta$ T cells or $\gamma\delta$ T cells-pretreated DCs aggravated the symptom of EAU. Thus, the interaction of DCs and $\gamma\delta$ T cells might be a factor to affect the activation of $\gamma\delta$ T cells, leading to the aggravated symptom of EAU. Whether the interaction between DCs and $\gamma\delta$ T cell could affect the mature of DC to influence the development of EAU, this problem would be another interesting problem for us to further study in the future.

Crosstalk between $\gamma\delta$ T cells and DCs via immunological synapse formation has been reported.³⁸ An immunological synapse is a platform formed between a T-cell and an APC. Multi-molecular activation clusters, involving TCR-MHC, ICAM-1-LFA-1, CD28-CD80, etc., accumulate at the T-cell-APC interface.³⁹ The immunological synapse

can control T-cell activation and regulate immune responses.^{39,40} In our study, ICAM-1, LFA-1, and $\gamma\delta$ TCR were found to be localized at the interface between DCs and $\gamma\delta$ T cells obtained from EAU mice. This indicated that immunological synapses were formed between the DCs and $\gamma\delta$ T cells. Such synapses might promote the crosstalk between DCs and $\gamma\delta$ T cells. The activation and movement of LFA-1 follows the movement of ICAM-1, and the binding of ICAM-1 and LFA-1 promotes contact between the two cells.⁴¹ Thus, ICAM-1-LFA-1 binding might be helpful for contact between DCs and $\gamma\delta$ T cells. The mechanism underlying the activation of $\gamma\delta$ T cells by DCs in EAU required further investigation.

We studied the mechanism of $\gamma\delta$ T-cell activation by flow cytometry and confocal microscopy. On flow cytometry, we found that the co-culture of $\gamma\delta$ T cells with mature DCs promoted the expression of CD69 and LFA-1 on $\gamma\delta$ T cells. Using imaging studies, we proved the contact between DCs and $\gamma\delta$ T cells and detected the accumulation of LFA-1 and ICAM-1 at the interface between the DCs and $\gamma\delta$ T cells. Furthermore, by cell transfer and the cell-cell contact imaging, the interaction of DCs and $\gamma\delta$ T cells might be a factor to promote the development of EAU through activating $\gamma\delta$ T cells. More detailed imaging studies will provide further information about immune responses and the molecular mechanism of DC- $\gamma\delta$ T cell contact.

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