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Effects of Specific Immunotherapy on the B7 Family of Costimulatory Molecules in Allergic Inflammation

Stefania Piconi, † Daria Trabattoni, † Marina Saresella, ‡ Enrico Iemoli, † Monica Schenal, † Alessandra Fusi, † Manuela Borelli, † Lieping Chen, § Ambra Mascheri, † and Mario Clerici ††

The effect of allergen-specific immunotherapy (IT) on Ag presentation and T lymphocyte stimulation was evaluated by verifying the expression of costimulatory molecules in allergic patients. Thus, CD28 and CTLA-4, B7, and B7-H molecules on immune cells, as well as cytokine production, were analyzed in and out of the pollingination period in 30 patients allergic to Betulaeae that had or had not undergone specific IT. Results showed that IT attenuated the increase in the percentage of CD28+ CD4 T cells and the decrease in the percentage of CTLA-4+ CD4+ T cells seen in untreated individuals. CD19+ /CD80, CD19+ /CD86+, and CD14+ /CD80+ APCs were significantly augmented during pollination in unvaccinated individuals. B7-H1-expressing monocytes (CD14+) and B lymphocytes (CD19) as well as CD14 and CD19 B7-H1+ /IL-10+ APC were augmented in Betulaeae Ag-stimulated cultures of vaccinated patients independently of pollination, and were further increased in these individuals during pollination. As a result, the IL-10-IFN-γ ratio in CD4+, CD14+, and CD19+ cells increased in vaccinated patients, but decreased in unvaccinated individuals during pollination. These data clarify the cellular and molecular basis underlying the recent observation that peripheral expansion of IL-10-producing cells is associated with successful IT. B7-H1 could be an optimal target for IT of allergic diseases using mAbs. The Journal of Immunology, 2007, 178: 1931–1937.

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2 This work was supported by grants from Istituto Superiore di Sanita’ "Programma Nazionale di Ricerca sull’ AIDS"; Centro di Eccellenza per Biomolecular Interdisciplinary Studies and Industrial Applications; the European Microbicides Project and AIDS Vaccine Integrated Project, European Community WP6 Projects; the Japan Health Science Foundation; and the "Fondo Incentivazione Ricerca di Base" Ministero dell’Istruzione dell’Università e della Ricerca.

3 Abbreviation used in this paper: IT, immunotherapy.

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formed about the aim of the trial and gave their consent. Luigi Sacco Hospital approved the study protocol; all patients were in-
cough, wheezing, asthma, and dyspnea. The Institutional Review Board of
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ing to a 0 –3 grading system (0, no symptoms; 1, mild symptoms; 2, mod-
the pollen season. To better understand the role of IT in inducing allergen-specific
tolerance, we analyzed the expression of a panel of costimulatory molecules and activation markers in allergic patients undergoing or not undergoing IT. In particular, to allow us to analyze the immune correlates of successful IT, we selected, a priori, patients who responded positively to IT. These parameters were analyzed in the same individuals before and during pollen season.

Materials and Methods

Study population

Thirty Italian patients were enrolled between September 2004 and January 2005 at the Allergy and Clinical Immunology Clinic of the Luigi Sacco Hospital (Milan, Italy). Inclusion criteria were a clinical history suggesting birch sensitization, age between 18 and 50 years, skin prick test positive to birch pollen, and clinical report of asthma and/or rhinoconjunctivitis. Exclusion criteria were a previous IT treatment, clinical sensitization to other inhalant allergens, severe atopic dermatitis, other severe acute or chronic diseases, and pregnancy. In vivo diagnosis was done by a skin prick test using a standard birch extract (Betula alba, 100 index of reactivity/ml, Alyostal; Stallergenes). Prick tests were scored positive if the wheal obtained was equal or larger than the wheal elicited by the positive control (histamine hydrochloride, 10 mg/ml). The patients were subdivided in two groups: group A, patients undergoing symptomatic therapy alone and who had never received IT (n = 16); group B, patients who had been undergoing efficacious specific sublingual IT for at least 2 years (Stalolar 300; Stallergenes) (n = 14). Patients were instructed to keep a diary during the pollen season for the daily evaluation of the intensity of symptoms according to a 0–3 grading system (0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms). Monitored symptoms were itching, tear flow, and redness of the eyes; sneezing, running and blocked nose; cough, wheezing, asthma, and dyspnea. The Institutional Review Board of Luigi Sacco Hospital approved the study protocol; all patients were informed about the aim of the trial and gave their consent.

Pollen counts

Birch pollen count for the 2005 season was obtained from Milan 3 Station Lofarma; the counts are shown in Fig. 1.

Blood sample collection

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (BD Biosciences). PBMCs were separated on lymphocyte separa-
tion medium (Organon Teknika), washed twice in PBS, and the number of viable leukocytes was determined by trypan blue exclusion and a hemocytometer. The samples were kept at room temperature and used within 12 h. Blood samples were obtained from all patients out of pollen season (January 2005) and at the peak of pollination (first decade of April 2005), when the patients were symptomatic.

Lymphocyte proliferation

PBMC (3 × 10^6) were placed in round-bottom wells of a microtiter culture plate (Costar) in a final volume of 0.2 ml along with: 1) no stimulation (BASAL); 2) major birch pollen allergen (Bet-v1, 1 μg/ml; provided by Dr. F. Frati, Stallergenes, Milan, Italy) (ALLE); and 3) CMV grade II Ag

![FIGURE 1. Birch pollen count (granules/mm³) for the 2005 season obtained from the Milano 3 Station.](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Symptoms</th>
<th>Months in Immunotherapy</th>
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<td>50</td>
<td>M</td>
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<td>RC+A</td>
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<td>F</td>
<td>RC+A</td>
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<td>M</td>
<td>RC+A</td>
<td>40</td>
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<td>41</td>
<td>M</td>
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<td>35</td>
<td>F</td>
<td>RC+A</td>
<td>46</td>
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<td>27</td>
<td>F</td>
<td>RC+A</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>32</td>
<td>F</td>
<td>RC+A</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup> R, Rhinitis; RC, rhinoconjunctivitis; A, asthma; nd, not done.
Incorporated [3H]thymidine was measured with a liquid scintillation beta counter anti-mouse B7-H3 (2.5 μg/ml). PBMCs were washed and fixed in Reagent A solution (FIX & PERM Cell Permeabilization kits), with mAbs specific for different cytokines (IL-10 PE, IFN-γ FITC, and IL-4 PE). After a 30-min incubation at 4°C in the dark, the cells were washed and fixed in 1% paraformaldehyde in PBS.

**Monoclonal Abs**

The following mAbs were used in this study: anti-human CD4 (mouse IgG2a isotype), anti-human CD14 (mouse IgG1 isotype), anti-human CD19 (mouse IgG1 isotype) coupled to R-PE-Cy5 (Caltag Laboratories); anti-human CD86 (mouse IgG1 isotype), anti-human CD28 (mouse IgG1 isotype) coupled to R-PE (Serootech); anti-human CD80 (mouse IgG1 isotype; Serotech) and anti-human CTLA-4 (mouse IgG2b; R&D Systems) coupled to FITC; anti-human B7-H1 (mouse IgG1 isotype; provided by Dr. L. Chen) and anti-mouse IgG (H+L) coupled to FITC (eBioscience); anti-mouse B7-H3 (hamster IgG isotype; provided by Dr. L. Chen) and anti-hamster IgG coupled to PE (BD Pharmingen). The intracellular molecule detection mAbs used were anti-human IL-10 (mouse IgG1 isotype; Caltag Laboratories), coupled to PE and anti-human IFN-γ (mouse IgG1 isotype; Caltag Laboratories) coupled to FITC.

**Cytometric analysis**

The cytometric analyses of phenotype and cytokine-secreting lymphocytes were performed using an EPICS XL flow cytometer (Beckman Coulter) equipped with a single 15 mW argon ion laser operating at 488 nm interfaced with 486 DX2 IBM computer. For each analysis, 20,000 events were acquired and gated on CD4 (or CD14, CD19) expression and side scatter properties. Green fluorescence from FITC (FL1) was collected through a 525-nm bandpass filter, orange-red fluorescence from R-PE (FL2) was collected through a 575-nm bandpass filter and deep-red fluorescence from PE-Cy5 (FL4) was collected through a 670-nm bandpass filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1, FL2, and FL4. Samples were first run using isotype control or single fluorochrome-stained preparations for color compensation.

**Statistical analysis**

Procedures were based on nonparametric analyses. Comparisons between the different groups were made using a two-tailed Mann-Whitney U test performed for independent samples. Possible relationships were evaluated using a Pearson’s correlation test. Statistical analysis was performed using the SPSS statistical package.

**Results**

**Patients**

Clinical, epidemiological, and therapeutic characteristics of all the patients enrolled in the study are presented in Table I. Sixteen patients (9 females and 7 males) were included in group A; 14 individuals (8 females and 6 males) who successfully underwent IT were included in group B. Immunological evaluations were performed for each condition. Pooled human AB serum (10%) was added to each well. After 6 days of incubation at 37°C and 7% CO₂, cultures were pulsed with 1 μCi [3H]thymidine and harvested 18 h later. Incorporated [3H]thymidine was measured with a liquid scintillation beta counter. Results were expressed as stimulation indexes; ratio of mean counts obtained in the presence of Ag to mean counts obtained without Ag.

**Immunophenotypic analyses**

Lymphocyte subsets were evaluated by flow cytometric analysis, using 50 μl of EDTA peripheral blood incubated for 30 min at 4°C with fluorochrome-labeled mAbs (CD4 R-PE-Cy5; CD14 PE-Cy5; CD19 PE-Cy5; CD28 PE; CD80 FITC; CD86 PE; CTLA-4 FITC). After incubation, erythrocyte lysis and fixation of marked cells was performed using the Immuno-Prep EPICS kit (Coulter Electronics) and Q-prep Work Station (Coulter Electronics).

**Stimulation of PBMC for FACS analysis**

A total of 5 x 10⁶ PBMCs were incubated for 18 h without any Ag or in the presence of ALLE or CMV at the doses specified above. Anti-CD28 Ab (clone 37407.111; R&D Systems) was added during incubation (1 μg/well) to facilitate costimulation. For cytokine analyses, 10 μg/ml Brefeldin A (Sigma-Aldrich) was added to the cell cultures during the last 6 h of stimulation to block protein secretion.

**Immunofluorescent staining**

PBMC were washed in PBS, split in different flow cytometry tubes, and stained with CD4, CD14, CD19, CD80, or CD86 mAbs for 30 min at 4°C in the dark. For indirect immunofluorescence staining, PBMC were first incubated with a mouse anti-human B7-H1 Ab (2.5 μg/ml) or with a hamster anti-mouse B7-H3 (2.5 μg/ml) (gifts of Dr. L. Chen). After 30 min at 4°C, the cells were washed and further incubated for 30 min at 4°C with a rat anti-mouse IgG (H+L) Ab conjugated with FITC (1 μg/ml) or with a mouse anti-hamster IgG conjugated with PE (1 μg/ml). For the analysis of cytokine-secreting cells, after phenotypic staining, PBMCs were washed and fixed in Reagent A solution (FIX & PERM Cell Permeabilization kits; Caltag Laboratories) for 10 min at room temperature in the dark. The cells were washed in PBS and resuspended in reagent B (FIX & PERM Cell Permeabilization kits), with mAbs specific for different cytokines (IL-10 PE, IFN-γ FITC, and IL-4 PE). After a 30-min incubation at 4°C in the dark, the cells were washed and fixed in 1% paraformaldehyde in PBS.

**Table II. Clinical score of patients enrolled in the study**

<table>
<thead>
<tr>
<th>Group</th>
<th>Location</th>
<th>No Symptoms (%)</th>
<th>Mild Symptoms (%)</th>
<th>Moderate Symptoms (%)</th>
<th>Severe Symptoms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Eyes</td>
<td>0/16 (0)</td>
<td>3/16 (19)</td>
<td>8/16 (50)</td>
<td>5/16 (31)</td>
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<tr>
<td></td>
<td>Nose</td>
<td>0/16 (0)</td>
<td>1/16 (6)</td>
<td>7/16 (44)</td>
<td>8/16 (50)</td>
</tr>
<tr>
<td></td>
<td>Chest</td>
<td>6/16 (37)</td>
<td>3/16 (19)</td>
<td>4/16 (25)</td>
<td>3/16 (19)</td>
</tr>
<tr>
<td>B</td>
<td>Eyes</td>
<td>10/14 (72)</td>
<td>3/14 (21)</td>
<td>1/14 (7)</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td></td>
<td>Nose</td>
<td>10/14 (72)</td>
<td>3/14 (21)</td>
<td>1/14 (7)</td>
<td>0/14 (0)</td>
</tr>
<tr>
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<td>Chest</td>
<td>11/14 (79)</td>
<td>3/14 (21)</td>
<td>0/14 (0)</td>
<td>0/14 (0)</td>
</tr>
</tbody>
</table>

* 0, No symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms.
performed before the initiation of symptomatic therapy. Clinical differences between group A and group B during the 2005 birch pollen season are shown in Table II. The efficacy of IT in group B was evaluated by a significant improvement of a 7-point visual analog scale applied to rhinitis, asthma, and conjunctivitis and need for therapy, according the guidelines of symptom severity assessment of allergic rhinitis (29). The numbers of CD4⁺, CD8⁺, CD14⁺, and CD19⁺ cells was comparable among both groups of patients at both time points.

Lymphocyte proliferation

T cell proliferation in response to Bet-v1 and CMV Ag was analyzed outside of the pollen season or at the peak of pollination in allergic patients who were only receiving symptomatic therapy (group A), or were undergoing allergen specific IT (group B). Bet-v1-specific T cell proliferation was comparable in the two groups of patients but was significantly reduced in unvaccinated individuals during pollination (in season vs out of season: $p = 0.023$)
In contrast, CMV-specific responses were reduced in both groups of patients during pollination (in season vs out of season: unvaccinated patients: \( p = 0.021 \), vaccinated individuals: \( p = 0.034 \)).

**Phenotypic analysis: CD28 and CTLA-4 expression on T lymphocytes**

CD28 is mainly an activating costimulatory molecule while CTLA-4 is an inhibitory costimulatory molecule. Activation markers (CD28 and CTLA-4) were evaluated on CD4\(^+\) T lymphocytes of all patients. CD28-expressing CD4\(^+\) T lymphocytes were increased (\( p = 0.007 \)), whereas CTLA-4-expressing CD4\(^+\) T cells were reduced (\( p = 0.031 \)) in unvaccinated patients during pollination (Fig. 3). In contrast, both lymphocyte subpopulations were similar over time in vaccinated individuals. These results indicate that a tendency favoring T cell activation is present in unvaccinated individuals during pollination.

**Phenotypic analysis: B7 family molecules expression on APC**

CD19\(^+\)/CD80\(^+\) (\( p = 0.042 \)), CD19\(^+\)/CD86\(^+\) (\( p = 0.006 \)), and CD14\(^+\)/CD80\(^+\) cells were increased (\( p < 0.001 \)) whereas
CD14⁺/CD86⁺ cells (p = 0.012) were diminished during pollination in unvaccinated patients. No significant modification was detected in vaccinated patients, suggesting that vaccination is associated with a stabilization of these populations of APC (data not shown).

**Allergen-induced modulation of phenotypic markers**

PBMC from all patients were incubated with Bet-v1 to evaluate the specific allergen-induced modulation of costimulatory molecules on B cells and monocytes. Results showed that incubation with Bet-v1 modifies the percentage of CD14⁺/CD86⁺ cells in samples collected during pollination. Thus, similar to results obtained ex vivo, CD14⁺/CD86⁺ cells were diminished (p = 0.022) in vaccinated and augmented (p = 0.047) in unvaccinated patients (data not shown).

Changes observed in B7-H1 and B7-H3 expression were more dramatic. During pollination, in vaccinated individuals CD19⁺/B7-H1⁺ (p = 0.041), CD14⁺/B7-H1⁺ (p = 0.033), and CD14⁺/B7-H3⁺ cells augmented whereas CD19⁺/B7-H3⁺ cells were reduced. These patterns mirrored those observed in unvaccinated individuals (Fig. 4) and were specific for Bet-v1 stimulation as these populations were not modified by incubation of PBMC with CMV (Fig. 5).

**Allergen-stimulated cytokine synthesis**

B7-H1-expressing cells preferentially produce IL-10; data presented herein show that B7-H1-expressing APC augment in vaccinated individuals during pollination. To verify whether these changes would be reflected in cytokine production, we analyzed the percentages of B7-H1-expressing and IL-10-producing CD14⁺ and CD19⁺ Bet-v1-stimulated cells. Results showed that CD14⁺/B7-H1⁺/IL-10⁺ as well as CD19⁺/B7-H1⁺/IL-10⁺ cells were increased (>2.5-fold) in vaccinated patients during pollination. As a result, during pollination, the IL-10/IFN-γ ratio in CD4⁺, CD14⁺, and CD19⁺ cells increased in vaccinated patients but decreased secondarily to a reduction in IL-10 production in unvaccinated individuals (Fig. 6). Again, changes in cytokine production were specific for Bet-v1 stimulation as none of these populations were modified by incubation of PBMC with CMV (data not shown). These data suggest that one of the main biological effects of specific IT is to up-regulate an APC-dependent and IL-10-mediated loop.

**Discussion**

In this study, we evaluated the biological effects of allergen-specific IT on Ag presentation and T lymphocyte stimulation. Thus, we analyzed the expression of CD28, CTLA-4, as well as B7 and B7-H molecules on immune cells in patients with allergy to *Betulaeae* that had or had nor undergone specific IT. These parameters were examined twice in the same individuals: in and out of the pollination period. Results shown herein demonstrate that IT has a stabilizing effect on the expression of costimulatory molecules and elicits an APC-dependent and IL-10-mediated loop at least partially responsible for disease control during pollination.

Proliferation to both Bet-v1 and an unrelated Ag (CMV) was reduced during pollination in unvaccinated patients, but was only slightly modified in vaccinated individuals. These data could be explained by the prevalence, in unvaccinated individuals, of Th2 clones of Th lymphocytes, clones that are characterized by a limited proliferative ability (30). The observation that successful IT is associated with a modulation of the Th2/Th1 imbalance, such that Th1 are favored, would justify the observation that proliferation is retained in vaccinated individuals. CD28 and CTLA-4 molecules are expressed on Th lymphocytes, bind the B7 receptors (CD80 and CD86) and elicit a stimulatory or an inhibitory effect on T cell activation, respectively. CD28 is constitutively expressed on cell surfaces whereas CTLA-4 is only present on activated lymphocytes. The reciprocal balance of CD28 and CTLA-4 is pivotal in modulating T cell responses. Expression of these markers on CD4⁺ T lymphocytes was comparable both out and in season in vaccinated individuals, suggesting that one of the effects of IT is to stabilize the reciprocal balance of these costimulatory molecules. In contrast with these results, CD4⁺/CD86⁺ cells were augmented and CD4⁺/CTLA-4⁺ lymphocytes were reduced during pollination in unvaccinated individuals, indicating that the immune response is less prone to suppression in these patients during pollination. These data are apparently in disagreement with the observed loss of Ag-specific proliferation detected in unvaccinated individuals during pollination. Ligation of CTLA-4 inhibits Ag-specific Th2 lymphocytes clones. Thus, the reduced proliferative ability detected in unvaccinated subjects could be explained by an increased activity of the Th2 Bet-v1-specific clones, which are endowed with a minimal proliferative capacity, and would avoid CTLA-4-mediated suppression (8).

To complete this part of the study, we analyzed B7.1 (CD80) and B7.2 (CD86)-expressing monocytes (CD14) and B lymphocytes (CD19) in all patients. The expression of these molecules was comparable out and in season in vaccinated individuals, confirming that IT stabilizes the CD28/CTLA-4⁺CD80/CD86⁺ T lymphocyte immunomodulatory circuit. In contrast, results obtained in unvaccinated individuals showed that CD19⁺/CD80⁺ and CD14⁺/CD80⁺, as well as CD19⁺/CD86⁺ cells are significantly increased during pollination. These results indicate that an increased susceptibility of the immune system to activation is present in unvaccinated patients during pollination (31, 32); specific IT down-modulates this activation loop.

B7-H1 and B7-H3 are additional co stimulatory molecules that have recently been identified; legation of B7-H1-expressing APCs results in the preferential production of IL-10, whereas triggering of B7-H3-expressing APC is associated with IFN-γ production. To evaluate changes in Ag-specific responses, we incubated PBMC of all individuals with Bet-v1, the main *Betulaeae* Ag, or a control Ag. Results showed that both CD14⁺/B7-H1⁺ and CD19/B7-H1 cells were increased in vaccinated individuals irrespective of pollination. Interestingly, whereas these cells diminished in unvaccinated subjects during pollination, they showed a further increase in patients undergoing allergen-specific IT. Thus, both types of B7-H1-expressing APC increased only in vaccinated patient whereas these cell types decreased in unvaccinated individuals. Data obtained by analyzing B7-H3-expressing cells were less univocal and a clear pattern could not be identified. This outcome, which needs to be further analyzed, could be a result of the weak pollinosis season that characterized the spring of 2005 in Lombardy.

The results shown herein suggest that the beneficial effect of allergen-specific IT is at least partially secondary to the increase of B7-H1-expressing cells. These data confirm and expand those recently reported by Kim et al. (33) who showed a pivotal role for B7-H1 in the modulation of delayed-type contact hypersensitivity response in primed animals. Thus, in this model: 1) blockade of B7-H1 enhanced the activity of hapten-specific T cells; 2) interaction with a dendritic cell line that expresses high cell surface levels of B7-H1 suppressed the proliferation of activated T cells; and 3) in vivo administration of hapten-carrying B7-H1/DC desensitized the response of sensitized animals to hapten challenge.

Ligation of B7-H1 results in IL-10 production. CD14⁺ and CD19, B7-H1⁺ and B7-H1⁺/IL-10⁺ cells were decreased during pollination in unvaccinated patients and, as a likely consequence, the Ag-specific IL-10/IFN-γ ratio was diminished in these patients.
during pollination. The observation that modifications of the IL-10–IFN-γ ratio were supported by decreases in IL-10 production, rather than by changes in the generation of IFN-γ suggests that one of the main biological effects of specific IT could be to up-regulate the main biological effects of specific IT.

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Disclosures

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References


