

ORIGINAL ARTICLE

Preventive effect of galectin-9 on double-stranded RNA-induced airway hyperresponsiveness in an exacerbation model of mite antigen-induced asthma in mice

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ABSTRACT

Background. Viral respiratory infection is the most common cause of acute asthma exacerbation in patients with stable asthma. The replication of most respiratory viruses requires the generation of double-stranded RNA (dsRNA), resulting in the activation of host immune responses. Synthetic dsRNA, polyinosinic-polycytidylic acid (PolyIC), mimics the effects of viruses in various cell types. To evaluate new therapies for mite antigen-induced chronic asthma, we developed an acute exacerbation model of mouse chronic asthma using mite antigen and PolyIC. We also examined the preventive effects of recombinant galectin-9 (Gal-9) on acute asthma exacerbation in this model. **Methods.** Airway hyperresponsiveness (AHR) was examined to evaluate the exacerbation of chronic asthma. To analyze airway inflammation, the numbers of inflammatory cells and concentrations of cytokines in the bronchoalveolar lavage fluid (BALF) were estimated by flow cytometry and enzyme-linked immunosorbent assay, respectively. **Results.** AHR was accelerated by intranasal administration of PolyIC in addition to mite antigen. Levels of cytokines that contribute to AHR, including interferon- γ , tumor necrosis factor- α , and RANTES (CCR5), and of Gal-9 in the BALF were elevated in this acute asthma exacerbation mouse model. Intranasal administration of recombinant Gal-9 reduced the PolyIC-induced AHR and levels of these cytokines in the BALF. Further, Gal-9 suppressed the production of cytokines induced by PolyIC in the alveolar macrophages. **Conclusions.** Our findings demonstrated that exogenous Gal-9 suppressed dsRNA-induced AHR in an acute exacerbation model of chronic asthma in mice, and suggest that recombinant Gal-9 could be therapeutically effective for preventing acute asthma exacerbation.

KEYWORDS asthma exacerbation, double-stranded RNA, galectin-9, mouse, Th1 cells

INTRODUCTION

Asthma is a chronic inflammatory airway disease characterized by airway hyperresponsiveness (AHR), reversible airway obstruction, and eosinophilic airway

inflammation. The majority of patients with asthma are well controlled by treatment with preferred controller drugs (inhaled corticosteroids with or without long-acting β -agonists and leukotriene receptor antagonists) [1–3]. Acute asthma exacerbations, however, are caused by respiratory viral infections and the resulting innate immune response in patients with stable asthma. Up to 80% of asthma exacerbations are triggered by viral infection, especially with the single-stranded RNA virus rhinovirus, which causes the common cold [4, 5]. Replication of most respiratory viruses requires the generation of double-stranded RNA (dsRNA), resulting in the activation

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of host immune responses. dsRNA elicits various cellular responses similar to those provoked by viral infection [6, 7].

Galectin-9 (Gal-9) is a member of the family of β -galactoside binding lectins that induces various biologic reactions, such as cell chemoattraction, activation, and apoptosis [8]. Gal-9 regulates immune responses by reducing helper T type 1 (Th1) cells, and the suppressive mechanism of Gal-9 is thought to be due to the induced apoptosis of activated Th1 cells via a Gal-9/T-cell immunoglobulin- and mucin domain-containing molecule (Tim-3) interaction [9]. Gal-9 was recently demonstrated to be involved in airway disease. Gal-9 expression is elevated in lung tissue in animal models of asthma, and its overexpression correlates with an increase in Th2 cytokines and increased eosinophil counts in the lung [10, 11]. The role of Gal-9 in allergic lung diseases, however, remains unclear.

In the present study, we developed an acute exacerbation model of chronic asthma using the *Der-matophagoides farinae* (Df)-induced murine model of chronic asthma and synthetic double-stranded RNA (dsRNA), polyinosinic-polycytidylic acid (PolyIC) to evaluate the effect of new therapies on acute asthma exacerbation. Our findings indicated that elevated levels of cytokines, including interferon (IFN)- γ , tumor necrotizing factor (TNF)- α , and RANTES (CCR5), contribute to AHR. Gal-9 levels in the bronchoalveolar lavage fluid (BALF) were also elevated in this acute asthma exacerbation model. Further, to evaluate the role of exogenous Gal-9 in the acute exacerbation of asthma, we used this model to examine the therapeutic effects of recombinant Gal-9.

METHODS

Animal Model

BALB/c mice were obtained from Charles River Laboratory (Yokohama, Japan). We used female mice (8–12 weeks old) in the experimental animal center of Kagawa University and Kawasaki Medical School. The mice were first sensitized by intraperitoneal injections of 500 μ g Df allergen (GREER Laboratories Inc., Lenoir, NC, USA) with 2 mg alum on days 0 and 14. Next, 400 μ g of Df solution was applied intranasally and the procedures were repeated on two consecutive days each week for 3 weeks (days 21, 22, 28, 29, 35, and 36). The mice were then challenged by intranasal administration of phosphate-buffered saline (PBS) or 80 μ g Df solution, or 80 μ g Df solution with 20 μ g PolyIC (InvivoGen, San Diego, CA, USA) on day 50. Negative control animals were

injected with PBS plus alum and exposed to PBS in a similar manner. In some experiments, mice were intranasally or subcutaneously administered either recombinant human stable Gal-9 (3 μ g/body) [12] or PBS 24 hours before the last antigen challenge. There was minimal endotoxin contamination in the Df and PolyIC solution, as previously described (<.25 EU/mg) [13]. All experiments in the present study were performed under a protocol approved by the Institutional Animal Care and Use Committee of Kagawa University and Kawasaki Medical School.

Bronchoalveolar Lavage and AHR

Bronchoalveolar lavage (BAL) was performed 24 hours after the last antigen administration, and BALF was obtained by washing the lungs with 4×1 mL of PBS and centrifuged. The supernatant of the first wash was stored at -80°C until use. Cell pellets of all washes were collected and resuspended in 1 mL PBS. The number of BALF cells was counted using a cell counter. Cytospin slides were stained with Diff-Quik (Sysmex, Kobe, Japan). Differential cell counts were performed on at least 400 cells. Airway resistance (sRaw) in awake mice was measured with a two-chambered, double-flow plethysmograph system (Pulmos; M.I.P.S, Osaka, Japan) as previously described [13, 14]. AHR is expressed as the concentration of methacholine required to provoke a doubling of sRaw (PC₂₀₀).

Enzyme-Linked Immunosorbent Assay (ELISA)

BALF concentrations of mouse Gal-9 were measured by ELISA as previously described [15]. BALF concentrations of interleukin (IL)-5, IL-13, IFN- γ , TNF- α , RANTES (CCL5), thymic stromal lymphopoietin (TSLP), and IFN- γ inducible protein (IP)-10 (CXCL10) in mice were measured using the respective ELISA kits (R&D Systems, Minneapolis, MN, USA). The detection limits (in pg/mL) were as follows: Gal-9, 62.5; IL-5, 7.0; IL-13, 1.5; IFN- γ , 2.0; TNF- α , 5.0; RANTES, 2.0; TSLP, 2.6; and IP-10, 4.2 in mice. Concentrations below the detection limits were assumed to be zero for statistical analysis. The Df-specific serum IgE was measured as previously described [14]. The Df-specific serum IgE levels were expressed as relative absorbance units (optical density at 450 nm [OD₄₅₀]).

Flow Cytometry

BALF cells were stained with fluorescein isothiocyanate (FITC)-anti-T1ST2 (MD Biosciences,

Zurich, Switzerland) as a Th2 cell surface marker [16], phycoerythrin (PE)-anti-Tim-3 (eBioscience, San Diego, CA, USA) as a Th1 cell surface marker [9], PerCP-anti-CD3, and APC-anti-CD4 (BD Biosciences, San Jose, CA, USA). Th1 and Th2 cells were evaluated by Tim-3 and T1/ST2 expression gating on CD3- and CD4-positive cells using a FACSCalibur[®] flow cytometer (BD Biosciences). Alveolar macrophages in the BALF from normal BALB/c mice were stained with PE-anti-Tim-3 pre-incubated with anti-CD16/CD32 (BD Biosciences) to block Fc-receptor mediated binding. Tim-3 expression was evaluated by flow cytometry.

Culture of Alveolar Macrophages

Alveolar macrophages were obtained by BAL from normal BALB/c mice. More than 95% of harvested cells from naïve mice were macrophages. The macrophages (1×10^5 /well) were cultured with medium alone (RPMI containing 5% heat-inactivated fetal bovine serum), PolyIC (1 μ g/mL), PolyIC (1 μ g/mL) + human stable gal-9 (1 μ g/mL), or PolyIC (1 μ g/mL) + human stable gal-9 (1 μ g/mL) + Tim3-Ig (10 μ g/mL) [9] for 18 hours at 37°C. We designed the experiment to clarify the involvement of Tim-3 in alveolar macrophages using Tim-3-Ig fusion protein and appropriate human IgG1 (Ancell, Bayport, MN, USA) as a control. Concentrations of TNF- α , RANTES, and IP-10 were determined in the culture supernatants by ELISA.

Statistical Analysis

All data are expressed as mean $\pm$ standard error (SEM). The Kruskal–Wallis test was used to compare values of different groups. When a significant difference between groups was detected, intergroup comparisons were assessed using the Mann–Whitney *U* test. Differences with probability values of less than 0.05 were considered significant.

RESULTS

Characterization of an Acute Exacerbation Model of Chronic Asthma in Mice

Two groups of mice were sensitized by intraperitoneal injection with either Df in PBS or PBS alone, and then intranasally administered either Df in PBS or PBS alone six times. Like in human patients with established asthma, this chronic asthma model already has an increased AHR to methacholine without Df challenge (Df/PBS) in comparison with PBS-

treated controls (PBS/PBS; $P = .0147$, Figure 1a). To develop an acute exacerbation model of mouse chronic asthma, PolyIC was used to mimic the viral infection in this chronic asthma model. In the present study, AHR was evaluated as one marker of acute asthma exacerbation. A greater degree of airway reactivity to methacholine was observed in the PolyIC challenge in the presence of the Df allergen (Df/Df+PolyIC) compared to Df alone (Df/Df; $P = .0006$, Figure 1a). Total leukocytes, macrophages, lymphocytes, eosinophils, and neutrophils were significantly increased in the BALF of Df-sensitized mice without Df challenge (Df/PBS) compared with PBS-treated controls (PBS/PBS; $P < .05$, Figure 1b). The numbers of these inflammatory cells in the BALF did not differ significantly between the Df- and PolyIC-challenged mice (Df/Df+PolyIC) and Df alone-challenged mice (Df/Df; Figure 1b).

To evaluate the mechanisms of the increase in PolyIC-induced AHR, we compared the number of CD4+ T cells, and cytokine levels in the BALF between the Df/Df+PolyIC and Df/Df groups 24 hours after intranasal PolyIC challenge. IFN- γ , TNF- α , RANTES, and IP-10 levels were increased, and IL-13 levels were decreased in Df/Df+PolyIC mice compared with Df/Df mice ($P < .05$, Figure 2a). TSLP was under the detection limit in both groups of mice (data not shown). The Gal-9 concentration in the BALF of Df/Df+PolyIC mice was higher than that in Df/Df mice ($P < .05$, Figure 2a). The number of Tim-3+CD4+ T cells was increased and the number of T1/ST2+CD4+ T cells was decreased in the BALF in Df/Df+PolyIC mice compared with Df/Df mice ($P < .05$, Figure 2b). The Th1-related airway inflammation was increased by PolyIC challenge in addition to Df.

Next, Df-specific serum IgE levels were determined by ELISA in both groups. Df-specific serum IgE levels were significantly increased in the Df/PBS group compared with the PBS/PBS group (Df/PBS, 0.778 ± 0.017 OD450; PBS/PBS, 0.016 ± 0.007 OD450; $P < .05$). Df or Df with PolyIC challenge did not affect the Df-specific serum IgE levels compared with the Df/PBS group (Df/Df, 0.726 ± 0.104 OD450; Df/Df + PolyIC, 0.652 ± 0.109 OD450, data not shown).

Effect of Gal-9 in an Acute Exacerbation Model of Chronic Asthma in Mice

We evaluated the effect of Gal-9 in this acute exacerbation model of chronic asthma. Recombinant human stable Gal-9 was intranasally or subcutaneously administered 24 hours before the final antigen challenge. At a dose of 3 μ g/body, intranasal, but not

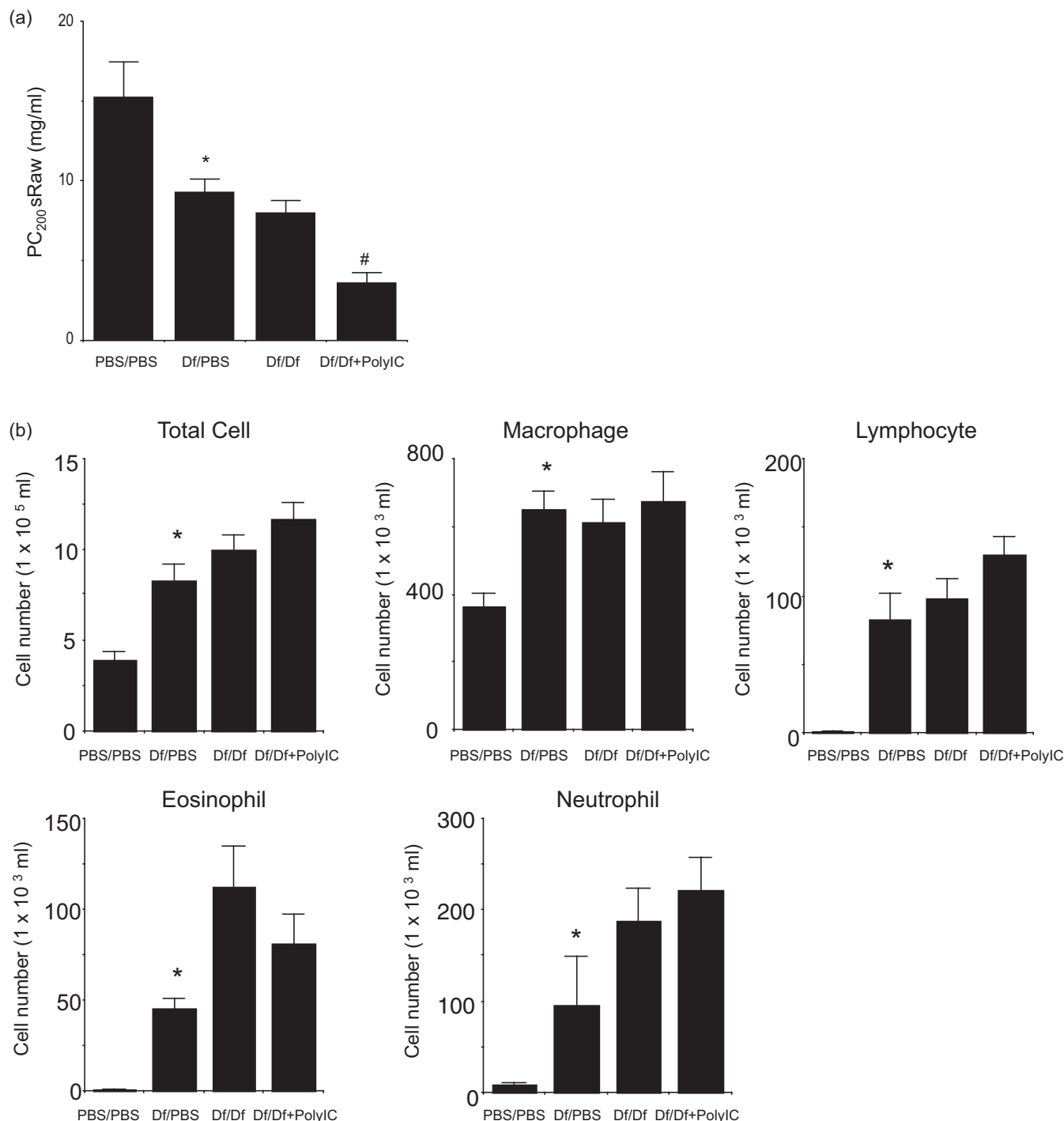


FIGURE 1. Characterization of an acute exacerbation model of Df-induced chronic asthma in mice. AHR was measured and expressed in PC₂₀₀ sRaw (mg/mL) as described in the Methods (a). Absolute numbers of inflammatory cells in the BALF were estimated as described in the Methods (b). Data represent means $\pm$ SEM. Values shown are means from at least three independent experiments with 6–10 mice per group. * $P < .05$ compared with PBS-intraperitoneally (ip) sensitized, PBS-intranasally (in) treated, and PBS-challenged mice (PBS/PBS); # $P < .05$ compared with Df-ip sensitized, Df-in treated, and Df-challenged mice (Df/Df). Df-ip sensitized, Df-in treated, and PBS-challenged mice (Df/PBS); Df-ip sensitized, Df-in treated, and Df+PolyIC-challenged mice (Df/Df+PolyIC).

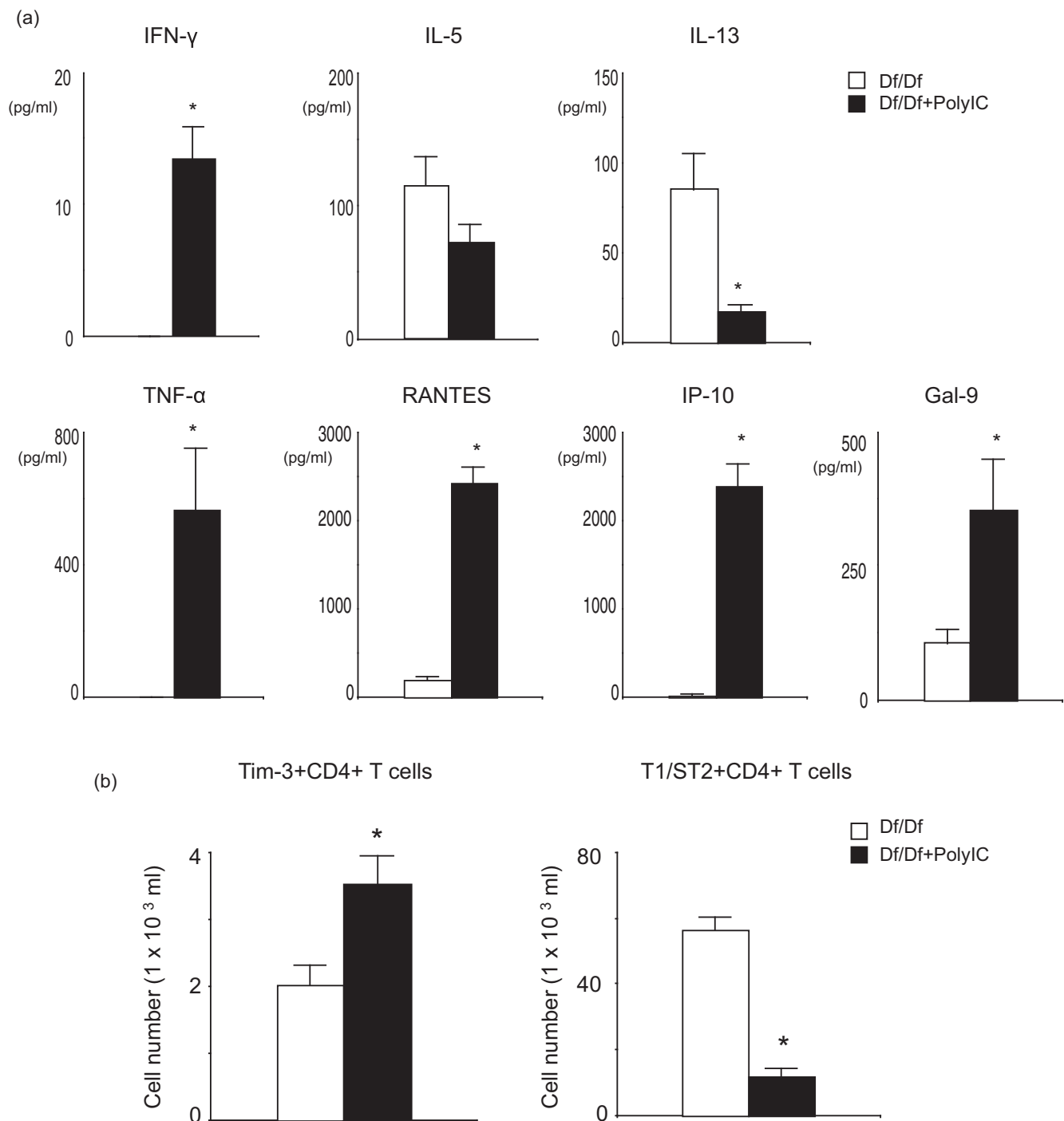


FIGURE 2. Levels of cytokines, chemokines, and Gal-9 in the BALF of an acute exacerbation model of Df-induced chronic asthma in mice. IFN- γ , IL-5, IL-13, TNF- α , RANTES, IP-10, and Gal-9 levels in the BALF were determined by ELISA (a). BALF cells were stained with FITC anti-T1/ST2, phycoerythrin anti-Tim-3, PerCP anti-CD3, and APC anti-CD4. Th1 and Th2 cells were evaluated by Tim-3 and T1/ST2 expression gating on CD3+ and CD4+ T cells (b). Data represent means $\pm$ SEM. Values shown are means from three independent experiments with 6 mice per group. * $P < .05$ compared with Df-ip sensitized, Df-in treated, and Df-challenged mice (Df/Df); Df-ip sensitized, Df-in treated, and Df+PolyIC-challenged mice (Df/Df+PolyIC).

subcutaneous, Gal-9 treatment reduced the PolyIC-induced AHR ($P < .05$, Figure 3a). The numbers of inflammatory cells in the BALF did not differ significantly between the Gal-9 treated groups and PBS-treated controls. Although the eosinophil number appeared to be substantially lower in the Df/Gal-9sc/Df+PolyIC group than in the other groups, the difference was not significant (Figure 3b).

The effect of intranasal Gal-9 treatment on the accumulation of CD4+T cells, and cytokine levels in the BALF were evaluated next. Concentrations of IFN- γ , TNF- α , RANTES, and IP-10, but not Th2 cytokines (IL-5 and IL-13) in the BALF were significantly lower in mice given Gal-9 intranasally (Df/Gal-9in/Df+PolyIC) ($P < .05$; Figure 4a). Furthermore, the number of Tim-3+CD4+ T cells, but not T1/ST2+CD4+ T cells, in the BALF was reduced by intranasal Gal-9-treatment ($P < .05$, Figure 4b).

Effect of Gal-9 on the Production of PolyIC-Induced Cytokines in Alveolar Macrophages

To evaluate the suppression mechanisms of Gal-9 on cytokine production in the BALF, we examined the effect of Gal-9 on the PolyIC-induced production of TNF- α , RANTES, and IP-10 in mouse alveolar macrophages *in vitro*. Concentrations of TNF- α , RANTES, and IP-10 in the supernatants of cultured alveolar macrophages were significantly higher in the presence of PolyIC, and Gal-9 significantly suppressed their production. Further, this inhibitory effect was abrogated in the presence of Tim-3-Ig, but not human IgG1 ($P < .05$, Figure 5). Tim-3 was expressed on mouse alveolar macrophages (Figure 5)

DISCUSSION

Viral respiratory infections are one of the most common illnesses in humans, and the most common cause of an acute asthma exacerbation [17]. Inhaled corticosteroids can reduce the frequency of exacerbations, but they cannot entirely prevent them. The efficacy of drugs currently available for the treatment of virus-induced asthma exacerbations is poor, and alternative therapies to modulate viral pathogenesis are required [18]. The role of PolyIC in the development of a mouse model of ova-induced asthma during the sensitization phase was previously evaluated. Airway eosinophilia and IL-13 production were augmented by PolyIC [19]. In the present study, we developed an acute exacerbation model of a mite

antigen-induced murine model of chronic asthma in which AHR is acquired like in human patients with established asthma. This mouse model could be useful for developing new therapies for chronic asthma patients induced by mite antigen. Using this model, we evaluated the PolyIC-induced asthmatic airway inflammation and AHR instead of viral infection-induced AHR in the challenge phase. Th1 cell responses were increased, leading to the production of IFN- γ , and cytokines and chemokines that are related to the induction of AHR, such as RANTES and TNF- α . We observed the opposite results in a previous study [19]. PolyIC might have different effects on asthmatic responses of an animal model between the sensitization phase and challenge phase, dependent on the antigen. Finally, we demonstrated that exogenous Gal-9 prevented PolyIC-induced AHR in an acute asthma exacerbation mouse model.

In the present study, we demonstrated that the production of TNF- α , RANTES, and IP-10 in alveolar macrophages was enhanced by PolyIC *in vitro*. Further, PolyIC-induced elevations in the levels of TNF- α , RANTES, and IP-10 in the BALF *in vivo*. These chemokines induced the accumulation of Th1 cells in the airway and increased IFN- γ levels in the BALF [20]. Viral infection increases the number of RANTES-producing cells [21]. Furthermore, IFN- γ stimulation of macrophages leads to steroid-resistant AHR in an allergen-induced model acute exacerbation of chronic asthma [22].

Previous studies demonstrated that viral infection increases vagal nerve-mediated bronchoconstriction caused by the loss of function of inhibitory M2 muscarinic receptors (M2R) present on parasympathetic neurons [23]. Viral infections might increase vagal nerve-mediated bronchoconstriction by inducing the release of IFN- γ , which inhibits M2R gene expression [24]. TNF- α causes M2R dysfunction thorough contributing to the adhesion of inflammatory cells on parasympathetic nerves and the reduced expression of M2R on airway nerves [25, 26]. Elevated levels of IFN- γ , RANTES, IP-10, and TNF- α in the BALF of Df/Df+PolyIC mice may worsen AHR and cause acute exacerbation in the Df-induced chronic asthma model.

A number of observations, including those from our laboratory, indicate that Gal-9 specifically induces apoptosis in Th1 cells in a Tim-3 dependent manner. Gal-9 triggering of Tim-3 on Th1 cells might serve to negatively regulate Th1 immunity [9]. In the present study, intranasal administration of Gal-9 reduced Th1-related airway inflammation and AHR induced by PolyIC, in association with a reduced number of Tim-3+CD4+ T cells as well as reduced levels of IFN- γ , TNF- α , RANTES, and IP-10 in the

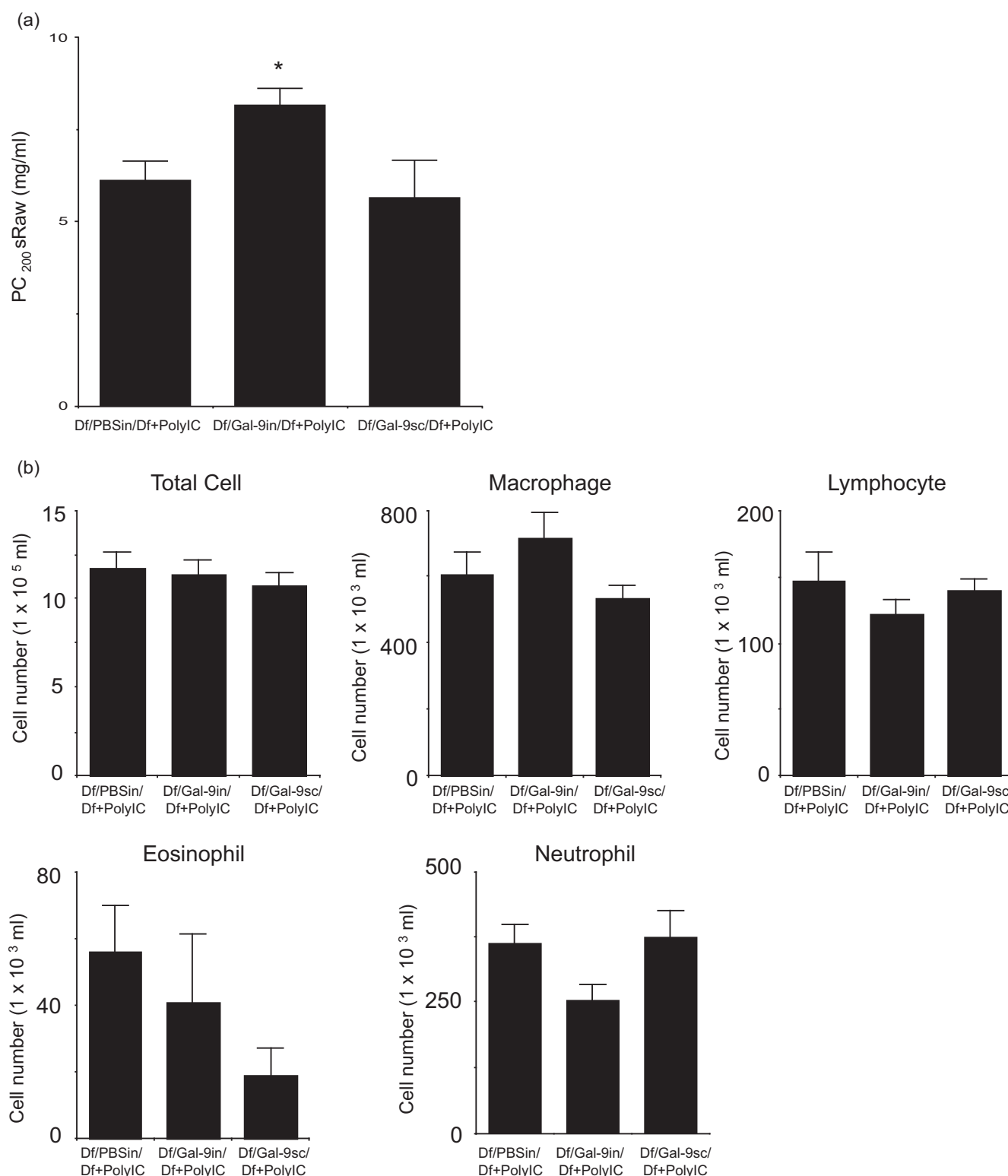


FIGURE 3. Effect of Gal-9 in an acute exacerbation model of Df-induced chronic asthma in mice. AHR was measured and expressed in PC₂₀₀ sRaw (mg/mL) as described in the Methods (a). Absolute numbers of inflammatory cells in the BALF were estimated as described in the Methods (b). Data represent means $\pm$ SEM. Values shown are means from three independent experiments with 6 mice per group. * $P < .05$ compared with Df-ip sensitized, Df-in treated, and intranasal PBS-pretreated and Df+PolyIC-challenged mice (Df/PBSin/Df+PolyIC). Df-ip sensitized, Df-in treated, and intranasal Gal-9-pretreated and Df+PolyIC-challenged mice (Df/Gal-9in/Df+PolyIC); Df-ip sensitized, Df-in treated, and subcutaneous Gal-9-pretreated and Df+PolyIC-challenged mice (Df/Gal-9sc/Df+PolyIC)

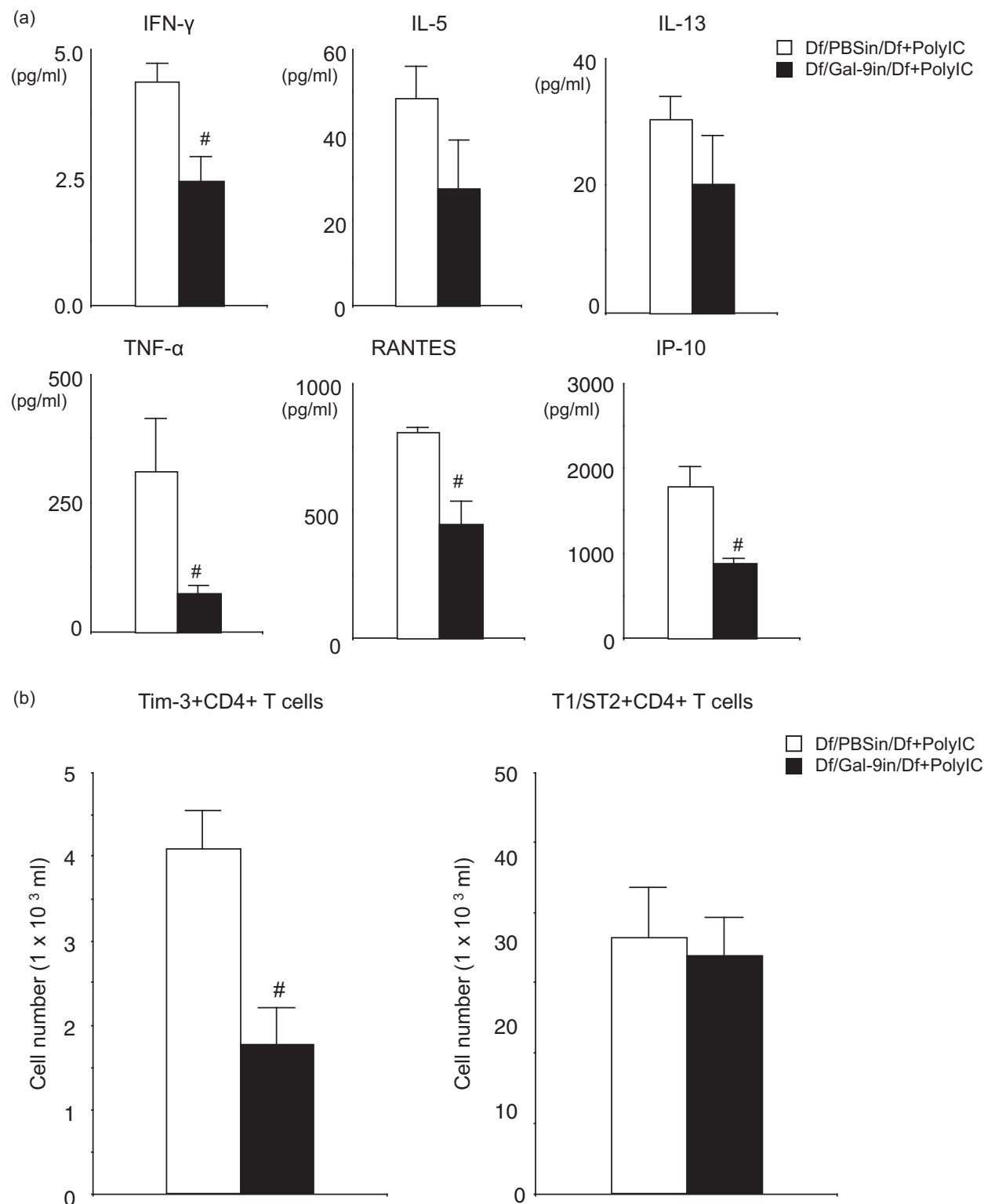


FIGURE 4. Effect of Gal-9 on Th1- and Th2-related inflammation in the lung. IFN- γ , IL-5, IL-13, TNF- α , RANTES, and IP-10 levels in the BALF were determined by ELISA (a). BALF cells were stained with FITC anti-T1/ST2, phycoerythrin anti-Tim-3, PerCP anti-CD3, and APC anti-CD4. Th1 and Th2 cells were evaluated by Tim-3 and T1/ST2 expression gating on CD3+ and CD4+ T cells (b). Data represent means $\pm$ SEM. Values shown are means from three independent experiments with 6 mice per group. [#] $P < .05$ compared with Df-ip sensitized, Df-in treated, and intranasal PBS-pretreated and Df+PolyIC-challenged mice (Df/PBSin/Df+PolyIC). Df-ip sensitized, Df-in treated, and intranasal Gal-9-pretreated and Df+PolyIC-challenged mice (Df/Gal-9in/Df+PolyIC).

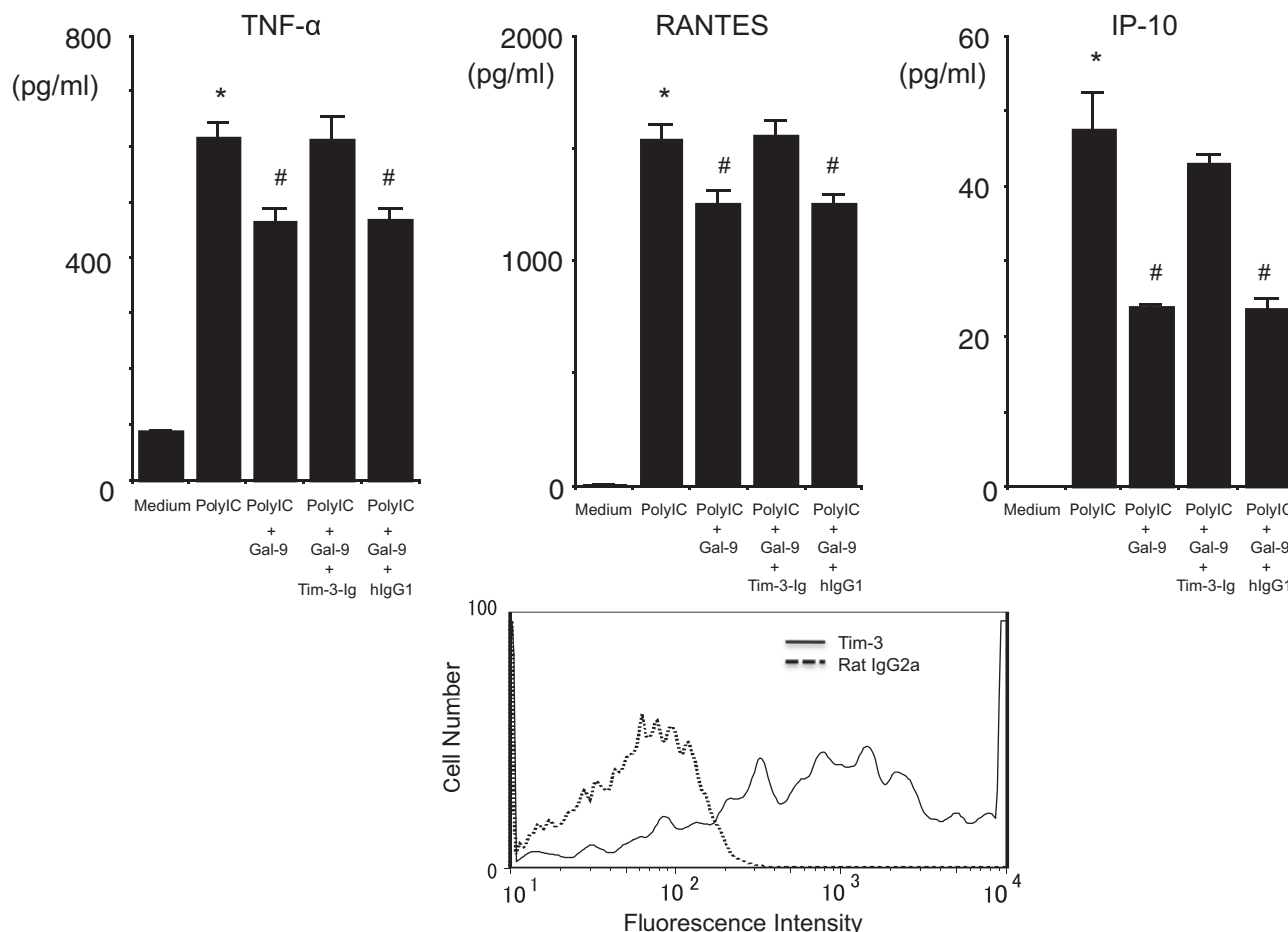


FIGURE 5. Gal-9 suppresses cytokine and chemokine production by alveolar macrophages. Alveolar macrophages were cultured in vitro as described in the Methods. TNF- α , RANTES, and IP-10 in the culture supernatants of alveolar macrophages were determined by ELISA. Data represent means $\pm$ SEM from three independent experiments. * $P < .05$ compared with medium alone. # $P < .05$ compared with PolyIC. Tim-3 expression on mouse alveolar macrophages was evaluated by flow cytometry as described in the Methods. Dotted line indicates background staining with control PE-rat IgG2a. Solid line indicates Tim-3 expression. Representative results of three independent experiments are shown.

BALF. These findings suggest that those cytokines and chemokines have a critical role in the acceleration of AHR in this model. Gal-9 might be directly involved in suppressing Th1 cells in the lung when administered intranasally. Tim-3 is expressed on peritoneal exudate macrophages, monocytes, and splenic dendritic cells [27]. We observed Tim-3 expression on mouse alveolar macrophages. Further, Gal-9 reduced the production of TNF- α , RANTES, and IP-10 induced by PolyIC in alveolar macrophages, and this inhibitory effect was abolished in the presence of Tim-3-Ig. Intranasal treatment with Gal-9 reduced PolyIC-induced AHR by inhibiting the production of TNF- α , RANTES, and IP-10 in the lung.

A previous study demonstrated that PolyIC enhances the expression of Gal-9 in vascular endothelial cells [28]. In the present study, the levels of Gal-9 were elevated in the BALF of PolyIC-challenged

mice. To downregulate the PolyIC-induced Th1-related inflammation, Gal-9 production might be enhanced by PolyIC by a negative feedback mechanism.

A limitation of this study was the use of AHR as a specific marker for acute asthma exacerbation, and the use of PolyIC instead of viral infection in this mouse model. Further studies are required to confirm the inhibitory effect of Gal-9 in viral-induced acute asthma exacerbation by respiratory virus infection and by using a direct indicator of allergen-specific responses in the airways in addition to AHR [29, 30].

CONCLUSIONS

Exogenous Gal-9 suppressed PolyIC-induced AHR and related cytokine production in an acute

exacerbation model of chronic asthma in mice. These findings suggest that recombinant Gal-9 might be therapeutically effective for preventing acute asthma exacerbation in patients with stable asthma.

Declaration of interest: The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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