

Anti-T-cell Ig and mucin domain-containing protein 1 antibody decreases T_H2 airway inflammation in a mouse model of asthma

Jeffrey A. Encinas, PhD,^a Edith M. Janssen, PhD,^b David B. Weiner, PhD,^c Sandra A. Calarota, PhD,^c Daisy Nieto,^a Thomas Moll, PhD,^a Dennis J. Carlo, PhD,^a and Ronald B. Moss, MD^a *San Diego, Calif, and Philadelphia, Pa*

Background: The T-cell Ig and mucin domain-containing (*TIM*) gene locus has been linked to differences in T_H2 responsiveness and asthma susceptibility in mice. The homologous locus in human subjects harbors the gene for TIM-1, which encodes a receptor for hepatitis A virus and has been linked with decreased susceptibility to atopic disease in hepatitis A virus-seropositive individuals.

Objective: We investigated the effects of administering antibodies against TIM-1 in a mouse model of allergic asthma to determine whether the treatment could downregulate T_H2 cytokines and reduce pulmonary inflammation.

Methods: BALB/c mice were sensitized and challenged with ovalbumin to induce airway inflammation. Before the ovalbumin challenge, mice were treated with anti-TIM-1 mAb or a control antibody.

Results: Administration of anti-TIM-1 antibody to mice after ovalbumin sensitization and before ovalbumin challenge results in a significant decrease in inflammatory cells in bronchoalveolar lavage fluid compared with administration of a control antibody. The decrease is accompanied by significantly lower antigen-specific production of the T_H2 cytokines IL-10 and IL-13 by cells from the draining lymph nodes. The T_H1 cytokine IFN- γ appears to be unaffected. Analysis of the lungs shows that goblet cell hyperplasia and mucus production and the expression of IL-10 are markedly decreased in anti-TIM-1-treated mice.

Conclusion: The results indicate that anti-TIM-1 might offer a novel approach to treating asthma. (*J Allergy Clin Immunol* 2005;116:1343-9.)

Key words: Asthma, T-cell Ig and mucin domain-containing protein, T_H2, cytokines

Abbreviations used

BAL: Bronchoalveolar lavage

HAV: Hepatitis A virus

TIM: T-cell Ig and mucin domain-containing protein

Asthma is a chronic debilitating disease with an increasing incidence that is characterized by airway inflammation, hyperresponsiveness, and reversible airway obstruction. The pathologic features of allergic asthma include inflammatory cell infiltration, edema, mast cell activation, denudation of airway epithelium, and collagen deposition. Although a variety of cell types are involved in allergic inflammation, there is substantial evidence that infiltration of T_H2-like T lymphocytes is common in the lungs of allergic asthmatic subjects, as are increased levels of T_H2 cytokines, including IL-4, IL-5, IL-10, and IL-13, particularly in the late phase.¹⁻³ Most notably, the level of T_H2 cytokines appears to correlate with severity of disease.⁴ These cytokines are responsible for the recruitment and activation of other cell types, including eosinophils, which have been associated with lung injury. They also mediate the production of mucus by the airway epithelium, contributing to the airway obstruction that is a major component of the pathology of asthma.⁵ The pivotal role of T_H2 cytokines in asthma-associated lung pathology is further supported by models of asthma in which T_H2 cytokines are overproduced in the lungs.⁶⁻⁹ Conversely, knockout mice that lack the T_H1 transcription factor T-bet experience spontaneous lower airway disease changes consistent with human asthma.¹⁰

Recent studies examining the relationship of asthma susceptibility genes and new T cell-associated proteins have led to the identification of a new family of T cell-associated proteins, the T-cell Ig and mucin domain-containing proteins (TIMs).^{11,12} The first *TIM* gene to be identified, *TIMI*, was found to be expressed by the kidney and liver and was initially cloned in the rat as kidney injury molecule 1 (*Kim-1*) and in monkeys as the hepatitis A virus cellular receptor (*HAVCR*).^{13,14} Later, a study defined a single chromosomal region in mice that conferred

From ^aTelos Pharmaceuticals LLC, San Diego, California; ^bthe Department of Developmental Immunology, La Jolla Institute for Allergy and Immunology, San Diego; and ^cthe Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia.

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Reprint requests: Jeffrey A. Encinas, PhD, Telos Pharmaceuticals LLC, 10150 Meanley Dr, San Diego, CA 92131. E-mail: jencinas@forestlifesciences.com.

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reduced T_H2 responsiveness and protected against the airway hyperreactivity characteristic of asthma.¹¹ The *TIM* gene cluster was located within this chromosomal region, and polymorphisms within *TIM1* were found to strongly associate with the strength of T_H2 responses.

On the basis of the potential association of *TIM1* with immune responses and its role as a cellular receptor for hepatitis A virus (HAV), several studies were conducted in both Europe and the United States that revealed that infection with HAV was associated with protection against the development of asthma in human subjects.^{15,16} Recent studies have characterized several *TIM1* polymorphisms in human subjects and also demonstrated that HAV seropositivity protects against atopy but only in individuals with a specific insertional mutation in the sequence corresponding to amino acid position 157 of *TIM1*.¹⁷ In addition, another study has suggested a direct correlation between certain *TIM1* allelic variants in exon 4 of the gene and susceptibility to atopic diseases.¹⁸ One variant in the study was found to be associated with the control of atopy but only in individuals with serologic evidence of hepatitis A infection. The relationship between *TIM1* and atopy in hepatitis A–seropositive individuals raised the question of whether it would be possible to simulate the HAV-mediated protection from T_H2 -associated diseases by targeting TIM-1 with anti-TIM-1 antibody.

This study therefore investigated the role of antibodies to TIM-1 in a mouse model of asthma. We speculated that anti-TIM-1 might downregulate T_H2 cytokines and limit the airway inflammation characteristic of asthma. Our results suggest that anti-TIM-1 might inhibit airway inflammation by decreasing the production of T_H2 cytokines. Therefore administration of anti-TIM-1 could prove to be a beneficial approach to the treatment of asthma.

METHODS

Animals

Female BALB/c mice (6–8 weeks of age) were purchased from Taconic (BALB/cAnNTac; Oxnard, Calif) and Jackson Labs (BALB/cJ; Bar Harbor, Me) and housed in a conventional facility. All animal experiments were performed in compliance with a protocol approved by an institutional animal care and use committee.

Disease induction and treatment protocol

An active sensitization protocol was used to induce experimental asthma. Mice were sensitized by administering a single intraperitoneal injection of 100 μ g of ovalbumin (grade V; Sigma, St Louis, Mo) adsorbed to 2 mg of alum (Imject Alum; Pierce, Rockford, Ill) on day 1. Antibody treatment was begun on day 11 by administering intraperitoneal injections of 50 μ g of anti-TIM-1 mAb (clone 222414; R&D Systems, Minneapolis, Minn) or an isotype-matched control rat IgG_{2B} antibody (clone 141945, R&D Systems) in 200 μ L of saline and repeating an additional 2 times on days 13 and 15. On the final day of the antibody treatment, mice began a challenge regimen in which they were exposed to ovalbumin (10 mg/mL) or PBS in an aerosol challenge for 10 minutes each day on days 15, 16, 19, and 20. Aerosols were performed in a Plexiglas exposure chamber coupled to an Aeroneb nebulizer (Buxco Electronics,

Wilmington, NC; particle size, 2.6 μ m) at a nebulization rate of 0.3 mL/min.

Analysis of bronchoalveolar lavage cells

Forty-eight hours after the final challenge, animals were killed by means of cervical dislocation, and a cannula was inserted into the trachea. The lungs were then lavaged 5 times with 1-mL aliquots of PBS. Recovered cells were washed and resuspended in 1 mL of PBS. A hemocytometer was used to count the total number of BAL cells. For differential BAL cell counts, cytospin preparations were made and stained with the Hema 3 Stat Pack (Fisher Scientific, Fairlawn, NJ). For each preparation, approximately 400 cells were counted and identified as macrophages, eosinophils, lymphocytes, or polymorphonuclear cells by means of standard morphology. The proportions of each cell type were multiplied by the total cell count to determine subset counts.

Cytokine analysis of lung-draining lymph node cells

Immediately after BAL, lung-draining mediastinal lymph nodes were collected, and single-cell suspensions were made. Cells (2×10^5 cells per well in 96-well, flat-bottom plates) were cultured in RPMI medium with 10% FCS in the presence of medium alone, various concentrations of ovalbumin, or, as a positive control, 1 μ g/mL concanavalin A (Amersham, Piscataway, NJ). After 120 hours of culture, supernatants were collected for cytokine analysis. Levels of the cytokines IL-4, IL-5, IL-10, IL-13, and IFN- γ in culture supernatants were determined by means of ELISA (R&D Systems), according to the manufacturer's protocols.

Lung immunohistochemistry

Lungs were removed from mice 48 hours after the final aerosol challenge, fixed in 10% zinc-formalin, and embedded in paraffin. Five-micrometer-thick sections were then made and stained with periodic acid–Schiff stain to detect mucus. IL-10 cytokine was detected immunohistochemically with goat anti-mouse IL-10 (R&D Systems). Staining was performed on an Autostainer Plus automated staining system (DAKO Cytomation, Carpinteria, Calif). Paraffin was cleared with xylene, and slides were rehydrated through descending concentrations of ethanol. Slides were then treated with 0.3% H₂O₂–methanol for 30 minutes. Slides were heated for 5 minutes in 0.01 M citrate buffer pH 7.6. After cooling, slides were rinsed in 0.1 M Tris Buffer and then blocked with 2% FBS for 5 minutes. Slides were then incubated with primary antibody at a 1:50 dilution for 30 minutes at room temperature. Slides were again rinsed and then incubated with biotinylated anti-goat IgG (Vector Laboratories, Burlingame, Calif) for 30 minutes at room temperature. After rinsing, slides were incubated with an avidin-biotin complex (Vector Laboratories) for 30 minutes at room temperature. Slides were then rinsed and incubated with DAB (DAKO Cytomation) for 10 minutes at room temperature. Slides were rinsed again and counterstained with hematoxylin (Fisher Scientific) for approximately 30 seconds, then rinsed, dehydrated through a series of ascending concentrations of ethanol and xylene, coverslipped, and examined. Grading of IL-10 staining intensity was carried out by 2 independent operators in a blinded fashion and based on the following grading system: 0, no staining; 1, weak staining; 2, mild staining; 3, moderate staining; 4, strong staining.

Statistics

The Student *t* test was used to compare treatment groups. *P* values of less than .05 were considered significant.

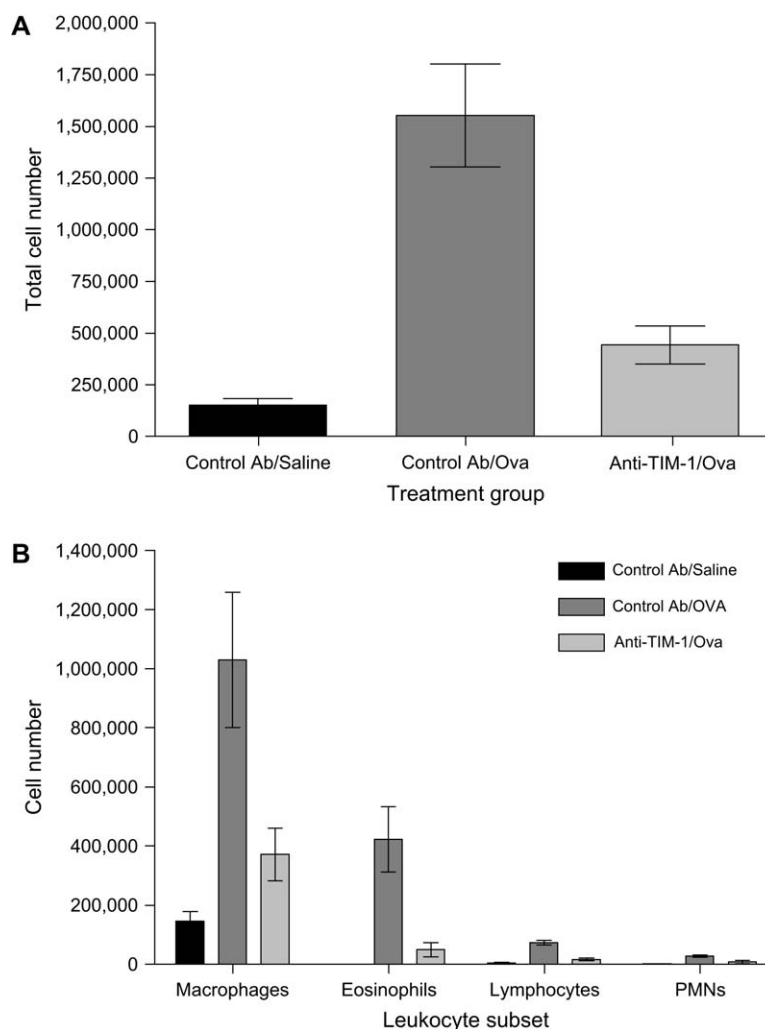


FIG 1. Anti-TIM-1 suppresses airway inflammatory cell infiltration. **A**, BAL leukocyte counts from mice sensitized to ovalbumin (OVA) and treated with control antibody (Ab) and challenged with PBS (Control Ab/Saline), treated with control antibody and challenged with ovalbumin (Control Ab/Ova), or treated with anti-TIM-1 and challenged with ovalbumin (anti-TIM-1/Ova; $n = 6$ for each group) are shown. **B**, Cellular composition of the airway infiltrates is shown. Data are shown as means $\pm$ SEM and are representative of 4 independent experiments.

RESULTS

Anti-TIM-1 treatment suppresses allergic inflammation and mucus production

BALB/c mice were sensitized with a single intraperitoneal injection of ovalbumin adsorbed to alum on day 1 to induce allergic airway inflammation. Mice were then challenged with 4 exposures to aerosolized ovalbumin over a 6-day period beginning on day 15. As a negative control, mice were sensitized in the same way but challenged only with PBS. All mice were treated with either anti-TIM-1 or an isotype-matched control antibody in 50- μ g on each of days 11, 13, and 15. Two days after the final challenge, the severity of allergic inflammation in the lungs was measured by retrieving inflammatory cells from the lungs by means of BAL.

As shown in Fig 1, A, administration of anti-TIM-1 resulted in a significant decrease in the inflammatory cell numbers recovered in the BAL fluid compared with those from the control antibody-treated ovalbumin-challenged mice ($P = .002$). Moreover, the number of inflammatory cells in the lavage fluid of TIM-1-treated animals was not significantly different from that in saline control group ($P > .05$). Administration of anti-TIM-1 instead of control antibody to the saline control group did not result in a significant increase in inflammatory cells in the BAL fluid (data not shown). The differential cell count revealed that macrophages and eosinophils were the predominant cell type in the BAL fluid (Fig 1, B) and that these cells were significantly decreased in the anti-TIM-1 group compared with in the positive control group ($P = .02$ and $P = .008$, respectively). Lymphocytes and polymorphonuclear cells

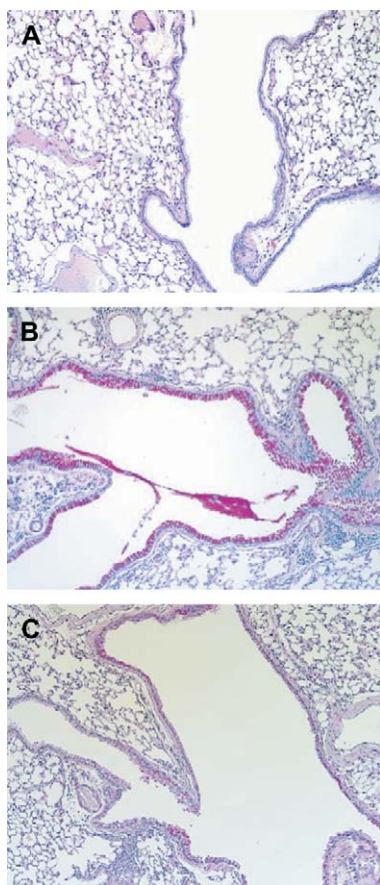


FIG 2. Effect of anti-TIM-1 on goblet cell hyperplasia and mucus production. Lung sections are shown from ovalbumin-sensitized mice that were treated with control antibody and challenged with PBS (**A**), treated with control antibody and challenged with ovalbumin (**B**), or treated with anti-TIM-1, and challenged with ovalbumin (**C**). Intense red staining indicates mucus. Airways of similar caliber were compared. Representative findings from 6 mice in each treatment group are shown.

were also significantly reduced in the anti-TIM-1-treated animals in BAL fluid ($P = .0001$ and $P = .015$, respectively). Among the cell types, eosinophil infiltration appeared to be the most strongly affected by the anti-TIM-1 treatment, showing approximately a 7-fold reduction in numbers compared with those seen in the control antibody-treated, ovalbumin-challenged control animals.

We next examined the lungs of mice histologically to determine whether anti-TIM-1 treatment reduces allergen-induced mucus production and goblet cell hyperplasia. Lungs from saline-challenged or ovalbumin-challenged mice or anti-TIM-1-treated ovalbumin-challenged mice were removed and fixed in 10% zinc formalin. They were then sectioned and stained with periodic acid-Schiff stain to detect mucus. As shown in Fig 2, saline-challenged mice demonstrate little detectable mucus production in the airways. Ovalbumin-challenged mice treated with control antibody, on the other hand, show a high degree of goblet hyperplasia, as indicated by intense red staining and a high

proportion of mucus-engorged goblet cells in the airway epithelium. Furthermore, plugs containing mucus and inflammatory cells can be readily found in the airway lumen of most of these mice. In contrast, ovalbumin-challenged mice treated with anti-TIM-1 showed a decrease in the proportion of mucus-engorged goblet cells in the bronchial epithelium, and no mucus plugs were found in the airway lumen.

Decreased cellular infiltration is accompanied by reduced T_H2 cytokine production

Because T_H2 cytokine production correlates with the magnitude of an allergic response, we measured the production of the T_H2 cytokines IL-13, IL-10, IL-4, and IL-5 and the T_H1 cytokine IFN- γ as markers of the immune response. Cells collected from the lung-draining mediastinal lymph nodes of control antibody-treated saline-challenged and ovalbumin-challenged mice or anti-TIM-1-treated ovalbumin-challenged mice were stimulated for 5 days with increasing concentrations of antigen, after which the cell culture supernatant was collected for analysis by means of ELISA. As shown in Fig 3, the anti-TIM-1-treated mice showed markedly decreased production of T_H2 -associated cytokines on ovalbumin stimulation *in vitro*. Production of both IL-13 and IL-10 was significantly decreased (both $P = .02$) when cells were stimulated with 100 μ g of ovalbumin. Reductions in the production of IL-4 and IL-5, although not reaching the level of significance, were seen consistently in 3 independent experiments. The production of the T_H1 cytokine IFN- γ in response to antigen stimulation was near or less than the lower limit of detection in the saline- and ovalbumin-challenged control animals and remained unchanged by anti-TIM-1 treatment (data not shown).

Anti-TIM-1 treatment decreases lung protein expression of IL-10

The decrease in T_H2 cytokines in the anti-TIM-1-treated mice was further supported by immunohistochemical staining of lung tissue. In the lungs the expression of the T_H2 cytokine IL-10 was found to be decreased in the group of animals treated with anti-TIM-1. In line with what has been reported previously,¹⁹ moderate staining of IL-10 protein in saline-challenged control mice (Fig 4, A) could be seen in the bronchial epithelium. However, in ovalbumin-challenged positive control mice, the staining is markedly increased (Fig 4, B). Uniformly intense staining can be seen in the bronchial epithelium, and moderate staining of alveolar macrophages can also be detected. In contrast, little staining of IL-10 in the bronchial epithelium can be detected in the anti-TIM-1-treated group (Fig 4, C). Blinded grading of the staining with a scale of 0 to 4 resulted in average staining intensities of 2.3 ± 0.2 (mean $\pm$ SEM) for the saline control group, 3.7 ± 0.2 for the ovalbumin control group, and 0.8 ± 0.3 for the anti-TIM-1-treated group. Overall, lung IL-10 expression was lowest in the anti-TIM-1-treated group, suggesting a downregulation to a level less than

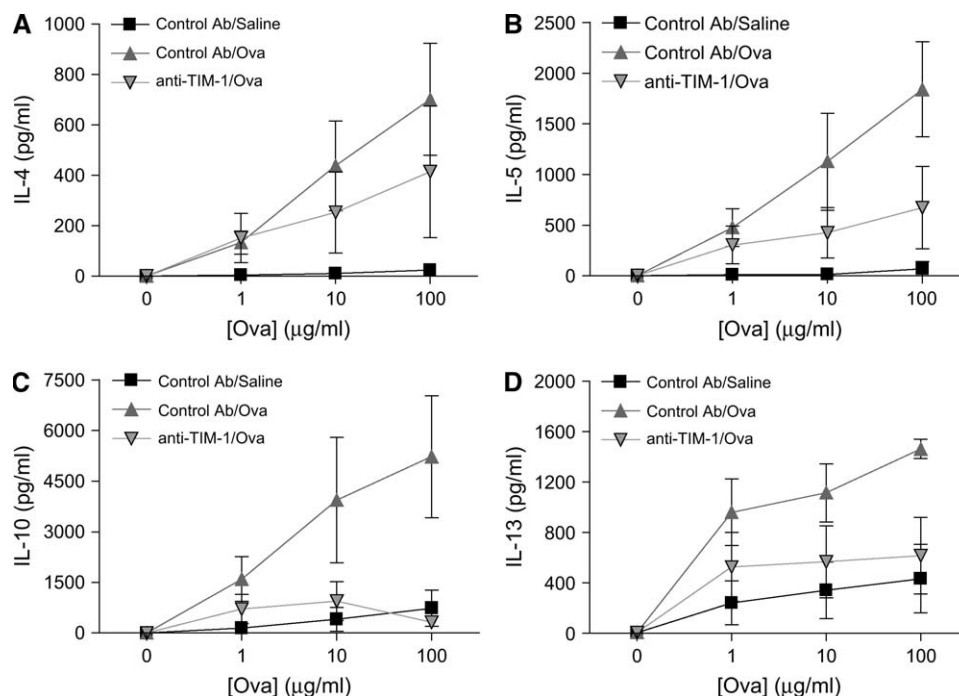


FIG 3. T_H2 cytokine production by inflammatory cells is reduced in anti-TIM-1-treated mice. Cells from lung-draining lymph nodes of mice treated as in Fig 1 were stimulated *in vitro* with ovalbumin ($n = 6$ for each group). Culture supernatants were tested for IL-4 (A), IL-5 (B), IL-10 (C), and IL-13 (D) by means of ELISA. Data are shown as means $\pm$ SEM and are representative of 3 independent experiments.

the normal basal level observed in the saline-challenged mice.

DISCUSSION

This is the first report of the therapeutic use of anti-TIM-1 in a mouse model of asthma. Overall, these results suggest that in this experimental model of asthma, lung inflammatory cell infiltration, T_H2 -associated cytokine production, and the pathologic sequelae of goblet cell hyperplasia and mucus overproduction can be reduced by treatment with anti-TIM-1. The decrease in lung inflammatory cells observed after anti-TIM-1 treatment appears to be the result of decreases in the infiltration of all of the major cells involved in allergic inflammation, indicating that a basic allergic inflammatory process is altered by the treatment.

Previously, the locus harboring the *TIM1* gene was described as being associated with decreased T_H2 responsiveness and found to be associated with decreased asthma susceptibility in mice. Furthermore, certain *TIM1* polymorphisms were determined to be associated with decreased atopic disease in HAV-seropositive individuals, possibly through a mechanism involving engagement of TIM-1 on the surface of cells by the virus. Although the expression of TIM-1 was first found in epithelial cells of the kidney, studies of the molecule in an immunologic context found TIM-1 to be expressed selectively on T_H2

cell lines but not on T_H1 cell lines.²⁰ If this selectivity also holds for T_H cells *in vivo*, an explanation of the effects observed from treatment with anti-TIM-1 might be that the antibody is able to cross-link TIM-1 on the surface of T_H2 cells and deliver an inhibitory signal into the cells. Conversely, the antibody might block a signal that would otherwise be transmitted through TIM-1 to T_H2 cells by antigen-presenting cells. The cellular site of action of anti-TIM-1 *in vivo*, however, has yet to be determined, and the possibility remains that TIM-1 expression on other cell types, such as antigen-presenting cells, might be the basis of anti-TIM-1 effects.

The observed decrease in inflammatory cells after anti-TIM-1 treatment was associated with significant decreases in the T_H2 cytokines IL-13 and IL-10, as well as reproducible decreases in IL-4 and IL-5, with no detectable change in T_H1 cytokines, such as IFN- γ . The decrease in IL-13, a predominantly T_H2 cell-produced mediator of allergic inflammation,²¹ supports the hypothesis that anti-TIM-1 might have direct effects on T_H2 cells. This decrease, measured in cells from lung-draining lymph nodes, also likely accounts for the reduced production of mucus in the lungs of the anti-TIM-1-treated mice. IL-13 overproduction has been shown to be the most critical factor in the induction of excess mucus production in experimental models of asthma.^{5,8,22}

IL-10, which in mice is produced primarily by T_H2 cells but is also produced by macrophages, B cells, and other cell types, was decreased in both draining lymph node

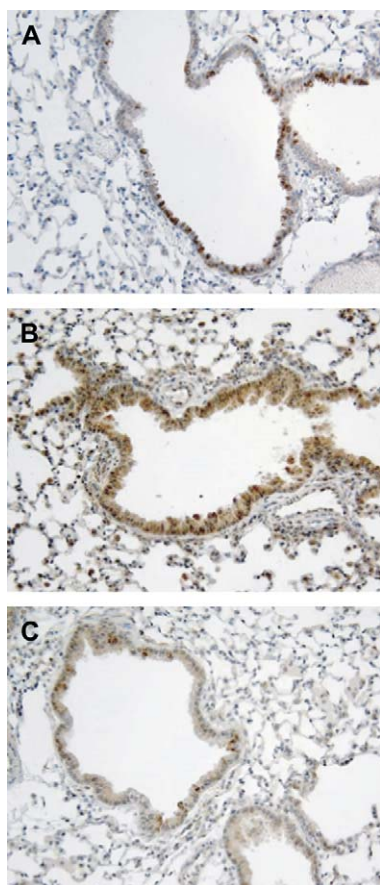


FIG 4. Lung sections from anti-TIM-1–treated mice show less IL-10 protein expression. Sections are shown from ovalbumin-sensitized mice that were treated with control antibody and challenged with PBS (**A**), treated with control antibody and challenged with ovalbumin (**B**), or treated with anti-TIM-1 and challenged with ovalbumin (**C**). Staining of IL-10 is seen on the bronchial epithelium and on alveolar macrophages. Representative findings from 6 mice in each treatment group are shown.

cell cultures and in the airway epithelium of the lungs, where IL-10 is known to be constitutively produced.^{19,23} Although the role of IL-10 in asthma has not yet been fully clarified, some studies suggest that this cytokine might contribute to pathology by enhancing airway hyperresponsiveness and allergic inflammation.^{24,25} It is possible that the decreased IL-10 production might result from a direct inhibitory effect of the antibody against cells that produce this cytokine, but the decrease might also be due to effects on cells, such as macrophages or dendritic cells, that play a role in polarizing the immune response toward T_H2 . Importantly, the observed decrease in T_H2 cytokines after anti-TIM-1 treatment was not accompanied by a detectable increase in T_H1 cytokines. These results contrast with other data suggesting that in unpolarized animal models anti-TIM-1 can be used as a T_H1 -stimulating adjuvant for vaccines (unpublished observations). The fact that mice were polarized to T_H2 before treatment with anti-TIM-1 in the current asthma experiments, compared with using naive animals in adjuvant experiments, might

account for these differences in T_H1 stimulatory effects. The lack of T_H1 deviation after anti-TIM-1 treatment in our asthma experiments, however, is noteworthy because some T_H1 cytokines might also be involved in allergic inflammation. Thus rather than counterbalancing the hyperpolarized T_H2 response with an enhancement of T_H1 cytokines, it appears that the anti-TIM-1 antibodies primarily affect the T_H2 arm of the response. This type of effect might be preferable because previous reports have indicated that increasing T_H1 responsiveness in asthma models might increase, rather than decrease, inflammation and disease pathology.²⁶

Our finding of an ameliorating effect of anti-TIM-1 on airway inflammation follows a recent report by Umetsu et al²⁷ that showed that anti-TIM-1 treatment prevents tolerance induction in a model of allergen-induced airway hyperreactivity. In that model respiratory tract exposure to ovalbumin before sensitization induces a state of tolerance to subsequent ovalbumin challenge. Tolerance induction, however, could be inhibited by administration of anti-TIM-1 before and after the tolerizing procedure, thereby allowing the induction of airway hyperreactivity. Because respiratory tract exposure to ovalbumin before sensitization produces an effect opposite to that of exposure after sensitization, it is perhaps not surprising that treatment with anti-TIM-1 during tolerizing versus stimulating exposures to antigen in the respiratory tract might result in differing outcomes. Interestingly, however, in both cases the anti-TIM-1 treatment inhibited the usual response to antigen in each of these contexts, preventing the generation of tolerance in one system while preventing a strong response to antigen in the other. In human allergic asthma, because patients as a rule are sensitized to antigens, perhaps through defective respiratory tolerance, the effects of anti-TIM-1 in our model might be more clinically relevant for the treatment of asthma compared with the previous study.

Initial reports on the mechanism of TIM-1 have suggested that ligation of TIM-1 *in vitro* has a costimulatory effect on T_H2 cell responses to antigen.^{27,28} Paradoxically, however, soluble TIM-1–Ig fusion protein, which potentially has the ability to block TIM-1 ligation by its endogenous ligand, also has a positive effect on T-cell proliferation and T_H2 cytokine production,²⁸ emphasizing the role of the TIM-1 ligand-expressing cell in the generation of the response. It therefore remains to be determined whether the differing effects of anti-TIM-1 on T_H2 immune responses *in vivo* and *in vitro* are related to the developmental state of the antigen-responding T cells on exposure to the antibody or related to the state of the antigen (solubilized or adsorbed to adjuvant) and antigen-presenting cells during the treatment with anti-TIM-1. Functional differences between the antibodies might also contribute to the differing effects. However, we have tested 3 different anti-TIM-1 antibodies in our model of asthma, and all 3 showed similar effects (data not shown), suggesting that the different outcomes *in vivo* might be primarily due to differences in study design rather than differences in the function of the

antibodies used. Nevertheless, it is clear from all of these studies that the therapeutic regulation of the TIM-1 molecule can result in a significant modulation of the immune response and therefore has wide-ranging potential in the treatment of disease.

In this study potent inhibition of the inflammatory response in a mouse model of asthma was observed with TIM-1 antibody administration. Additional studies are warranted to further examine the effects of TIM-1 antibody and its utility to treat immune-mediated inflammatory diseases, such as asthma.²⁹

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