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# Suppression of Th2-Driven Airway Inflammation by Allergen Immunotherapy Is Independent of B Cell and Ig Responses in Mice

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**Allergen-specific immunotherapy (IT) uniquely renders long-term relief from allergic symptoms and is associated with elevated serum levels of allergen-specific IgG and IgA. The allergen-specific IgG response induced by IT treatment was shown to be critical for suppression of the immediate phase of the allergic response in mice, and this suppression was partially dependent on signaling through FcγRIIB. To investigate the relevance of the allergen-specific IgG responses for suppression of the Th2-driven late-phase allergic response, we performed IT in a mouse model of allergic asthma in the absence of FcγRIIB or FcγRI/FcγRIII signaling. We found that suppression of Th2 cell activity, allergic inflammation, and allergen-specific IgE responses is independent of FcγRIIB and FcγRI/FcγRIII signaling. Moreover, we show that the IT-induced allergen-specific systemic IgG or IgA responses and B cell function are dispensable for suppression of the late-phase allergic response by IT treatment. Finally, we found that the secretory mucosal IgA response also is not required for suppression of the Th2-driven allergic inflammation by IT. These data are in contrast to the suppression of the immediate phase of the allergic response, which is critically dependent on the induced allergen-specific serum IgG response. Hence, IT-induced suppression of the immediate and late phases of the allergic response is governed by divergent and independent mechanisms. Our data show that the IT-induced suppression of the Th2 cell-dependent late-phase allergic response is independent of the allergen-specific IgG and IgA responses that are associated with IT treatment.** ***The Journal of Immunology*****, 2010, 185: 3857–3865.**

Allergen exposure in sensitized individuals results in cross-linking of IgE bound to FcεRI on the mast cell surface and induces the immediate type I hypersensitivity response, which is driven by inflammatory mediators released from the mast cells (1). This immediate-phase allergic response is strictly IgE dependent and is treated by pharmacotherapy aimed at blocking mast cell effector molecules, such as histamine. The late response to allergen exposure is characterized by an influx of eosinophils, neutrophils, basophils, and activated $CD4^+$ T cells and is sensitive to immunosuppressive drugs, such as steroids (1). Allergen-specific immunotherapy (IT) is the only disease-modifying treatment for allergic disorders and consists of a series of s.c. injections with increasing doses of allergen, which induces long-term desensitization and relief of symptoms (2, 3). IT is commonly used for the treatment of allergic rhinitis and insect venom hypersensitivity, yet its efficacy in allergic asthma is controversial. The main drawbacks of IT are the long-term treatment protocols and the risk for eliciting anaphylactic reactions (2). Improvement of safety and efficacy can only be achieved by characterizing the immunological mechanisms that contribute to IT-induced suppression of allergic inflammation in more detail.

Although the critical mechanisms by which IT suppresses allergic symptoms have not been fully identified, several potential mechanisms have been postulated to contribute to IT, converging on the modulation of T and B cell responses to the allergen (4). With regard to the T cell responses, IT was postulated to divert the allergen-specific Th2 response into Th1 responses and induce regulatory T cell activity (5). With regard to the B cell responses, IT was shown to induce an IgG1 and IgG4-dominated Ab response to the allergen with a mild increase in IgA levels, whereas the serum levels of allergen-specific IgE showed an early increase followed by a long-term gradual decline (1). However, the relationship between the efficacy of IT and induction of allergen-specific IgG remains controversial, with serum concentrations of allergen-specific IgG not consistently correlating with clinical improvement (1, 6, 7).

The increase in IgG serum levels, and especially IgG4, following IT treatment led to the formulation of the blocking Ab hypothesis, which states that the IgG Abs compete with IgE for binding to the

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; Fel d1, *Felis domesticus* allergen 1; IT, immunotherapy; pIgR, polymeric IgR.



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allergen (8). As a result, the allergen is neutralized, preventing IgE cross-linking by the allergen and degranulation of mast cells (9). In addition, signaling through the low-affinity IgGR (FcγRIIB) was shown to directly suppress IgE cross-linking–induced mast cell activation (10). Cross-linking of FcγRIIB receptors on memory B cells by the allergen-specific IgG4 induced by IT was shown to inhibit IgE production (11, 12). In the mouse, allergen–IgG complexes can also induce IL-10 production by macrophages (13, 14) and induce deviation of the Th cell response, resulting in increased IgG1 serum levels (13). Allergen-specific IgA responses in serum are also induced by IT for grass pollen (15) and house dust mite (16), although not consistently (17). The possible role for allergen-specific IgA responses induced by IT is thought to encompass the induction of IL-10 expression in monocytes by FcαRI cross-linking and allergen capture at mucosal surfaces (15).

We developed a mouse model for allergen-specific IT based on the OVA-induced mouse model for allergic asthma. In this mouse model, IT treatment by s.c. application of gradually increasing doses of OVA inhibits allergen-induced allergic airway inflammation and hyperresponsiveness in OVA-sensitized mice and significantly suppresses the OVA-specific IgE response in serum (18). Subcutaneous delivery of three high-dose (1 mg) OVA injections was shown to suppress asthma manifestations as efficiently as the gradual up-dosing of OVA in the mouse model (18). The suppressive effects on asthma manifestations were found to last for ≥5 wk after IT treatment and to be dependent on IL-10 production (19). Moreover, IT treatment also induces increased serum levels of OVA-specific IgG and IgA Abs, mirroring the observations in human subjects. It is unknown whether the induction of allergen-specific IgG and IgA responses contributes to the IT-mediated suppression of Th2-driven allergic inflammation that is responsible for the late-phase allergic response in vivo. Interestingly, very recent data from a mouse model of *Felis domesticus* allergen 1 (Fel d1)-specific IT indicate that suppression of the immediate allergic reaction is dependent on the allergen-specific IgG response (20). Our protocol of OVA-IT specifically allows analysis of the IT-induced suppression of the Th2-mediated late-phase allergic response, because the allergen-induced eosinophilic airway inflammation (21, 22) and airway hyperresponsiveness (AHR) (23) are independent of allergen-specific IgE responses and the immediate phase of the allergic response in the OVA/alum mouse asthma model.

In this article, we tested whether the IT-induced suppression of the Th2 cell-driven late-phase response is also critically dependent on the induction of allergen-specific IgG and IgA responses. Surprisingly, we found that suppression of allergic inflammation by OVA-IT was efficiently induced in the absence of FcγRIIB signaling and even in the absence of an adaptive IgG or systemic IgA response in B cell-deficient mice. Finally, we show that mucosal IgA production is dispensable for suppression of allergic inflammation by IT. Our data show that suppression of the Th2-driven late-phase allergic response by allergen-specific IT is independent of B cell function and the induction of allergen-specific Ig responses.

## Materials and Methods

### *Animals*

Animal housing and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Groningen. All mice used in the experiment were 6–8-wk-old males. Mice deficient for FcγRI/III and FcγRIIB and polymeric IgR (pIgR)-deficient mice were on the BALB/c background. Wild-type BALB/c control mice were obtained from Charles River Laboratories (Maastricht, The Netherlands). B cell-deficient *Igh-6*$^{tm1Cgn}$ (μMT) and wild-type littermates (both on a C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in Makrolon cages in a laminar flow cabinet and provided with food and water ad libitum.

### *Sensitization, challenge, and IT protocol*

The short-term protocol (OVA-IT in OVA-sensitized mice, Fig. 1*D*) was the same as previously described (19). Briefly, mice received two i.p. injections of 10 μg OVA (endotoxin-free, <0.5 U/ml; Seikagaku Kogyo, Tokyo, Japan) adsorbed onto 2.25 mg alum (ImjectAlum, Pierce, Rockford, IL) in 100 μl pyrogen-free saline (B. Braun, Melsungen, Germany) on days 0 and 7. Two weeks after the second sensitization, mice were treated with three s.c. injections of 1 mg OVA in 200 μl pyrogen-free saline (IT) or 200 μl saline (sham) on alternate days. One week after the last OVA or sham treatment, mice were challenged three times every third day with OVA aerosols in saline (1% w/v) for 20 min in a Plexiglas exposure chamber coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 l/min. The long-term protocol (OVA-IT in OVA-sensitized and -challenged mice; Fig. 1*A*) was identical to the short-term protocol, except for three additional OVA inhalation challenges (or PBS control treatment) given 2 wk prior to sham/IT treatments. The effects of possible endotoxin contamination in the OVA preparations on the efficiency of IT was previously tested in our laboratory (A.J.M. van Oosterhout and B.C.A.M. van Esch, unpublished data), and all OVA preparations used in these experiments were in the lower range of endotoxin levels that were shown to efficiently induce IT-dependent suppression of allergic inflammation (0.5–500 endotoxin unit/ml).

Airway responsiveness to methacholine, serum levels of Igs, cellular infiltration, and Th2 cytokine levels in bronchoalveolar lavage (BAL) fluid were measured 24 h after the last OVA inhalation challenge in each mouse.

### *Measurement of airway responsiveness*

Airway responsiveness was assessed by measuring airway resistance in response to i.v. administration of increasing doses of methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich, St. Louis, MO), as described previously (24). Briefly, anesthetized (ketamine/domitor) mice were attached to a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada). Mice were ventilated at a breathing frequency of 280 breaths/min and a tidal volume of 10 ml/kg, which was pressure limited at 300 mm $H_2O$. Airway resistance in response to increasing doses of methacholine was calculated from the pressure response to a 2-s pseudorandom pressure wave, as described previously (24).

### *Determination of serum levels of OVA-specific Igs*

Blood for assessment of serum Igs was collected 7 d before the first OVA aerosol challenge (via orbital puncture) and 24 h after the last challenge (via cardiac puncture). Serum was collected, and OVA-specific IgE, IgG1, IgG2a, and IgA levels in serum were measured by ELISA, as described previously (18). A reference standard was used with arbitrary units of each isotype of 1000 experimental unit/ml. The detection levels of the ELISAs were 0.05 experimental unit/ml for IgG2a, 0.5 experimental unit/ml for IgE and IgA, and 0.005 experimental unit/ml for IgG1.

### *Analysis of the cellular composition in BAL fluid*

Immediately after bleeding, the lungs were lavaged through a tracheal cannula with 1 ml saline at 37°C containing 5% BSA and 2 mg/ml aprotinin (F. Hoffman-La Roche, Basel, Switzerland). Cells were pelleted, and supernatants were used for measurement of cytokines by ELISA. Subsequently, lungs were lavaged with 4 ml saline, and BAL cells were pooled and counted. For differential BAL cell counts, cytospin preparations were stained with Diff-Quick (Merz & Dade, Dudingen, Switzerland) and evaluated by one observer in a blinded fashion. Cells were differentiated into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standard morphology. At least 200 cells were counted per cytospin preparation.

### *Measurement of cytokines*

IL-5 and IL-13 in the BAL fluid were determined by ELISA, according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The lower detection limits of the ELISAs were 32 pg/ml for IL-5 and 15 pg/ml for IL-13.

### *Statistical analysis*

All data are expressed as mean ± SEM. The Mann–Whitney *U* test was used to analyze the results, and $p < 0.05$ was considered significant.

## Results

### *OVA-specific IT suppresses airway inflammation and hyperresponsiveness in asthmatic mice*

We previously developed and used a mouse model for OVA-specific IT in the classical OVA/alum mouse asthma model (18). In this

model, gradual up-dosing or application of three high doses of OVA by s.c. injections in OVA/alum-sensitized mice renders these mice protected against clinically relevant asthma manifestations, such as eosinophilic airway inflammation and AHR upon subsequent OVA inhalation challenge. In this model, the mice have not experienced an asthmatic response prior to the IT treatment, which is in contrast to the routine clinical situation in which a patient is treated with allergen-specific IT only upon presenting with clinical manifestations of an atopic disease, such as rhinitis or allergic asthma. Hence, we first tested whether OVA-specific IT would also be protective in mice that had experienced a prior OVA-induced asthmatic response. To address this question, we designed the experimental layout depicted in Fig. 1*A*. Before IT treatment, mice were given an OVA/alum sensitization followed by OVA inhalation challenges (asthmatic mice) or PBS control treatment (sensitized-only mice). Two weeks after the last OVA/PBS inhalation challenge, both groups (sensitized only and asthmatic) were sham (saline) or IT (OVA) treated. After an additional 2 wk, all groups received a series of three OVA inhalation challenges to induce the asthma phenotype, and AHR to methacholine was assessed by direct measurement of airway resistance, followed by section as described in *Materials and Methods*.

As shown in Fig. 1*B*, OVA-inhalation challenges in sham-treated mice that had not received prior OVA-inhalation challenges (sensitized only) induced a dose-dependent increase in AHR to methacholine, which was increased compared with PBS-challenged control mice. In concordance with the data from our previous studies, OVA-inhalation challenges in OVA-IT–treated sensitized-only mice induced a significant reduction in airway responsiveness, almost to the level of PBS-challenged control mice (Fig. 1*B*). These data are in agreement with our previous studies (18, 24) in which we showed that OVA-specific IT efficiently repressed the induction of an asthmatic phenotype by OVA-inhalation challenges in OVA/alum-sensitized mice.

Importantly, OVA challenges in sham-treated mice that had received prior OVA-inhalation challenges (asthmatic mice) induced a similar level of AHR compared with sham-treated sensitized-only mice, indicating that the AHR to methacholine was similar between the first and second series of OVA-inhalation challenges. Remarkably, OVA-IT also strongly suppressed the AHR to methacholine in the asthmatic mice (Fig. 1*B*), supporting the notion that OVA-IT in our mouse model suppresses AHR, irrespective of the presence of a pre-existing asthmatic phenotype in the treatment group.

Analysis of the eosinophilic airway inflammation yielded similar results (Fig. 1*C*). OVA-IT treatment in sensitized-only mice reduced the BAL numbers of eosinophils compared with sham treatment, as we reported in several previous studies (18, 19, 24–26), although the effect was not statistically significantly ($p = 0.161$) in this specific experiment. Importantly, however, OVA-IT in asthmatic mice strongly and significantly suppressed the levels of BAL eosinophils (Fig. 1*C*), indicating that OVA-IT also renders protection against the induction of eosinophilic airway inflammation in mice that received prior OVA-inhalation challenges. Taken together, these data indicate that OVA-IT in the mouse model of OVA-induced asthma efficiently suppresses the induction of clinically relevant asthma phenotypes upon OVA-inhalation challenges, irrespective of the presence or absence of a prior asthma phenotype at the time of OVA-IT treatment. Therefore, the remaining experiments were performed using the short protocol as our model for IT, using sensitized-only mice as the recipient for the IT injections (Fig. 1*D*).

### *OVA-specific IT suppresses the late-phase allergic response in FcγRIIB-deficient mice*

IT treatment in human subjects, as well as in mouse models, induces strong allergen-specific IgG responses (1, 18), which were shown to be critically required for suppression of the immediate response in a mouse model of Fel d1 IT (20). We hypothesized that induction of an allergen-specific IgG response by IT is critical for suppression of the Th2-dependent late-phase allergic response in an FcγRIIB-dependent fashion. To test our hypothesis, we assessed whether IT treatment was able to suppress Th2 cell-driven allergic inflammation in the absence of FcγRIIB signaling. To this end, we performed our

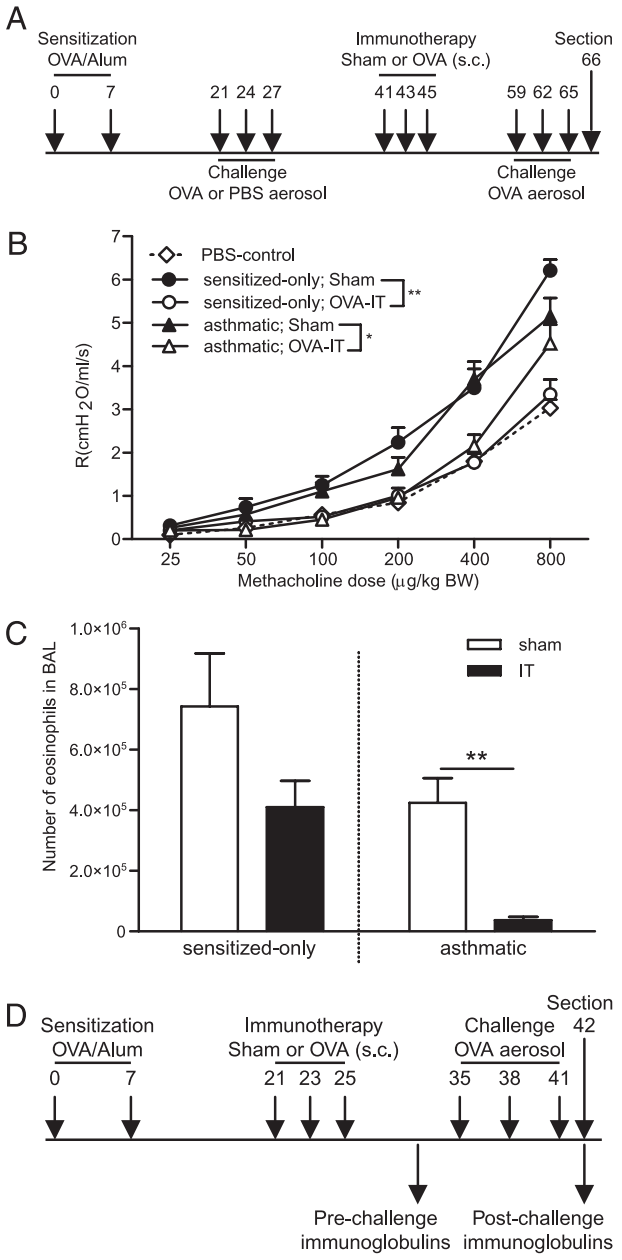


**FIGURE 1.** Allergen-specific IT protocol in a murine model of allergic asthma. *A*, Long-term protocol of OVA-IT in the mouse model: OVA/alum i.p.-sensitized mice received a series of three PBS- (referred to as sensitized-only mice) or OVA- (referred to as asthmatic mice) inhalation challenges at week 3. Two weeks later, mice were treated with three s.c. injections of saline (sham treatment) or OVA (OVA-IT treatment). After an additional 2 wk, all mice received a series of three OVA-inhalation challenges to induce an asthma phenotype. AHR was measured, and BAL was collected 24 h after the last challenge. *B*, Airway responsiveness to methacholine by sham- (solid symbols) or OVA-IT– (open symbols) treated sensitized-only mice (● and ○) or asthmatic mice (▲ or △) in comparison with PBS-challenged control mice (-◇-) and eosinophil counts in BAL fluid of sham- and OVA-IT–treated mice (*C*). Values are expressed as mean ± SEM of six mice per group. $*p < 0.05$; $**p < 0.01$. *D*, Short-term protocol of OVA-IT in the mouse model.

experimental mouse model of allergen IT (schematically depicted in Fig. 1*D*) using FcγRIIB-deficient mice.

In OVA-sensitized and sham-treated wild-type or FcγRIIB$^{-/-}$ mice, OVA aerosol challenges induced a significant OVA-specific IgE response (Fig. 2*A*, pre- versus postchallenge). In addition, the OVA challenges induced moderate IgG1, IgG2a, and IgA responses in sensitized and sham-treated wild-type and FcγRIIB$^{-/-}$ mice (Fig. 2*B–D*). OVA-IT treatment in sensitized wild-type mice induced a strong IgG1 and IgG2a response, which was not further upregulated by subsequent OVA challenges as we showed previously (18). In FcγRIIB$^{-/-}$ mice, OVA-IT also induced strong IgG1 and IgG2a responses (Fig. 2*B*, 2*C*), in agreement with the recent observations in the mouse model of Fel d1 IT (20). Finally, OVA-IT strongly suppressed the OVA-specific IgE response in wild-type mice (Fig. 2*A*). Surprisingly, FcγRIIB$^{-/-}$ mice also displayed a strong reduction in the OVA-specific IgE response upon OVA-IT, indicating that the IT treatment efficiently suppressed allergen-specific IgE, independent of FcγRIIB signaling. Remarkably, the OVA-specific IgA response induced in wild-type mice by OVA-IT was not observed in FcγRIIB mutant mice (Fig. 2*D*).

Because OVA-IT efficiently suppressed the allergen-specific IgE response in mice deficient for FcγRIIB, we next analyzed Th2-driven eosinophilic airway inflammation. In line with the observed loss of the OVA-specific IgE response after OVA-IT treatment, wild-type mice displayed an aborted eosinophilic airway inflammation upon OVA-IT, accompanied by strongly decreased local Th2 cell activity, as measured by IL-5 and IL-13 levels in BAL fluid (Fig. 3). Intriguingly, FcγRIIB mutant mice showed an identical suppression of eosinophil cell counts, as well as IL-5 and IL-13 levels, in BAL fluid after OVA-IT. Taken together, these data indicate that OVA-specific IT is fully capable of suppressing allergen-specific IgE responses, as well as Th2-driven eosinophilic airway inflammation, in the absence of FcγRIIB-dependent signaling.

### *OVA-specific IT suppresses IgE responses and Th2-driven inflammation in FcγRI/FcγRIII$^{-/-}$ mice*

Although the suppressive activities of FcγRIIB signaling on mast cell activation and APC function are well established (10), cross-linking of FcγR by OVA–IgG immune complexes was also shown to induce IL-10 expression in macrophages through FcγRI (14), resulting in deviation of the T cell phenotype and induction of an OVA-specific IgG response (13). Hence, we next aimed to assess whether signaling of IgG–allergen immune complexes through FcγRI or FcγRIII contributed to the IT-induced IgG and IgA responses or the suppression of the late-phase allergic response. To this end, we performed OVA-IT in double-mutant FcγRI$^{-/-}$/FcγRIII$^{-/-}$ mice. As observed in wild-type mice, the OVA-specific IgE response was strongly suppressed by OVA-IT treatment in FcγRI/FcγRIII$^{-/-}$ mice and was accompanied by strongly increased OVA-specific IgG1 and IgG2a responses (Fig. 2*A–C*). These data indicate that neither the increased IgG responses induced by IT nor the suppression of IgE responses are dependent on FcγRI or FcγRIII signaling. Interestingly, we found that although OVA-IT induced significantly increased OVA-specific IgA levels in wild-type mice, decreased levels of OVA-specific IgA were detected in serum from FcγRI/FcγRIII$^{-/-}$ mice after OVA-IT.

To evaluate the suppression of allergic airway inflammation by OVA-IT in FcγRI/FcγRIII$^{-/-}$ mice, we also analyzed eosinophil counts and Th2 cytokine levels in BAL after sham or OVA-IT treatment followed by OVA challenge. Similar to wild-type mice, BAL

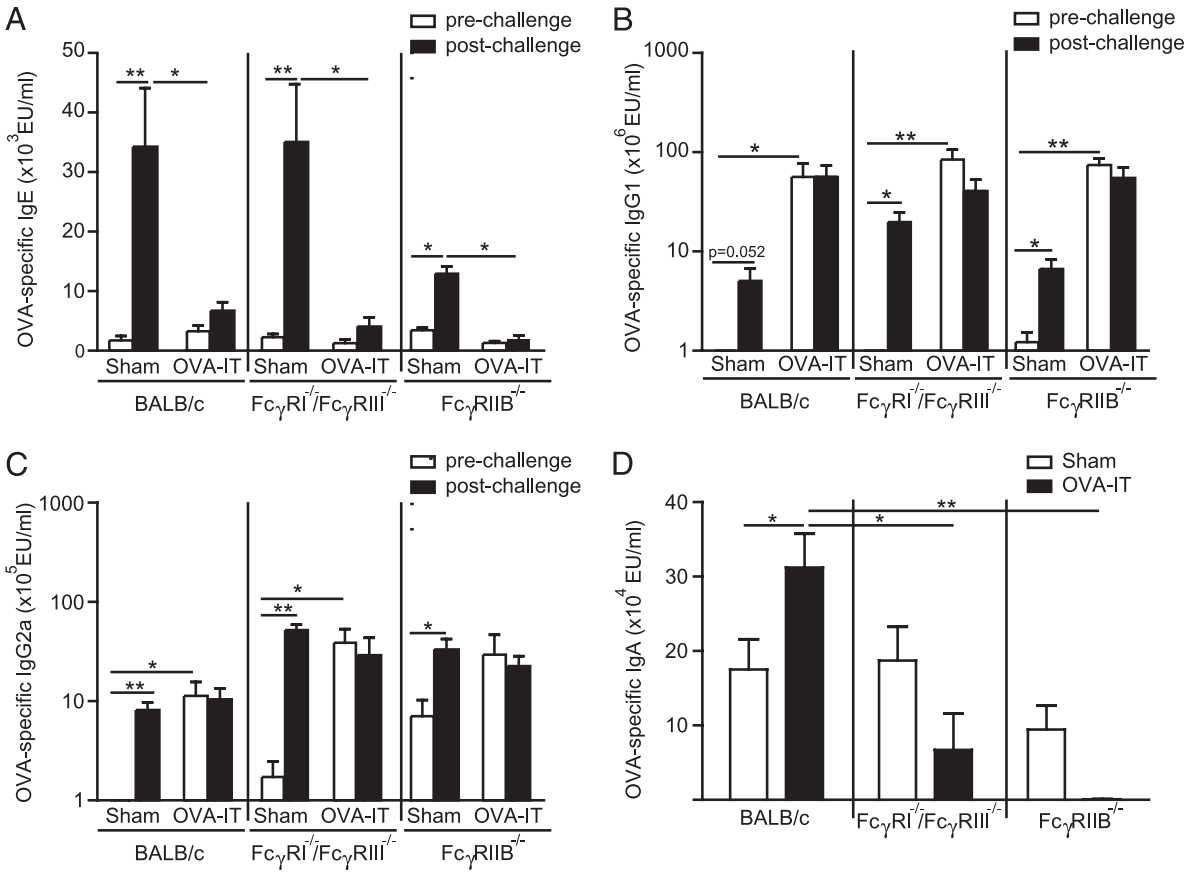


**FIGURE 2.** OVA-specific Ig responses to IT treatment in FcγRI/FcγRIII$^{-/-}$, FcγRIIB$^{-/-}$, and wild-type mice. Levels of OVA-specific IgE (*A*), IgG1 (*B*), IgG2a (*C*), and IgA (*D*) in serum were measured before (prechallenge) and after (postchallenge) OVA aerosol challenges in sensitized and IT/sham-treated mice. Values are expressed as mean ± SEM of six mice per group. $*p < 0.05$; $**p < 0.01$.

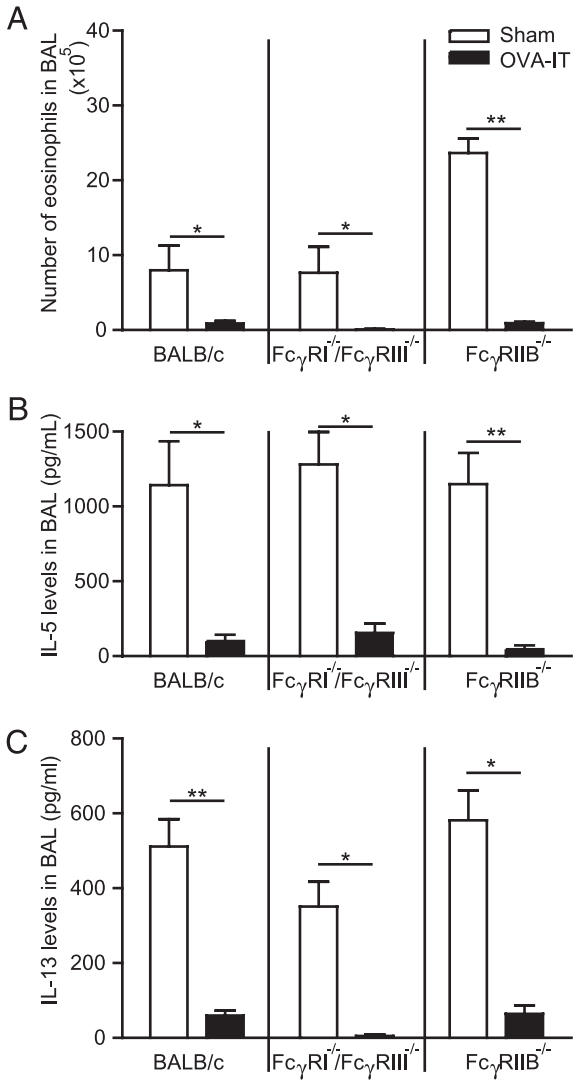


**FIGURE 3.** IT-induced suppression of allergic inflammation in FcγRI/FcγRIII$^{-/-}$, FcγRIIB$^{-/-}$, and wild-type mice. BAL fluid was obtained from OVA-sensitized and IT/sham-treated mice 24 h after the last inhalation challenge. Cytospin slides were prepared for differential cell counts, and cytokines were measured by ELISA. Number of eosinophils (*A*) and levels of IL-5 (*B*) and IL-13 (*C*) in BAL fluid. Values are expressed as mean ± SEM ($n$ = 6). $*p < 0.05$; $**p < 0.01$.

eosinophil counts in FcγRI/FcγRIII$^{-/-}$ mice were significantly decreased after OVA-IT compared with sham-treated mice (Fig. 3*A*). In addition, OVA-IT significantly reduced levels of IL-5 and IL-13 in BAL fluid compared with those of sham-treated FcγRI/FcγRIII$^{-/-}$ mice (Fig. 3*B*, 3*C*). Taken together, these data indicate that FcγRI/FcγRII-mediated signaling is not required for the suppression of the late-phase allergic response by IT. Hence, we next asked whether the allergen-specific Ig responses were required for the suppression of the Th2-driven allergic inflammation by allergen IT.

### *IT suppresses allergic airway inflammation in B cell-deficient mice*

Because we showed that IT efficiently suppresses Th2 cell activity and eosinophilic airway inflammation in mice deficient for FcγRI and FcγRIII, as well as in mice deficient for FcγRIIB, FcγR-mediated signaling seems to be redundant for the suppression of the late-phase allergic response by IT. Nevertheless, IT treatment induces a strong OVA-specific IgG response in FcγRIIB$^{-/-}$ mice and FcγRI$^{-/-}$/FcγRIII$^{-/-}$ mice (Fig. 2*B*, 2*C*). Therefore, we aimed to test whether the IgG serum responses contribute to IT by a non–FcγR-dependent mechanism. Moreover, we wanted to assess the putative role of IL-10–producing B cells (27) in the IT-dependent suppression of Th2 cell activity. Therefore, we subjected B cell-deficient μMT mice to our mouse model of IT in allergic asthma (Fig. 1*D*).

As a result of the functional inactivation of the H chain of IgM in μMT mice, B cell differentiation is arrested at the pro-B cell stage, and these mice lack most mature B cells capable of producing Ig (28). Except for some residual mucosal IgA production (29), IgM and IgG serum responses are absent in μMT mice. Interestingly, in

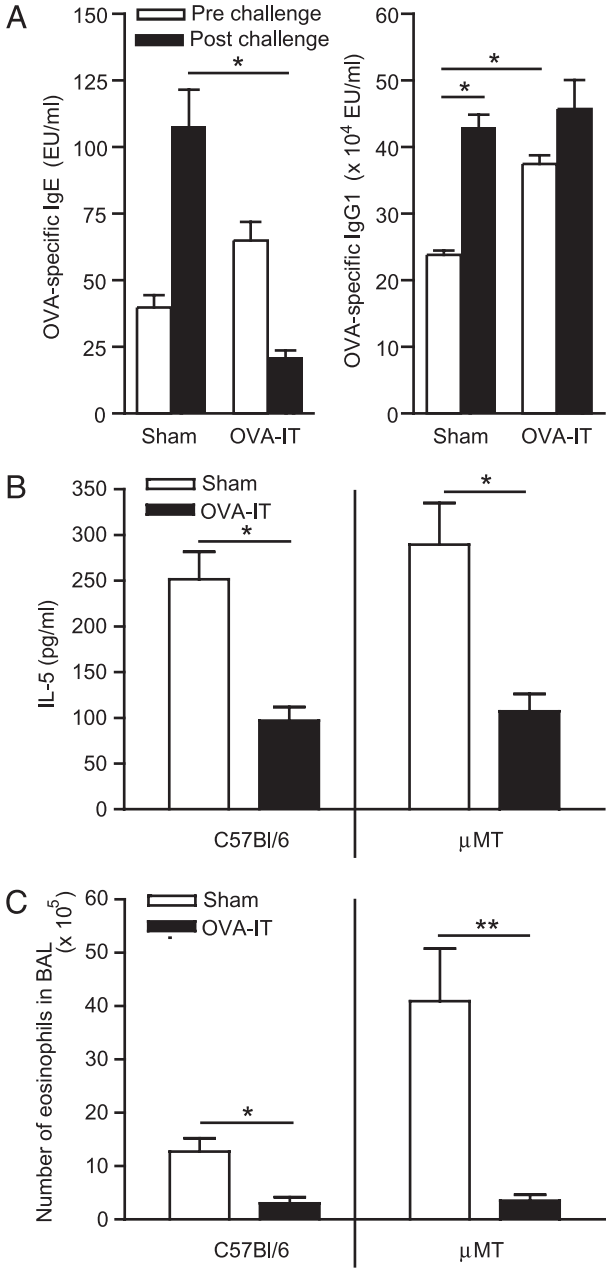


**FIGURE 4.** OVA-IT suppresses allergic inflammation in B cell-deficient mice. OVA-sensitized C57BL/6 μMT mice and wild-type controls were IT/sham treated and challenged with OVA aerosols. *A*, OVA-specific IgE was measured before (prechallenge) and 24 h after (postchallenge) the last inhalation challenge in wild-type control mice. BAL fluid was collected 24 h after the last inhalation challenge. IL-5 levels (*B*) and number of eosinophils (*C*) in BAL were determined. Values are expressed as mean ± SEM ($n$ = 6–8). $*p < 0.05$; $**p < 0.01$.

the OVA/alum mouse model of allergic asthma, μMT mice were shown to display Th2 cytokine production and airway eosinophilia and hyperresponsiveness, despite the absence of OVA-specific IgE responses (30). Therefore, the μMT mouse model allows analysis of the regulation of the allergen-specific Th2 cell-driven late-phase allergic response by IT in the absence of allergen-specific IgE and IgG and systemic IgA responses and the immediate phase of the allergic response.

To confirm that our IT protocol was successful, we first evaluated serum levels of OVA-specific IgE in wild-type controls. As expected, OVA-IT significantly inhibited the OVA-specific IgE response induced by OVA inhalation in sham-treated mice (Fig. 4*A*). Furthermore, the IT treatment itself induced a strong upregulation of OVA-specific IgG1 levels, in agreement with our typical IT-induced Ig responses.

To evaluate the suppression of Th2-driven airway inflammation after OVA-IT in μMT mice, we measured eosinophil cell counts and Th2 cytokine levels in BAL fluid. After sham treatment, OVA challenge resulted in high IL-5 levels in BAL fluid and the infiltration of a high number of eosinophils into the airways in wild-type and μMT mice (Fig. 4*B*, 4*C*). As expected, BAL levels of IL-5 and airway eosinophilia were strongly suppressed by OVA-IT in wild-type mice. Interestingly, the same level of suppression of BAL IL-5 and airway eosinophilia was observed in IT-treated μMT mice (Fig. 4*B*, 4*C*), indicating that the suppression of Th2 cell activity and allergic inflammation by allergen-specific IT is exerted independently of the allergen-specific IgG and systemic IgA responses induced by IT.

### *Suppression of allergic inflammation by IT in pIgR-deficient mice*

Our data in the μMT mouse model indicate that the IgG and systemic IgA responses induced by OVA-IT are dispensable for the suppression of Th2-driven allergic inflammation. Nevertheless, μMT mice were shown to retain a functional mucosal B cell population that is capable of producing luminal IgA (29). Because airway delivery of allergen-specific IgA to sensitized mice was shown to suppress airway responses upon subsequent allergen challenge and to induce a systemic allergen-specific IgG2a response (31) resembling some IT-induced phenotypes, we wanted to test whether modulation of the airway luminal IgA response by IT could contribute to the suppression of allergic inflammation. To this end, we performed our mouse model of OVA-IT in pIgR-deficient mice, which are incapable of transporting polymeric IgA across the airway epithelium and, therefore, lack luminal IgA (32). As shown in Fig. 5, pIgR-deficient mice displayed a strong IgG1 response upon OVA-IT treatment (Fig. 5*B*). In contrast, the OVA-specific IgG2a response induced by OVA-IT in wild-type mice was not observed in pIgR$^{-/-}$ mice, even though

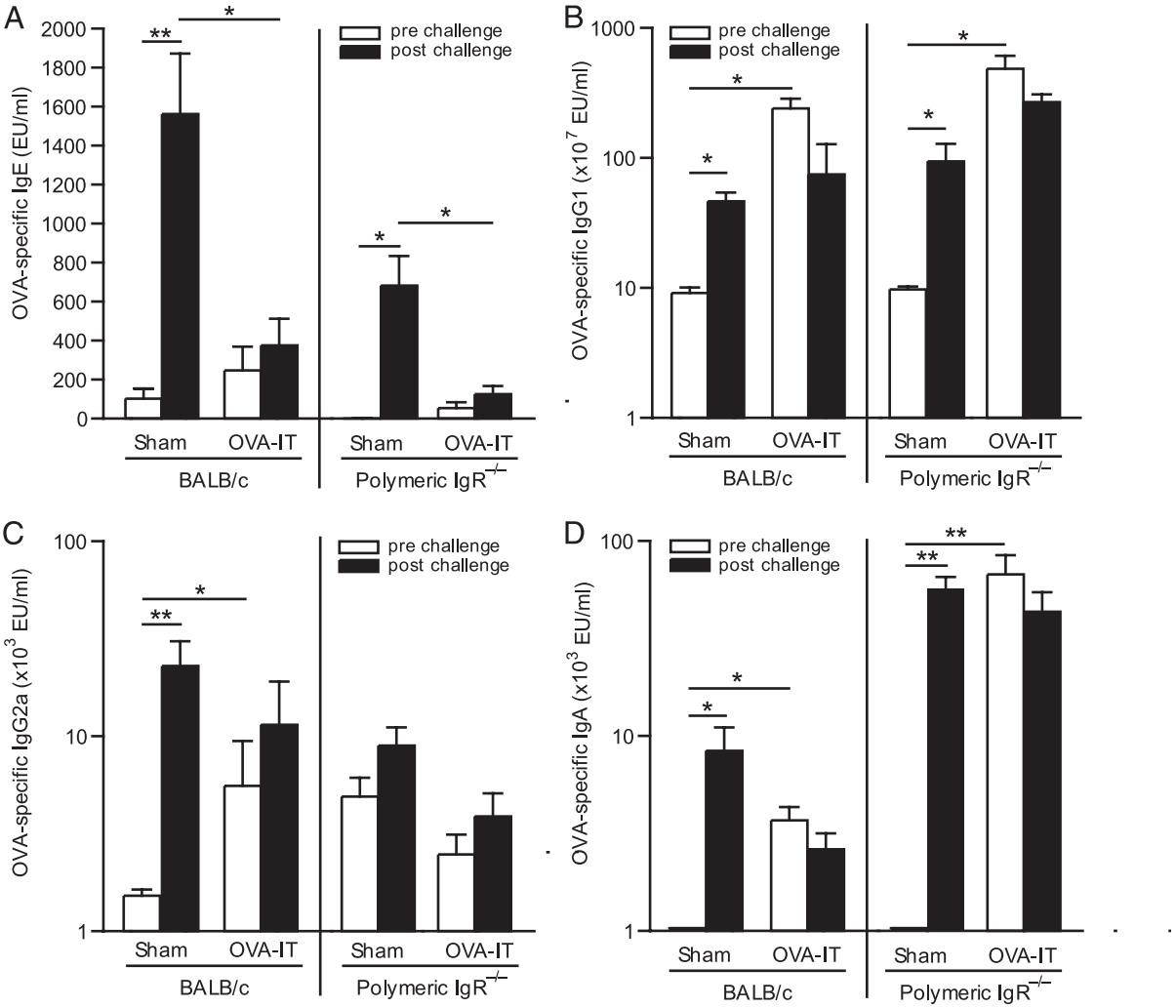


**FIGURE 5.** OVA-specific Ig responses after IT treatment in pIgR$^{-/-}$ mice. OVA-sensitized mice received IT/sham treatments. Levels of OVA-specific IgE (*A*), IgG1 (*B*), IgG2a (*C*), and IgA (*D*) in serum were measured before (prechallenge) and 24 h after (postchallenge) the last OVA aerosol challenge. Values are expressed as mean ± SEM of six mice per group. $*p < 0.05$; $**p < 0.01$.

these mice have a higher basal level of OVA-specific IgG2a after sensitization (Fig. 5*C*). Nevertheless, OVA-specific IgE responses are suppressed by OVA-IT in pIgR$^{-/-}$ and wild-type mice to a similar degree (Fig. 5*A*). Finally, the OVA-specific serum IgA responses were strongly increased in pIgR$^{-/-}$ mice, in agreement with previous observations (32, 33), but no differential effect of IT treatment on serum IgA was seen between wild-type and pIgR$^{-/-}$ mice (Fig. 5*D*). Surprisingly, measurement of OVA-specific IgA levels in BAL of the nontransgenic littermates indicated that, in our model, OVA inhalations in IT-treated mice resulted in a reduced level of BAL IgA compared with OVA inhalations in sham-treated control mice, although the differences were not statistically significant (1504.5 ± 673.49 experimental unit/ml in IT versus 5978 ± 1545.4 experimental unit/ml in sham-treated controls; $p = 0.157$).

To extend our findings to the effects of IT on the suppression of Th2-driven airway inflammation in pIgR$^{-/-}$ mice, we also analyzed BAL Th2 cytokine levels and eosinophil cell counts (Fig. 6). IL-5 levels and eosinophil cell counts in BAL were equally suppressed by OVA-IT in wild-type and pIgR$^{-/-}$ mice, indicating that the suppression of allergic inflammation by IT is independent of airway luminal IgA levels.

## Discussion

We present a comprehensive set of data assessing the relevance of allergen IT-induced Ig responses for the suppression of the Th2-driven allergic inflammation that underlies the late-phase allergic response. We show evidence that cardinal features of the late-phase allergic response in the OVA-driven mouse model of allergic asthma are suppressed by IT via a mechanism that does not depend on the induction of IgG or IgA responses, FcγRIIB signaling, or B cell function. The use of s.c. injections of high-dose OVA in the classical OVA-driven mouse model of asthma was previously established as a mouse model that phenocopies the human response to IT treatment in considerable detail (18, 19). Nevertheless, OVA as a model allergen lacks several aspects of natural allergens and consequently requires i.p. immunization adsorbed to alum to achieve sensitization. Moreover, repeated airway administrations with OVA were shown to induce a gradual loss of the asthmatic phenotype in sensitized mice (34–36), although this effect is not Ag specific. We observed a limited reduction in eosinophilic airway inflammation in mice receiving two series of three OVA inhalations compared with mice that received only a single series of three OVA-inhalation challenges (Fig. 1*C*; compare sham-treated asthmatic mice with sham-treated sensitized-only mice), which might be attributed to the repeated OVA inhalations in this extended IT protocol. This observation underscores the notion that the data from these mouse studies can only be extrapolated to the human situation with great care. Nevertheless, the suppressive effect of repeated airway administrations with OVA was not observed on the AHR to methacholine, and, importantly, OVA-IT still suppressed AHR and airway eosinophilia in the asthmatic group very efficiently, indicating that despite the side effects of repeated OVA exposure, IT is still fully operational. Importantly, the three high doses of OVA used in our model were previously shown to be as efficacious as a gradual up-dosing of OVA in the mouse model (18), and suppression of clinically relevant parameters of the asthmatic phenotype, such as AHR and airway eosinophilia, are as efficiently suppressed by the IT treatment in mice that were only sensitized to OVA as in mice that had experienced a prior asthmatic phenotype at the time of IT treatment, the latter reflecting the clinical practice more closely.

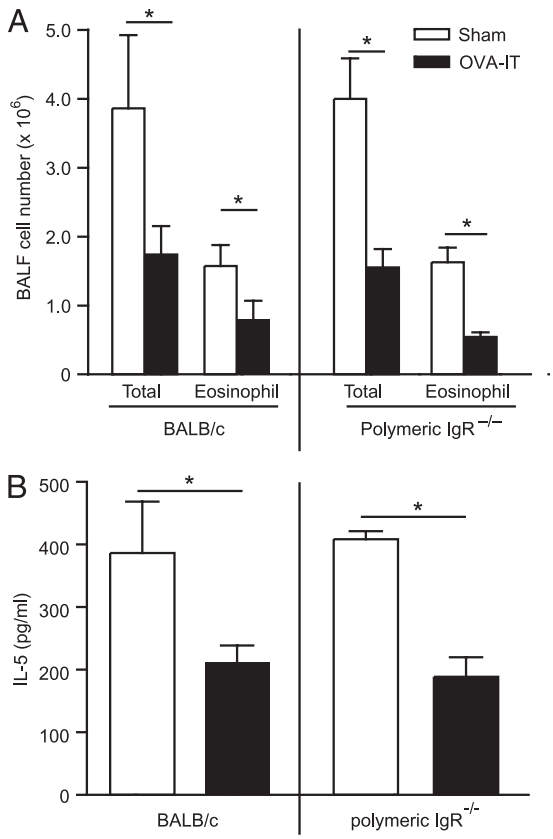


**FIGURE 6.** IT suppresses allergic inflammation in pIgR$^{-/-}$ mice. OVA-sensitized and IT/sham-treated pIgR-deficient and wild-type mice were challenged with OVA aerosols. At 24 h after the last OVA challenge, BAL fluid was recovered and the total number of cells and eosinophils (*A*) and the levels of IL-5 (*B*) were determined. Values are expressed as mean ± SEM ($n = 6$). $*p < 0.05$.

In human allergic individuals, allergen IT was shown to efficiently suppress the late phase of the allergic response (37–39), associated with reduced numbers of infiltrating T cells, basophils, neutrophils, and eosinophils. Intriguingly, the effects of IT on the immediate phase of the allergic response are modest, although a reduction in the magnitude of the immediate-phase allergic response was reported (39, 40). The most prominent response to allergen IT treatment in allergic individuals is the induction of allergen-specific IgG1 and IgG4 responses, yet the relevance of the IT-induced IgG Ab response has remained controversial (1). Although some studies reported observations that indicate a contribution of blocking Abs to IT (41), several groups failed to show a correlation between the presence of neutralizing IgG Abs and improved clinical outcome (6, 42). The allergen-specific IgG Abs might contribute to IT by blocking access of the allergen to IgE on mast cells or basophils or by preventing FcεRI-dependent mast cell activation by binding to FcγRIIB (reviewed in Ref. 1). Upon cross-linking of the FcεRI–IgE complex on the mast cell surface with the FcγRIIB–IgG complex by the same multivalent ligand, FcγRIIB ITIM phosphorylation by FcεRI-associated kinases can directly suppress FcεRI signaling and mast cell activation (43, 44). In the mouse model of Fel d1-specific IT, both mechanisms have been postulated to contribute to the suppression of the immediate phase of the allergic response (20). Although suppression of systemic anaphylaxis by IT was critically dependent on FcγRIIB signaling, suppression of tissue mast cell degranulation was maintained in FcγRIIB-deficient mice. Remarkably, the suppression of the systemic and the local immediate-phase allergic response could be passively transferred by serum or the serum IgG fraction, indicating the requirement for allergen-specific IgG (20). The allergen-induced Th2 cell-driven airway inflammation (21, 22) and AHR (23)

in the OVA/alum mouse model of experimental allergic asthma were previously shown to be independent of allergen-specific IgE responses and the immediate-phase allergic response induced by cross-linking of IgE on mast cells and basophils. This is in marked contrast to the human situation, in which allergic asthma is characterized by a clear immediate and late-phase allergic response, depending on allergen-specific IgE and Th2 cells, respectively (45). Our mouse model of OVA-IT exclusively allows the analysis of the mechanism by which the treatment suppresses the late-phase allergic response. We found that the allergen-specific IgG response induced by IT is not critically required for suppression of the Th2 cell-dependent late-phase allergic response or the allergen-specific IgE responses.

The FcγRIIB-independent suppression of allergen-specific IgE responses was also observed in the Fel d1 virus-like particle vaccination study (20). Although the investigators hypothesized a contribution of the ssRNA within the virus-like particles to suppression of the IgE response through TLR3/7 signaling, our data replicate their findings on the FcγRIIB-independent suppression of the IgE response by IT, even in the absence of TLR signaling. Therefore, the mechanism by which allergen IT represses the IgE response seems to be independent of direct suppression of IgE-producing B cells via FcγRIIB (46, 47). In fact, the IT-induced suppression of the Th2-driven allergic inflammation and the allergen-specific IgE responses seem to correlate, indicating that inhibition of Th2 cell activity might be critical for the reduced IgE levels after IT.

In serum, OVA-IT induces a specific IgA response in our mouse model (Fig. 2*D*), in agreement with human studies (15, 16). Nevertheless, the use of a reference serum containing an arbitrary level of OVA-specific IgA as a standard in these assays precludes the absolute quantification of the OVA-specific IgA levels induced by OVA-IT or a direct comparison with the magnitude of the OVA-specific IgG response. Consequently, the biological relevance of this response remains to be determined. Moreover, the secreted form, rather than the serum levels, of IgA is critical for mucosal protective activity of this Ig isotype. For instance, direct nasal installation of allergen-specific IgA was shown to have systemic effects, reflected in an altered allergen-specific $CD4^+$ T cell response and increased serum IgG2a levels (31), indicating that increased luminal IgA levels can contribute to suppression of allergic manifestations. We did not directly measure OVA-specific IgA levels in BAL after OVA-IT but prior to inhalation challenge. However, after OVA challenge, specific IgA levels were not significantly reduced in the serum (Fig. 5*D*) and BAL fluid of OVA-IT–treated mice compared with sham-treated mice. These data seem to indicate that in our mouse model, the luminal IgA levels might not be critical for the suppression of the late-phase response by IT treatment. Our data in the $pIgR^{-/-}$ mice lend further support to the notion that mucosal IgA responses are dispensable in IT-induced suppression of local allergic inflammation. In addition, our data from the μMT mice strongly indicate that the systemic IgA responses induced by IT are also dispensable for suppression of the late phase of the allergic response.

Remarkably, we found that pIgR deficiency selectively abrogates the IT-induced OVA-specific IgG2a response, whereas other Ig responses and suppression of allergic inflammation remain unaffected, in line with the observations of Schwarze et al. (31). These data seem to indicate the presence of a feedback mechanism by which luminal IgA levels regulate the systemic Ag-specific IgG2a response. Given the Ag specificity of this process, we would assume the involvement of a Th1 cell population, as was previously postulated (31). Nevertheless, we find that this mechanism does not contribute to the IT-induced suppression of the late-phase allergic response or allergen-specific IgE.

Our experiments in μMT mice also indicated that allergen-specific IT can efficiently suppress Th2-driven eosinophilic airway inflammation in sensitized mice in the absence of B cells. This is in contrast to the critical role for B cells in tolerance induction in naive mice by mucosal application of allergen (48, 49), but is in agreement with earlier observations that T cells can efficiently be tolerized in vivo in the absence of B cells (50). Because FcγRIIB-dependent signaling was shown to be required for the induction of mucosal tolerance in naive mice by intranasal installation of OVA (51), oral-tolerance induction in naive mice seems to be at least partially dependent on the Ig response. Because we found that suppression of Th2 cell activity can be induced independently of B cells, it remains of interest to determine which APC is required for the observed suppression of Th2 cell activity by IT. We recently showed that IT treatment is partially dependent on IDO (24), the rate-limiting enzyme in tryptophan metabolism that contributes to the induction of adaptive regulatory T cells by plasmacytoid dendritic cells (52). Because we and other investigators showed a dependency of the suppression of allergic inflammation by IT on IL-10 (19, 53), it is tempting to speculate that IDO-competent dendritic cells induce adaptive regulatory T cells during IT treatments that then suppress Th2 activity upon allergen exposure. Alternatively, the high-dose OVA treatment might induce Th2 cell anergy, although the IL-10 dependence of the IT-induced suppression of allergic inflammation would not seem to support anergy induction as a major mechanism for IT in our mouse model. In this article, we show that, irrespective of the involvement of regulatory or anergic T cell populations, the IT-induced suppression of the late-phase allergic response is induced independently of allergen-specific IgG and IgA responses and FcγR signaling.

In summary, to our knowledge, we demonstrate for the first time that the allergen-specific IgG and IgA responses induced by allergen-specific IT in a sensitized host are not critically required for the suppression of allergen-specific IgE responses and the Th2 cell-driven late-phase allergic response upon allergen provocation. This is in marked contrast to recent data showing that allergic desensitization by an Fel d1 vaccination strategy caused suppression of the immediate phase of the allergic response that could be passively transferred by serum or the serum IgG fraction and which was, in part, dependent on FcγRIIB signaling (20). We postulate that allergen-specific IT suppresses the immediate and late-phase allergic response by divergent and independent mechanisms. Other mechanisms that could contribute to the IT-induced suppression of allergic inflammation, such as local IL-10 production, induction of regulatory T cells, or immune deviation to Th1 or Tr-1 cells, need to be addressed in future studies.

## Disclosures

The authors have no financial conflicts of interest.

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