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J Immunol 2007; 178:3924-3931; doi: 10.4049/jimmunol.178.6.3924
http://www.jimmunol.org/content/178/6/3924

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Costimulation Blockade Inhibits Allergic Sensitization but Does Not Affect Established Allergy in a Murine Model of Grass Pollen Allergy

Birgit Linhart,* Sinda Bigenzahn,† Arnulf Hartl,‡ Christian Lupinek,* Josef Thalhamer,§ Rudolf Valenta,2* and Thomas Wekerle2,3*†

Type I allergy is characterized by the development of an initial Th2-dependent allergen-specific IgE response, which is boosted upon a subsequent allergen encounter. Although the immediate symptoms of allergy are mainly IgE-mediated, allergen-specific T cell responses contribute to the late phase as well as to the chronic manifestations of allergy. This study investigates the potential of costimulation blockade with CTLA4Ig and an anti-CD154 mAb for modifying the allergic immune response to the major timothy grass pollen allergen Phl p 5 in a mouse model. BALB/c mice were treated with the costimulation blockers at the time of primary sensitization to the Phl p 5 allergen or at the time of a secondary allergen challenge. Costimulation blockade (CTLA4Ig plus anti-CD154 or anti-CD154 alone) at the time of sensitization prevented the development of allergen-specific IgE, IgM, IgG, and IgA responses compared with untreated but sensitized mice. However, costimulation blockade had no influence on established IgE responses in sensitized mice. Immediate-type reactions as analyzed by a rat basophil leukemia cell mediator release assay were only suppressed by early treatment but not by a costimulation blockade after sensitization. CTLA4Ig given alone failed to suppress both the primary and the secondary allergen-specific Ab responses. Allergen-specific T cell activation was suppressed in mice by early as well as by a late costimulation blockade, suggesting that IgE responses in sensitized mice are independent of T cell help. Our results indicate that T cell suppression alone without active immune regulation or a shifting of the Th2/Th1 balance is not sufficient for the treatment of established IgE responses in an allergy.

The Journal of Immunology, 2007; 178: 3924–3931.

*Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology, Center of Physiology and Pathophysiology and †Division of Transplantation, Department of Surgery, Medical University of Vienna, Austria; ‡Department of Physiology and Pathophysiology, Paracelsus Medical University, Salzburg, Austria; and ‡Department of Molecular Biology, University of Salzburg, Salzburg, Austria.

Received for publication October 11, 2006. Accepted for publication December 12, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This study was supported by Austrian Research Promotion Agency (Forschungs- förderungsgesellschaft) Bridge Grant 810105-SCK/SAI, Christian Doppler Research Association (to R.V.) and Austrian Science Fund Grants SFB F2310-B13 (to T.W.), F1815 (to R.V.), and S8811 (to J.T.).

R.V. and T.W. are cosenior authors of this article.

1 Address correspondence and reprint requests to Dr. Thomas Wekerle, Medical University of Vienna, Division of Transplantation, Department of Surgery, Vienna General Hospital, Wachinger Guertel 18-20, A-1090 Vienna, Austria. E-mail address: thomas.wekerle@meduniwien.ac.at

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CTLA4Ig (abatacept) has recently been approved for the treatment of rheumatoid arthritis (18), and a second generation version (LEA29Y (belatacept)) is currently being evaluated in renal transplantation (19). Little is known, however, about the potential effect of these costimulation blockers on allergic immune responses; in particular, no studies dissecting the humoral response to defined clinically relevant allergens have been reported. Our results demonstrate that costimulation is essential for the establishment of an allergen-specific immune response at the time of allergic sensitization. In contrast, costimulation blockade did not affect an already established IgE Ab response. These findings have important implications for the development of therapeutic strategies for allergic diseases.

Materials and Methods
Recombinant allergens and antibodies

Purified recombinant timothy grass pollen allergens (rPhl p 2 and rPhl p 5) (20) were obtained from Bioarray. The hamster anti-mouse CD154 (MR1) Ab was purchased from Bioexpress. Human CTLA4Ig was generously provided by Bristol-Myers Squibb. Both reagents have been used to successfully block T cell responses in several mouse models of transplantation (14, 25). Intraperitoneal treatment with corresponding (14). Treatment (anti-CD154 mAb; human CTLA4Ig) (0.5 mg/mouse) was performed in the presence of 100 μg/ml CTLA4Ig. Spleen cells were harvested by centrifugation, and resuspended in 10 mM MgSO4. Cells were dissolved in 0.6% (w/v) agarose and plated onto Luria-Bertani plates containing 50 μg/L ampicillin. Two-microliter aliquots of phage lysates were dotted onto the plates. Plates were incubated at 43°C until plaques became visible and protein synthesis was induced by overlay with nitrocellulose filters (Schleicher & Schuell) soaked with 10 mM isopropyl β-D-thiogalactopyranoside for 37°C. Filters described (27) were adsorbed to Al(OH)3 (Alu-Gel-S; SERVA Electrophoresis) overnight with nitrocellulose filters (Schleicher & Schuell) soaked with 10 mM isopropyl β-D-thiogalactopyranoside for 37°C. Filters described (27) were adsorbed to Al(OH)3 (Alu-Gel-S; SERVA Electrophoresis) and stimulated with or without Con A (0.5 μg/well), rPhl p 2 (3 μg/well), and rPhl p 5. After 30 min, Ag-agglutinated by mouse sera diluted 1/1000 overnight, a monoclonal rat anti-mouse IgM (BD Pharrmingen) diluted 1/1000 for 5 h, and a 125I-labeled goat anti-rat IgG Ab (Sigma-Aldrich) diluted 1/2000 for 2 h. Reactivity with the allergen fragments was detected by autoradiography. The intensities of the signals were determined by densitometry using the AlphaEaseFC ChemiImage 4400 software.

ELISA experiments
To measure Ag-specific Abs in the sera of immunized mice, an ELISA was performed as described earlier (14, 26). Plates were coated with rPhl p 5 (5 μg/ml), sera were diluted 1/10 for IgE, 1/100 for IgM, IgA, and IgG2a, and 1/1000 for IgG1, and bound Abs were detected with monoclonal rat anti-mouse IgM, IgG1, IgG2a, and IgG2b Abs (BD Pharrmingen) diluted 1/1000 and a HRP-coupled goat anti-rat antisemur (Amersham Biosciences) diluted 1/10000.

T cell proliferation assay
Spleens were removed under aseptic conditions (day 100) and homogenized. After the lysis of erythrocytes, cells were washed and resuspended in complete medium (RPMI 1640, 10% FCS, 0.1 mg/ml gentamicin, and 2 mM glutamine). Single cell suspensions were plated into 96-well round-bottom plates at a concentration of 2 × 105 cells/well (200 μl) in triplicates and stimulated with or without Con A (0.5 μg/well), rPhl p 2 (3 μg/well), and rPhl p 5 (3 μg/well) for 4 days. The cultures were pulsed with 0.5 μCi/well tritiated thymidine for 16 h and harvested. The proliferation responses were measured by scintillation counting. The ratio of the mean proliferation after Ag stimulation and medium control values, i.e., the stimulation index (SI), was calculated.

Rat basophil leukemia (RBL) assay
For the quantification of IgE Ab-mediated, immediate-type reactions, RBL cell mediator release assays were performed as previously described (27). RBL-2H3 cells were cultivated in 96-well tissue culture plates (4 × 104 cells/well) for 24 h at 37°C using 7% CO2. Passive sensitization was performed by incubation with 1/30 diluted murine sera for 2 h. Cells were washed twice with Tyrode’s buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl2, 1.8 mM CaCl2, 0.4 mM NaH2PO4, 5.6 mM d-glucose, 12 mM NaHCO3, 10 mM HEPES, and 0.1% w/v BSA (pH 7.2)) to remove unbound Abs. Degranulation of RBL cells was induced by adding 0.3 μg/ml rPhl p 5. After 30 min, β-heomaminidase release was analyzed. Results are expressed as percentages of total β-heomaminidase released after the addition of 1% Triton X-100 and represent the mean of triplicate determinations.

Mixed lymphocyte reaction
As a positive control for the biological activity of CTLA4Ig, an MLR was performed in the presence of 100 μg/ml CTLA4Ig. Spleen cells were

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Table I. Immunization and treatment protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensitization</th>
<th>Treatment</th>
<th>CTLA4Ig</th>
<th>Early/Late</th>
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<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>Days 0, 2, and 4</td>
<td>Days 0, 2, and 4</td>
<td>Early/Late</td>
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<tr>
<td>B</td>
<td>+</td>
<td>Days 0, 2, and 4</td>
<td>Days 0, 2, and 4</td>
<td>Early/Late</td>
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<tr>
<td>C</td>
<td>+</td>
<td>Days 0, 2, and 4</td>
<td>Days 0, 2, and 4</td>
<td>Early/Late</td>
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<tr>
<td>D</td>
<td>+</td>
<td>Days 21, 23, and 25</td>
<td>Days 21, 23, and 25</td>
<td>Late/Late</td>
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<tr>
<td>E</td>
<td>+</td>
<td>Days 21, 23, and 25</td>
<td>Days 21, 23, and 25</td>
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<tr>
<td>F</td>
<td>+</td>
<td>Days 21, 23, and 25</td>
<td>Days 21, 23, and 25</td>
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<tr>
<td>G</td>
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<td>Days 21, 23, and 25</td>
<td>Days 21, 23, and 25</td>
<td>Late/Late</td>
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*Abbreviations used in this paper: SI, stimulation index; RBL, rat basophil leukemia.
washed with PBS and resuspended in MLR medium (42.5 ml of RPMI 1640 (BioWhittaker), 7.5 ml of controlled processed serum replacement type 2 (CPSR-2; Sigma-Aldrich), 0.5 ml of HEPES buffer (ICN Biomedicals), 1.55 ml of nutrient mixture (10 ml of distilled water, 5 ml of non-essential amino acids, 0.055 g of sodium pyruvate, 0.146 g of L-glutamine, 5000 U of penicillin, and 5000 μg of streptomycin), and 100 μl of 2-ME (dilution 1/3000)). B6 responder cells (4 × 10^5) were incubated at 37°C and 5% CO_2 with 4 × 10^5 irradiated (30 Gy) stimulator cells of either BALB/c or B6 mice or with medium in triplicates. After 3 or 4 days, cells were pulsed with [3H]thymidine and incubated for 12–18 h. After harvesting on filter plates, the cells were analyzed with a MicroBeta 1450 beta counter (Wallac Products; HVD Life Science). SI values were calculated by dividing the mean counts per minute from responses against self (B6), and against the allogeneic stimulator (BALB/c) by mean background counts per minute (i.e., counts per minute with no stimulator population).

Statistical analysis

Ab levels are illustrated by box-and-whisker plots. Differences between groups B–G and the control, group A, were assessed with a Wilcoxon-Mann-Whitney U test and exact significances were determined and adjusted using the Bonferroni-Holm method. SPSS statistical software system 14.0 was used for calculations. The reported p values are results of two-sided tests. p ≤ 0.05 was considered statistically significant.

Results

Establishment of a murine model for grass pollen allergy by sensitization of mice with the timothy grass pollen allergen Phl p 5

Mice sensitized with Phl p 5 developed an IgE Ab response specific for Phl p 5 already at day 21 (Fig. 1A, group A). Passive transfer of serum IgE from the sensitized mice but not of their preimmune sera (PS) and challenged with the allergen. The mean β-hexosaminidase release ± SD of each group of mice is shown on the y-axis. *, p < 0.05; indicates a significant reduction compared with the control, group A. 

FIGURE 1. Costimulation blockade with CTLA4Ig plus anti-CD154 inhibits allergic sensitization but does not affect an already established allergic secondary IgE response. A. Serum levels of rPhl p 5-specific IgE measured by ELISA on days 0, 21, and 42 are shown in box-and-whiskers plots. B. The biological activity of allergen-specific IgE was studied by RBL degranulation assays. RBL cells were loaded with serum IgE of Phl p 5-sensitized mice (groups A–G) obtained on day 21 and 42 or with the corresponding preimmune sera (PS) and challenged with the allergen. The mean β-hexosaminidase release ± SD of each group of mice is shown on the y-axis. *, p < 0.05; indicates a significant reduction compared with the control, group A.
patients (Fig. 3). As determined by densitometry, >75% (average 78%) of Phl p 5-specific Abs bound to the Lol p 5-derived fragments. Most of the sensitized mice developed Ab responses against the clone 81-defined Lol p 5B fragment (Fig. 3), which corresponds to an N-terminal fragment of Phl p 5 (timothy grass, *P. pratense*) and is highly homologous to group 5 allergens from other grasses and group 6 allergens from timothy grass. The clone 81-defined fragment corresponds to a highly allergenic domain of Phl p 5 that is recognized by most grass pollen allergic patients (25), underscoring the clinical relevance of our model.

**Costimulation blockade inhibits allergic sensitization**

The effects of costimulation blockade on allergen-specific primary immune responses (i.e., allergic sensitization) and on the secondary allergen-specific immune responses (i.e., boosting of an allergen-specific immune response) was investigated by the administration of costimulation blockers CTLA4Ig and/or an anti-CD154 mAb at the time of sensitization or at the time of the secondary allergen challenge (i.e., 3 wk after sensitization) (for group descriptions see Table I).

First, we investigated the effect of costimulation blockade on allergic sensitization in our murine allergy model (“early” treatment). In BALB/c mice that had been sensitized to the major grass pollen allergen Phl p 5 (group A), allergen-specific IgE could be detected 3 wk after sensitization by ELISA. Almost no Phl p 5-specific IgE responses could be detected in groups B and D, which had received treatment with anti-CD154 or anti-CD154 plus CTLA4Ig at the time of sensitization. The effect of early costimulatory blockade at days 0, 2, and 4 was limited, because it was possible 3 wk later to sensitize mice with a second injection of Phl p 5. This second immunization induced an allergen-specific IgE Ab response in groups B and D similar to that observed for the untreated group A.

![FIGURE 2](https://example.com/figure2.png)
CTLA4Ig alone (group C) did not suppress allergic sensitization to rPhl p 5 (Fig. 1A). Despite treatment with CTLA4Ig the induction of a Phl p 5-specific IgE response was comparable to that of the untreated, sensitized control group A, although the activity of the CTLA4Ig fusion protein had been confirmed in a MLR using the same batch of protein (data not shown).

The impact of costimulation blockade on IgE AB-mediated immediate allergic reactions was also demonstrated using RBL cell degranulation assays. When mouse sera of the sensitized but untreated group A were loaded on RBL cells, a strong degranulation was observed with the RBL assay was in agreement with the measured levels of IgE in the serum. Administration of CTLA4Ig at the time of sensitization failed to elicit degranulation. The data obtained with the RBL assay were confirmed by mouse sera from groups A, D, and G obtained before sensitization (lanes 1), on day 21 (lanes 2), and on day 42 (lanes 3).

Costimulation blockade has no influence on a secondary IgE response

To analyze the effect of costimulation blockade on an already established allergic immune response (i.e., secondary immune response or boosting of an established response), 3 wk after sensitization the groups of mice that had been sensitized to Phl p 5 were injected with anti-CD154 Ab alone (group E), CTLA4Ig alone (group F), or a combination of anti-CD154 plus CTLA4Ig (group G) (Table I). In Fig. 1A the “late” row shows the development of Phl p 5-specific IgE AB titers in these groups 3 and 6 wk after sensitization. On day 21 after the first allergen contact, mice of groups E, F, and G had developed Phl p 5-specific IgE Abs comparable to those of the sensitized and untreated controls of group A. Upon secondary allergen contact (i.e., second injection of Phl p 5 at day 21), the IgE Ab levels in the treated groups E, F, and G were almost identical with those in the untreated sensitized control group A, indicating that the IgE response to Phl p 5 was not influenced by costimulation blockade after sensitization. We again determined the allergenic activity of these Phl p 5-specific IgE Abs in an RBL degranulation assay, confirming that there was no effect of late costimulation blockade on allergic immune responses because there was no significant difference in the β-hexosaminidase releases from rat basophils between the untreated group A and mice of groups E, F, and G (Fig. 1B).

Costimulation blockade prevents the initiation of allergen-specific IgM, IgG, and IgA responses but does not affect allergen-specific secondary Ab responses

BALB/c mice, which had been sensitized with the major grass pollen allergen Phl p 5 on days 0 and 21, had Phl p 5-specific IgM, IgG1, IgG2A, and IgA Ab responses already at day 21 (Figs. 2 and 4). Treatment with the anti-CD154 Ab alone and with the combination of both costimulation blockers given at the time of sensitization blocked the development of Phl p 5-specific IgM as well as the IgG1, IgG2A, and IgA responses. As observed for IgE, CTLA4Ig alone was not able to suppress allergen-specific Ab responses. The suppressive effect of costimulation blockade on humoral responses had disappeared at day 21, because the second injection of Phl p 5 at day 21 could induce Phl p 5-specific Ab responses in mice of groups B and D (Figs. 2 and 4).

Similarly as observed for IgE, costimulation blockade given after the development of an allergen-specific Ab response did not suppress allergen-specific IgM, IgG, and IgA secondary immune responses in mice of groups E, F, and G (Figs. 2 and 4). Animals from groups E, F, and G had already developed a Phl p 5-specific Ab response comparable to that of the untreated but sensitized control group A. This allergen-specific Ab response could be boosted upon subsequent immunization with Phl p 5, which resulted in an increase of allergen-specific Ab levels 6 wk after primary immunization (Figs. 2 and 4). Thus, no strong effect of costimulation blockade on secondary Ab responses became apparent.

FIGURE 3. Epitope-mapping of the Phl p 5-specific IgG antibody response in immunized mice. A, Schematic representation of Lol p 5A and Lol p 5B fragments expressed by the phage clones. B, Lol p 5A- and Lol p 5B-derived clones (11–120) and a phage clone without an insert (Agg11) were immobilized to a nitrocellulose filter in the order given and probed with mouse sera from groups A, D, and G obtained before sensitization (lanes 1), on day 21 (lanes 2), and on day 42 (lanes 3).
Possible subtle differences between the Ab levels on day 42 were not analyzed in detail. Mice that were not sensitized to Phl p 5 but had been treated with CTLA4Ig and anti-CD154 or isotype control Abs did not mount any detectable humoral response to the Phl p 5 allergen (data not shown).

Costimulation blockade induces allergen-specific nonresponsiveness of T cells

We further investigated whether spleen-derived T cells isolated from the various groups of mice were able to proliferate upon stimulation with rPhl p 5 (Fig. 5). Spleen cells from untreated Phl p 5-sensitized mice strongly proliferated in response to Phl p 5 (mean SI: 18.5). Phl p 5-specific proliferation of splenocytes was substantially inhibited in treated groups B, D, E, and G, resulting in mean stimulation indices between 1.5 and 2. In our model, CTLA4Ig treatment alone (groups C and F) was not sufficient for the complete suppression of allergen-specific T cell responses, although T cell responses were considerably reduced (mean SI: 2.6 and 3.6). Spleen cells of mice from the various groups did not proliferate in response to an immunologically unrelated allergen, Phl p 2 (20) (Fig. 5).

**FIGURE 4.** Effects of costimulation blockade on Phl p 5-specific IgA and IgG 2A Abs responses. Serum samples, collected on days 0, 21, and 42 from the mice of groups A–G were analyzed for Phl p 5-specific IgA and IgG 2A Abs by ELISA. Ab levels (OD values) are displayed as box-and-whiskers plots, *p < 0.05; indicates a significant reduction compared with the untreated but sensitized group A.

**FIGURE 5.** Treatment with CTLA4Ig and/or anti-CD154 reduces lymphoproliferative responses to Phl p 5. T cell proliferation was measured at day 100 in spleen cell cultures after in vitro stimulation with timothy grass pollen allergens Phl p 5 (filled bars) or Phl p 2, an immunologically unrelated grass pollen allergen (negative control) (gray bars). The bars represent the mean stimulation indices (SI ± SD) for the different groups of mice.
Discussion
Sensitization, i.e., the induction of allergen-specific IgE Abs, is the initial event in the establishment of allergic disease. Subsequent allergen contact leads to a boost of allergen-specific IgE and T cell responses as well as to allergic inflammation (28). Allergic inflammation can be elicited via IgE-mediated activation of different inflammatory cells but may also occur via IgE-independent T cell activation (11, 28).

In this study, we investigated the effects of costimulation blockade on allergic sensitization and allergen-specific secondary IgE responses. The CTLA4Ig and anti-CD154 mAb that we used for suppression of T cell help have previously been described as pot-ent costimulatory blockade reagents and were effective in numerous models of transplantation and autoimmunity, but also in clinical trials for rheumatoid arthritis and psoriasis (18, 21–23, 29). When these reagents were given in combination in the course of allergic sensitization, they efficiently prevented the induction of a primary IgE response to the major timothy grass pollen allergen Phl p 5. This result confirms the pivotal role of the CD40-CD40L interaction (30–32) in the induction of an allergen-specific IgE response and also in T cell-dependent B cell responses in general, as the induction of an IgM and IgG response to rPhl p 5 was also inhibited. CTLA4Ig, when given alone at the time of sensitization with an allergen, did not effectively prevent a primary IgE response while dampening allergen-specific T cell reactivity to some degree. Several studies have suggested that CD28 blockade through CTLA4Ig can have distinct effects on Th1 vs Th2 re- sponses, but results differ depending on the specific experimental system investigated (13). In several models Th2 responses were less susceptible to CTLA4Ig treatment (33–35), which is compat-ible with our observation that in the presented allergy model a Th2-driven humoral response was not blocked by CTLA4Ig.

Interestingly, CTLA4Ig and anti-CD154 failed to inhibit sec-ondary IgE responses and IgE-mediated allergen-specific degran-ulation of basophils in mice with an already established allergy even though T cell responses had been strongly suppressed (as revealed by the results of the proliferation assays). Our data thus suggest that established secondary IgE responses are difficult to control via T cell-based therapeutic approaches. In fact, it is well established that the priming of naive T cells during sensitization is crucial to provide help for B cell differentiation, but the role of T cells in the secondary immune responses of memory B cells is a matter of controversy (36–40). Previous studies in mice by using T cell-depleting anti-CD4 Abs suggested that memory B cell per-sistence needs little or no Ag-mediated T cell help (36). Also, the influence of T cells on the activation of memory B cells has re-cently been investigated. Using a model of T and B cell deficient RAG−/− mice for adoptive transfer experiments, the activation of virus-specific memory B cells to secrete IgG was shown to be independent of cognate or bystander T cell help (37).

The lack of effect of costimulation blockade on secondary IgE responses and IgE-mediated allergic responses in already sensi-tized mice does not contradict earlier studies showing that costi-mulation blockade can suppress T cell-mediated allergic inflam-mation, because several reports have provided convincing evidence for the coexistence of IgE-mediated as well as IgE-inde-pendent, T cell-mediated reactions in allergic patients (4, 11). This may also explain why attempts to treat allergic symptoms by down-regulating allergen-specific T cell responses in asthmatic pa-tients using allergen-derived T cell peptides led to controversial results (41, 42).

In fact, the blockade of the CD28/CTLA4-CD80/CD86 pathway could block allergen-induced peripheral blood T cell proliferation as well as the IL-5 production of asthmatic subjects (43) and abrogate airway hyperresponsiveness in a murine model of allergic asthma (44). Furthermore, it has been demonstrated that the aero-sol-induced effector functions of memory T cells could be inhib-ited by blocking the function of the B7-1 or B7-2 ligands (45–47). As a consequence, the blockade of costimulation at the time of allergen exposure has been proposed as a potential strategy for the treatment of atopic disease (48).

Costimulation blockers exert their effects through several mech-anisms. Both anti-CD154 and CTLA4Ig have been shown to be able to induce anergy, deletion, and regulation (24, 49). The contribu-tion of each of these mechanisms in vivo depends on the specif-ics of the model used (13). It is likely that regulation was in-duced by anti-CD154 mAb and CTLA4Ig in the present study but, if so, it was not sufficient to suppress the secondary IgE response.

We think that the differential effects of costimulation blockade on allergic inflammation can be explained by the existence of at least two pathomechanisms, one involving IgE-mediated allergic inflammation that seems to be less susceptible to T cell-mediated control, and the other involving non-IgE-mediated but T cell-de-pendent allergic inflammation, a mechanism that can be controlled by T cell epitope-derived peptides, anti-CD4 Abs, cyclosporine, and perhaps regulatory T cells (4, 11). In addition, there may be a link between the latter two mechanisms because it has been shown that T cell activation in allergic patients can be regulated by IgE-facilitated Ag presentation (8).

Our data therefore suggest that it will be difficult to treat all facets of established allergic disease using strategies focusing only on the induction of T cell tolerance. It will rather be necessary to regulate established allergic immune responses by using strategies that actively antagonize the established immune response (e.g., regulatory immune responses, shifting the balance toward Th1, or the induction of counterimmune responses) (11, 50). The dissec-tion of the various pathways and their importance for allergic inflam-mation as well as the determination of their sensitivity for various immunological treatment strategies should conceivably re-sult in the selection of optimal immunological strategies for the treatment of allergic diseases.

References


