



Adjuvants determine the contribution of basophils to antigen sensitization *in vivo*

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ABSTRACT

Purpose: Basophils expressing FcεRIα, the alpha chain of the total IgE high affinity receptor, appear to play a role in antigen (Ag) sensitization, although dendritic cells (DCs) are the principal Ag-presenting cells (APCs). To investigate whether these two types of APCs are involved differentially in Ag-sensitization when distinct types of adjuvants are utilized.

Methods: To investigate whether basophils and DCs serve as APCs *in vitro*, whole splenocytes, FcεRIα⁺ basophil-depleted splenocytes, and CD11c⁺ DC-depleted splenocytes from naïve DO11.10 mice were stimulated *in vitro* with ovalbumin (OVA) or OVA peptide 323–339 to evaluate Ag-induced proliferation. To investigate whether basophils function as APCs *in vivo*, BALB/c mice were actively immunized with ragweed (RW) emulsified in alum or Complete Freund's Adjuvant (CFA) followed by an RW challenge in eye drops. An anti-FcεRIα antibody (Ab) or a control Ab was injected intraperitoneally during the sensitization phase. Twenty-four hours after the RW challenge, conjunctivas and spleens were harvested for histological analysis to evaluate conjunctival eosinophilia and cytokine production, respectively.

Results: Depletion of basophils or DCs from naïve DO11.10 splenocytes significantly suppressed proliferative responses to either OVA or OVA peptide. Treatment with the anti-FcεRIα Ab suppressed the conjunctival eosinophilia when alum, but not CFA, was utilized as the adjuvant. Similarly, the anti-FcεRIα Ab inhibited cytokine production by splenocytes when alum was used as the adjuvant.

Conclusion: Adjuvants determine which APCs are utilized in Ag-sensitization.

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1. Introduction

To sensitize animals to an antigen (Ag) *in vivo*, T cells need to recognize the antigenic peptides presented by Ag-presenting cells (APCs) [1]. For many years, macrophages were thought to be the cells most likely to have the necessary capabilities for acting as APCs, because they express major histocompatibility complex (MHC) class II molecules [2,3]. However, there have been a number of studies in mice, rats and humans, that have highlighted the fact that macrophages have a low Ag presenting capability [4]; for example, the presence of alveolar macrophages was unnecessary for effective Ag presentation and macrophages may actually be unfavorable to this process [5]. Later, dendritic cells (DCs), first described by Steinman and Cohn in 1973 [6], were identified hav-

ing Ag presenting properties, being able to initiate T cell-dependent immune responses, and being more developed and considerably more potent than other types of APCs [7].

Recently, the roles of basophils in Ag-sensitization have been investigated. Treatment with the basophil-depleting monoclonal Ab (mAb), Ba103, before allergen challenge prevented the development of an immunoglobulin E (IgE)-mediated anaphylaxis [8]. More recently, three groups [9–11] reported that MHC class II-positive IL-4 producing basophils are the 'professional' APCs necessary and sufficient for the generation of T-helper type 2 (Th2) immune responses, using three different experimental settings (helminthic parasites [9], Ag–IgE complexes [10] or protease allergens [11]). Thus, basophils appear to be the key players in Ag sensitization, especially with regard to Th2-mediated allergic inflammation.

The Th1/Th2 balance is controlled by DCs [12]. The localization of DC subsets, the duration of DC activation and environmental factors (Ag and tissue) may contribute to the commitment of T helper cells *in vivo* [13]. However, it is generally accepted that DCs preferentially induce Th1 cells, while basophils preferentially induce Th2 cells [14], as described above. To reinforce the idea that DCs and basophils are used preferentially in the induction of Th1 and Th2 cells, respectively, we investigated whether these two types of

Abbreviations: Ab, antibody; Alum, aluminum hydroxide; Ag, antigen; APC, antigen-presenting cell; CFA, complete Freund's adjuvant; DC, dendritic cell; mAb, monoclonal antibody; OVA, ovalbumin; RBC, red blood cell; RW, ragweed; Th1, T-helper type 1; Th2, T-helper type 2.

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APCs are involved differentially in Ag-sensitization by using murine experimental allergic conjunctivitis. As we have previously shown that aluminum hydroxide (alum) and complete Freund's adjuvant (CFA) induce different characteristics of Th1/Th2-mediated immunopathology in rat experimental allergic conjunctivitis [15], we used these two adjuvants to induce Th1- and Th2-mediated experimental allergic conjunctivitis in mice and investigated the role basophils play in the development of experimental allergic conjunctivitis.

2. Methods

2.1. Mice

Inbred wild-type (WT) BALB/c mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan. BALB/c DO11.10 mice, which express a T cell receptor (TcR) transgene specific for the immunodominant epitope of OVA peptide 323–339, were kindly provided by Dr. Iwabuchi (Hokkaido University, Japan). The mice were kept in specific pathogen-free conditions at the Kochi Medical School's animal facility, and age- and gender-matched mice were used when they were 6–12-weeks-old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Reagents

Short ragweed (RW) pollen was purchased from Polysciences Inc., Warrington, PA. RW extract was obtained from LSL Co. Ltd., Tokyo, Japan. Alum (13 mg/ml) was purchased from Sigma, St. Louis, MO. CFA containing 2.5 mg/ml *Mycobacterium tuberculosis* was obtained from Difco Laboratories (Baltimore, MD). A hybridoma producing a mAb specific for Fc ϵ RI α (MAR-1), which had been used elsewhere [11,16], was established by immunizing an Armenian hamster with CHO transfectants, stably expressing mouse Fc ϵ RI, and fusing the immune splenocytes with a P3U1 myeloma. The Abs for *in vivo* treatments were purified from ascites using a protein G column and the Ab preparations contained less than 100 pg/ml of endotoxin. The following Abs were purchased: normal hamster IgG (nhlgG) (MP Biomedicals Inc., Aurora, OH), fluorescein isothiocyanate (FITC)-labeled anti-MHC class II, FITC-labeled anti-CD49b (DX5), biotin-labeled anti-Fc ϵ RI α (MAR-1), phycoerythrin-Cy5-labeled streptavidin (SAPECy5) and phycoerythrin-Cy7-labeled streptavidin (SAPECy7) (eBioscience, San Diego, CA), FITC-labeled anti-CD11c and phycoerythrin (PE)-labeled streptavidin (SAPE) (BD Biosciences, Franklin Lakes, NJ), and PE-labeled anti-CD11c and allophycocyanin (APC)-labeled DX5 (Miltenyi Biotec, Auburn, CA).

2.3. Negative selection of Fc ϵ RI α ⁺ and CD11c⁺ cells from splenocytes

The negative selection of Fc ϵ RI α ⁺ and CD11c⁺ cells from splenocytes was conducted using biotinylated MAR-1 or biotinylated anti-CD11c and streptavidin Microbeads on AutoMACS columns (Miltenyi Biotec) following the manufacturer's recommendation.

2.4. Flow cytometric analysis

Splenocytes were incubated for 30 min on ice with FITC-, APC- or biotin-labeled Abs at optimal concentrations. After the incubation, the cells were washed with cold 2% fetal calf serum in phosphate buffered saline (FCS-PBS). In the case of biotin-labeled Abs, the cells were then incubated with SAPE for 10 min and washed again. When cells were stained with three different Abs, SAPECy5 and SAPECy7 were used for biotinylated MAR-1. Dead cells were excluded using

7-amino-actinomycin D (7-AAD) or 4',6-diamidino-2-phenylindole (DAPI). These cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and a MACSQuant analyzer (Miltenyi Biotec). Data are presented as histograms or dot-plots.

2.5. Cellular proliferation assay

Red blood cell (RBC)-depleted splenocytes (3×10^5 cells/well) from naïve DO11.10 mice were cultured in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 5% FCS and 50 μ M 2-mercaptoethanol (ME). Alternatively, Fc ϵ RI α ⁺ or CD11c⁺ cells were depleted by Auto MACS and then cultured as above. The cells were stimulated with either OVA peptide 323–339 (0.01, 0.1 and 1 μ g/ml) or OVA (1, 10 and 100 μ g/ml). After an 80 h incubation at 37 °C in a humidified atmosphere containing 5% CO₂, the cultures were pulsed for 16 h with 0.5 μ Ci/well [³H]thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). The cultures were then harvested and the incorporated radioactivity was measured using standard techniques. The data were expressed as counts per minute [cpm].

2.6. RW sensitization, RW challenge and treatment with Abs

RW sensitization and challenge were performed as previously described [17]. Briefly, RW emulsified in alum or CFA was injected subcutaneously into left leg and tail base; 50 μ l of the emulsion containing 50 μ g of RW (RW:adjuvant = 1:1 volume) was injected into each site. The mice were injected intraperitoneally with 100 μ g of purified MAR-1 ($n = 5$ [CFA] and $n = 10$ [alum]) or nhlgG ($n = 5$ [CFA] and $n = 10$ [alum]) on six occasions (three, four and five days prior to immunization, on the day of immunization, and one and two days after immunization). Ten days after immunization, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes and spleens were harvested for histological analysis and cytokine assays, respectively.

2.7. Histological analysis

The eyes, including the conjunctivas, were harvested and fixed in 10% buffered formalin. Vertical 2 μ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by two blinded observers. The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head. The data are presented as the average $\pm$ SEM of all the mice examined.

2.8. Measurement of cytokines in the culture supernatants

RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h with RW extract (25 μ g/ml) in 96-well flat-bottom plates in 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. Levels of IL-4, IL-10, IL-12 and IFN- γ were measured using the Bioplex system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Data are shown as the average $\pm$ SEM (pg/ml) of three individual experiments.

2.9. Statistical analysis

Differences between the two groups with respect to their splenocyte proliferation, cytokine production, and infiltrating eosinophil numbers were tested for significance using the Student's *t*-test. *P*-values less than 0.05 were considered significant compared to the control group.

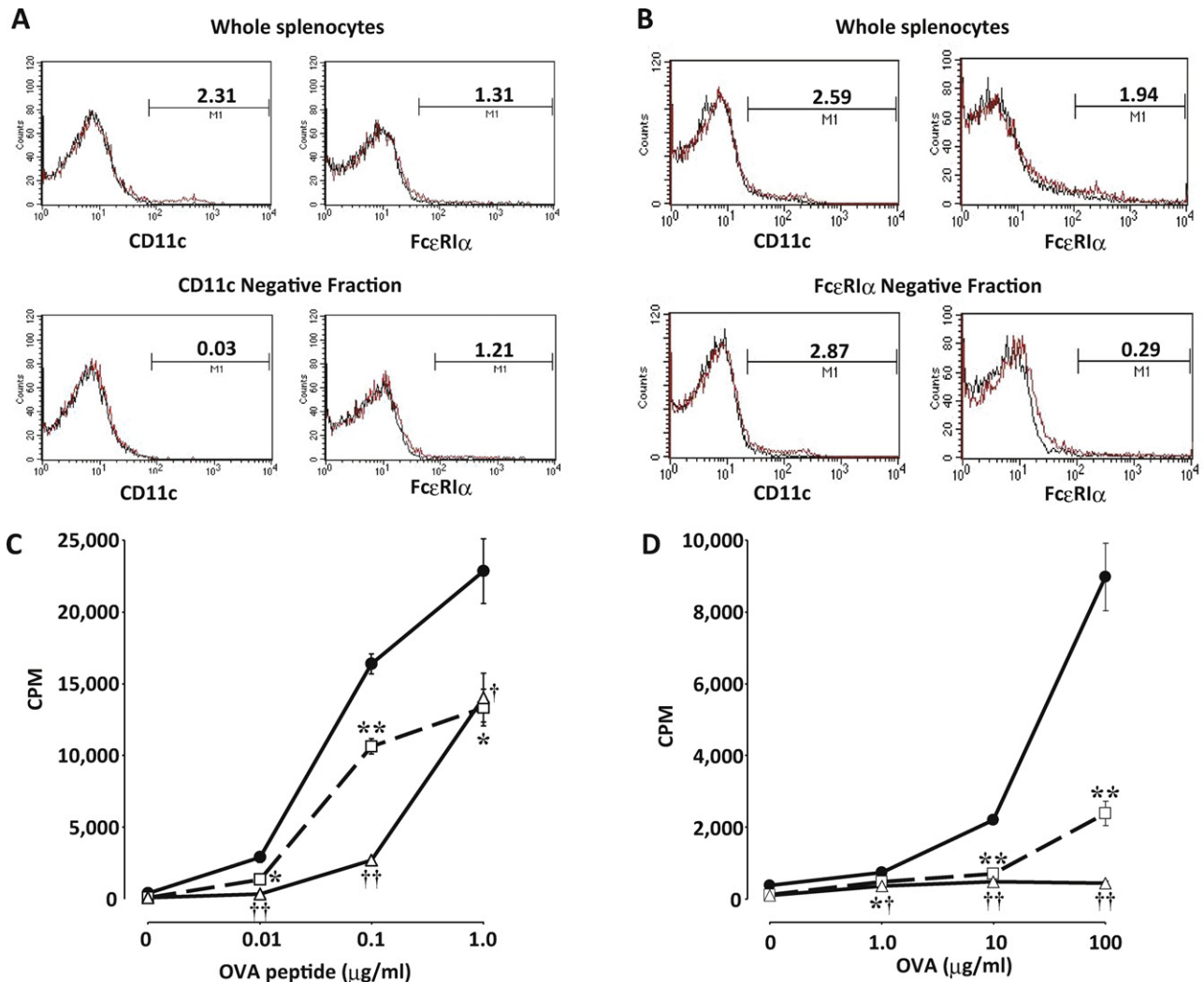


Fig. 1. Both basophils and DCs participate in Ag-induced splenocyte proliferation. CD11c⁺ and FcεRIα⁺ cells were depleted from naïve DO11.10 splenocytes using Auto MACS. (A, B) Flow cytometric analysis of whole splenocytes, CD11c⁺ DC-depleted splenocytes (A), and FcεRIα⁺ basophil-depleted splenocytes (B). Upper panels: whole splenocytes before negative selection. Lower panels: splenocytes depleted of CD11c⁺ or FcεRIα⁺ cells. Red and black lines indicate with or without staining with the indicated Ab. The number depicted in each column indicates percentage positive for the indicated Ab. One representative of three experiments is shown. (C, D) Proliferative responses of splenocytes. Whole, basophil-depleted or DC-depleted splenocytes from naïve DO11.10 mice were stimulated *in vitro* with OVA peptide 323–339 (C) or OVA (D). Closed circle: whole splenocytes. Open square: basophil-depleted splenocytes. Open triangle: DC-depleted splenocytes. **P* < 0.05 and ***P* < 0.01 between whole splenocytes and basophil-depleted splenocytes. †*P* < 0.05 and ††*P* < 0.01 between whole splenocytes and DC-depleted splenocytes. One representative of three experiments is shown.

3. Results

3.1. Basophils and DCs are required for Ag-induced splenocyte proliferation

To confirm that basophils and DCs are necessary for T cell activation, whole splenocytes, CD11c⁺ DC-depleted splenocytes and FcεRIα⁺ basophil-depleted splenocytes from naïve DO11.10 mice were tested for proliferative responses to OVA peptide 323–339 and OVA. Depletion was performed using AutoMACS and the specific depletion of each population was confirmed as shown in Fig. 1A and B. Proliferation against the OVA peptide (Fig. 1C) and OVA protein (Fig. 1D) was observed in a dose-dependent manner when whole DO11.10 splenocytes were stimulated. However, proliferative responses were significantly impaired at three different concentrations of Ag in basophil- and DC-depleted DO11.10 splenocytes, compared to whole DO11.10 splenocytes (Fig. 1C and D). To examine whether basophils expressed MHC class II molecules under our experimental conditions, we performed flow cytometric analysis. The proportion of FcεRIα-expressing cells out of

whole splenocytes was 0.52% (0.05 + 0.47%) and 10% of the FcεRIα-expressing cells were basophils, which concomitantly expressed CD49b (Fig. 2A). Most of the basophils expressed MHC class II molecules (Fig. 2B).

3.2. Basophils are required for Ag sensitization with alum, a Th2-inducing adjuvant

Next, to investigate the roles of basophils in Th2 skewed sensitization *in vivo*, mice were immunized with RW adsorbed on alum followed by a challenge with RW in eye drops to induce conjunctival eosinophilia. These mice were injected intraperitoneally with MAR-1 (100 μg/injection) for six times during the sensitization phase. Treatment with MAR-1 markedly suppressed conjunctival eosinophil numbers when compared to control mice that received nhlgG (Fig. 3A–C). Splenocytes from the mice treated with MAR-1 produced significantly less IL-4 and IL-10 when they were re-stimulated with RW extract, while Th1 cytokines were not affected (Fig. 3D).

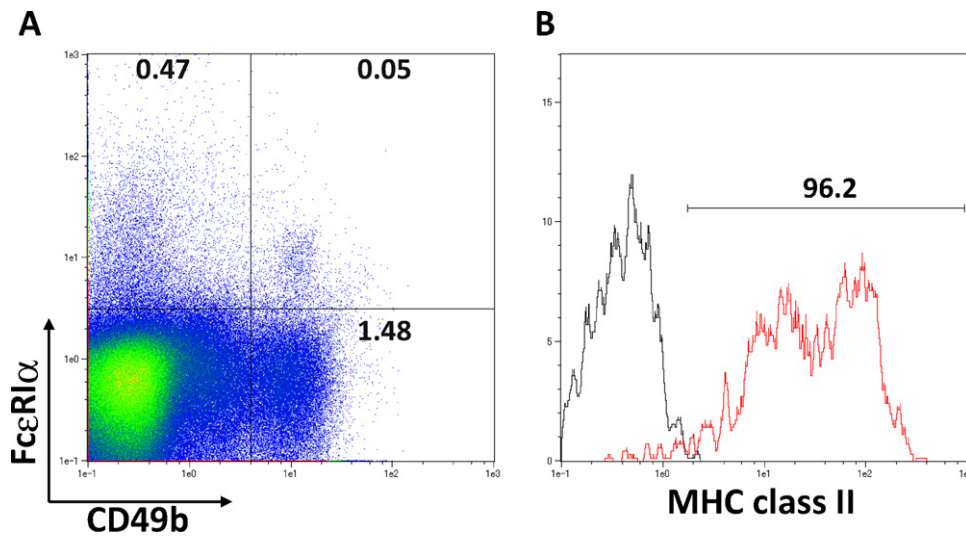


Fig. 2. Splenic basophils express MHC class II molecules. Splenocytes from naïve BALB/c mice were subjected to flow cytometric analysis by using MACSQuant. (A) Expression of FcεRIα (MAR-1) and CD49b (DX5). Percentage shown in each quadrant indicates % out of total cells examined. (B) Expression levels of MHC class II molecules on splenocytes expressing both FcεRIα and CD49b (basophils). Red and black lines indicate with or without staining with the Ab against MHC class II, respectively. The number depicted indicates % positive for MHC class II in basophil compartment. One representative of four experiments is shown.

3.3. Basophils are not required for Ag sensitization with CFA, a Th1-inducing adjuvant

Finally, to investigate the roles of basophils in Th1 skewed sensitization *in vivo*, the mice were immunized as above, except that CFA was used, instead of alum, as the adjuvant. In contrast to the data using an alum adjuvant, MAR-1 treatment affected neither conjunctival eosinophilia (Fig. 4A–C), nor Th2 cytokine production (Fig. 4D). On the contrary, IFN- γ was significantly upregulated by MAR-1 treatment (Fig. 4D).

4. Discussion

In this study, we have demonstrated that both basophils and DCs function as APCs when naïve T cells are stimulated *in vitro*. Furthermore, we found that treatment with the anti-FcεRI α Ab, MAR-1, suppressed the conjunctival eosinophilia induced by RW

when mice were sensitized with RW in alum, but not RW in CFA. Our data demonstrate, for the first time, that the contribution of basophils to Ag-sensitization is determined by the adjuvant used.

A previous study comparing alum and CFA [18] demonstrated that after immunization with Ags in alum, IL-4 production was required for the proliferation of the elicited T-cells, whereas the proliferative responses of T-cells elicited by Ags in CFA were not inhibited by anti-IL-4. Thus, the same Ag administered in different adjuvant preparations elicited functionally different subpopulations of CD4 $^{+}$ cells; Th2 cells were produced after immunization with Ags in alum [19] and Th1 cells after immunization with Ags in CFA [20]. As described in the Introduction, three research groups have demonstrated recently that DCs are not required for the development of Th2 responses to helminthic parasites [9], complexes of Ag and IgE [10], or protease allergens [11]. Furthermore, it was demonstrated that the administration of Ag-pulsed basophils, but not Ag-pulsed DCs or mast cells, selectively induced Th2 cells in

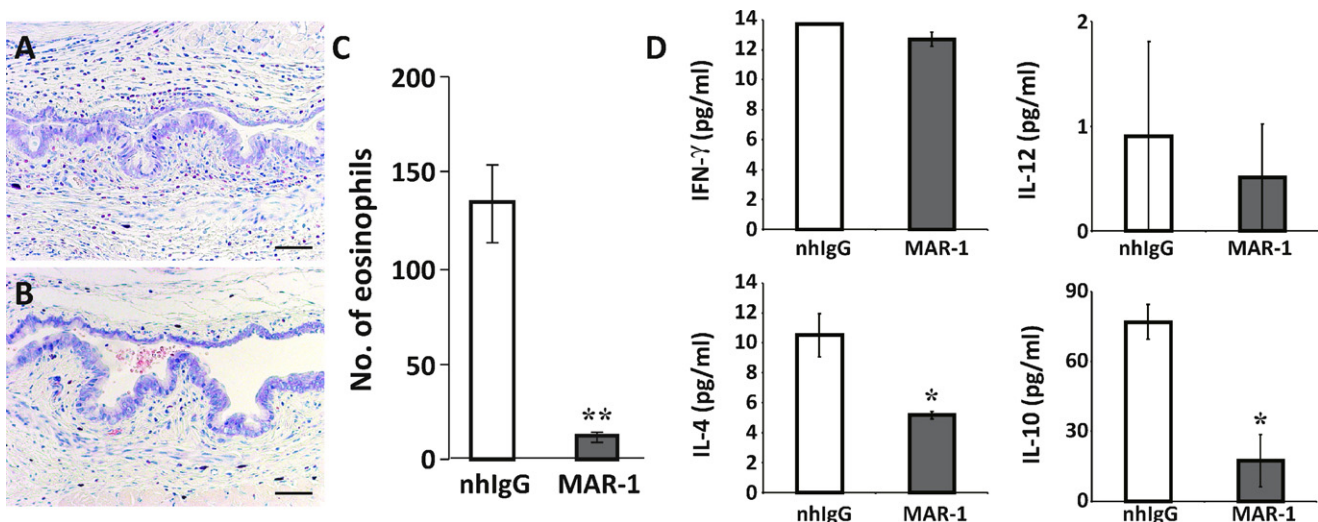


Fig. 3. Effects of MAR-1 treatment on conjunctival eosinophilia and splenocyte cytokine production in RW/alum-sensitized mice BALB/c mice were immunized with RW adsorbed on alum. Ten days later, the mice were challenged with RW in eye drops to induce conjunctival eosinophilia. These mice were injected intraperitoneally with 100 μ g of nhlgG (A) or MAR-1 (B) six times during the induction phase. Microphotographs of conjunctiva (A, B). Eosinophils were counted and the data are presented as eosinophil numbers per section (C). RW-specific cytokine production by splenocytes (D). Open bars and filled bars indicate nhlgG- and MAR-1-treated group, respectively. * P < 0.05 and ** P < 0.01.

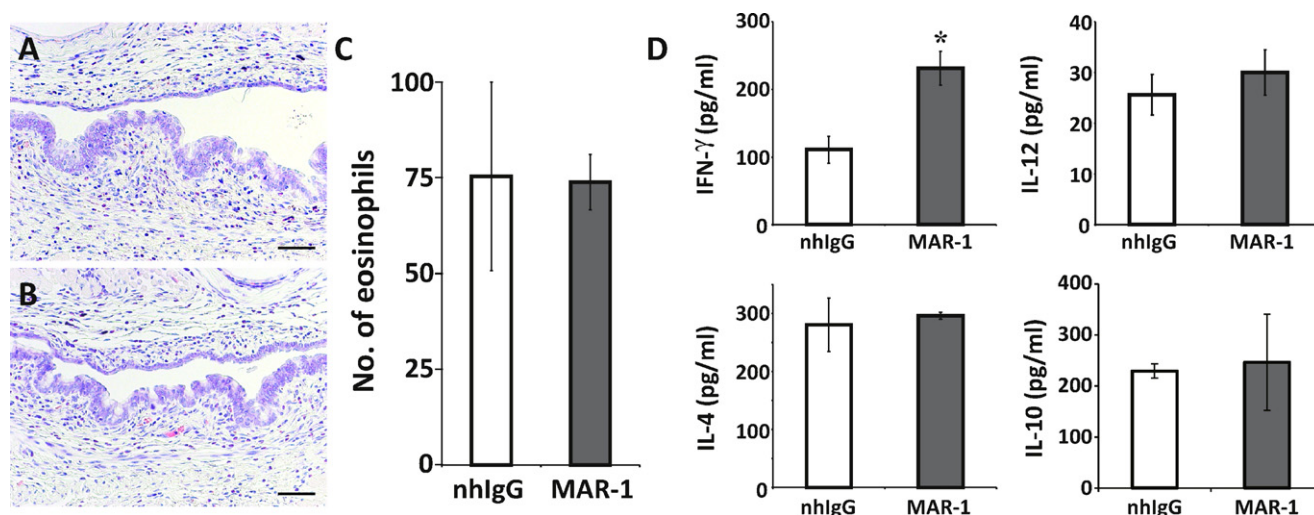


Fig. 4. Effects of MAR-1 treatment on conjunctival eosinophilia and splenocyte cytokine production in RW/CFA-sensitized mice BALB/c mice were immunized with RW emulsified in CFA. Ten days later, the mice were challenged with RW in eye drops to induce conjunctival eosinophilia. These mice were injected intraperitoneally with 100 μ g of nhlgG (A) or MAR-1 (B) six times during the induction phase. Microphotographs of conjunctiva (A, B) Eosinophils were counted and the data are presented as eosinophil numbers per section (C). RW-specific cytokine production by splenocytes (D). Open bars and filled bars indicate nhlgG- and MAR-1-treated group, respectively. * $P < 0.05$.

vivo [10]. Together with our data which showed that treatment with MAR-1 suppressed conjunctival eosinophilia in mice sensitized with RW in alum, basophils appear to be the principal APCs in conditions that induce Th2 cells.

It was reported that inoculation with the nematode parasite, *Trichuris muris* (*T. muris*), failed to develop a Th2 response and to expel helminths in mice whose MHC class II expression was restricted to CD11c⁺ DCs [9]. Furthermore, in those mice, it was found that a *T. muris* infection induced Th1 cells [9]. Thus, DCs preferentially promote a Th1 immune response and appear to be the predominant APCs during Th1 development. To examine whether basophils exert their APC function under Th1-inducing conditions, we examined the effects of MAR-1 treatment *in vivo* under Th1-inducing conditions using CFA as the adjuvant. In contrast to experiments in which alum was used as the adjuvant, the suppression of conjunctival eosinophilia was not observed. Additionally, Th2 cytokine production was not affected by MAR-1 treatment. Furthermore, IFN- γ , a Th1 cytokine, was significantly upregulated by MAR-1 treatment. Based on the fact that Th1 and Th2 cells regulate each other by their producing cytokines [21], it could be considered that depletion of basophils by MAR-1 treatment attenuates Th2 immune responses, thereby augmenting Th1 immune responses. Our data together with the previous reports [9–11,14] further suggested that basophils function as APCs under Th2-inducing conditions. However, it should be kept in mind that a controversial report demonstrated that depletion of basophils did not affect Th2 immunity induced by *Nocardia brasiliensis* [22]. More recently, Hammad et al. reported that basophils are not necessary for the induction of Th2 immunity to inhaled house dust mite allergen [23]. Thus, further studies such by using different types of adjuvants are necessary to clarify which factors determine that basophils act as APCs *in vivo*.

It remains unclear how adjuvants determine the types of APCs involved in immune responses. It was reported that CFA-derived pathogen-associated molecular patterns (PAMPs), which are agonist against Toll-like receptor (TLR), participate in proinflammatory cytokine expression by myeloid DCs [24]. Recent studies demonstrated that alum exerts its adjuvant activity by stimulating inflammatory DCs through the activation of the NALP3 inflammasome, a member of nucleotide-binding oligomerization domain-like receptors (NLRs) [25,26]. Thus, TLR-dependent and independent pathways appear to play a role in the adjuvant activity

of CFA and alum, respectively. Future studies will focus on whether the TLR-dependence in each adjuvant determines the selection of APC types.

Conflict of interest

All the authors have declared that they have no conflict of interest.

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