

CLINICAL REPORT

Expression and Functional Role of Co-stimulatory Molecules in CD40 + IL-4-Stimulated B Cells from Atopic and Non-atopic Donors

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As immunological dysregulation is a possible key defect in atopic diseases, we have studied the expression and function of costimulatory molecules in atopic dermatitis (AD) patients compared with normal controls. Using flow cytometry, we showed that CD80 and CD86 are expressed at increased levels on human peripheral B cells in both groups after stimulation with anti-CD40 and interleukin 4 (IL-4), but to a significantly higher extent in the AD group. Furthermore, baseline expression of CD80 and CD86 on peripheral B cells was low in normal donors and increased in AD donors. To study the functional role of the costimulatory molecules in CD40 + IL-4-stimulated peripheral mononuclear cells from normal and atopic donors, proliferation and IgE production were analysed in the presence of antibodies against the receptors of the costimulatory molecules. In the presence of either anti-CD28 or anti-CTLA-4, cell proliferation and IgE synthesis were significantly enhanced in the atopic group in anti-CD40 + IL-4-stimulated peripheral mononuclear cells. These findings suggest that interaction of CD80 and CD86 with their receptors CD28 and CTLA-4 selectively promotes cell activation, including proliferation and IgE production in CD40 + IL-4-stimulated peripheral blood mononuclear cells from atopic donors. It remains to be elucidated whether these changes are primary, based on the genetic background of atopics, or whether they are induced secondarily in the context of atopic pathology. **Key words:** co-stimulatory molecules; atopic dermatitis; IgE-production.

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In recent years, major advances have been made in the understanding of the regulation of IgE synthesis, which represents a key event in the induction of immediate-type allergic diseases. A dysregulation of this process is also thought to constitute the major pathology in genetically transmitted atopic diseases.

For the induction of the IgE isotype switch, stringent requirements have been elaborated, namely an interaction of CD40 with its ligand (CD40L), resulting in B-cell activation and, in conjunction with interleukin-4 (IL-4), in IgE production by these cells (1, 2). Furthermore, the expression of major co-stimulatory molecules, like CD80 and CD86, not only plays an important modulatory role in this process but

these molecules have also been shown to be upregulated in human B cells in the presence of CD40 and IL-4 (3, 4).

The co-stimulatory molecules CD80 and CD86 are not only expressed on B cells, but also on monocytes, macrophages and dendritic cells (5). Although CD80 is not expressed by resting B cells, it is upregulated on these cells by many molecules, including CD40 and cytokines such as IL-2 and IL-4 (6). For CD86, low-level expression is already detectable in unstimulated B cells and various cytokines, such as IL-5, IL-4, interferon-gamma (IFN- γ), granulocyte/macrophage colony-stimulating factor (GM-CSF) and, to a lesser extent, IL-2, upregulate its expression (6).

Both CD80 and CD86 interact with their receptors on activated T cells, namely with CTLA-4 and CD28 (7). The interaction of the co-stimulatory molecules with their receptors has been implicated as an important event during T cell activation and clonal expansion (8). Activation of T cells via the T cell receptor and anti-CD28 results not only in proliferation of the cells, with concomitant IL-2 production, but also in upregulation of IL-4, IL-5, IL-13, IFN- γ , tumour necrosis factor-alpha (TNF- α) and GM-CSF production (9, 10). Furthermore, expression of CD40L is upregulated by anti-CD28 in human T cells (11).

CTLA-4, however, is only expressed on activated T cells and stimulation via this molecule results in inhibition of T cell proliferation and IL-2 production and the induction of apoptosis, indicating a negative regulatory function of CTLA-4 (12). This has been confirmed by studies showing that CTLA-4-deficient mice suffer from a lethal lymphoproliferative disease (13). Because of the known dysregulation of IgE synthesis in patients with atopic dermatitis (AD), we reasoned that a key to the understanding of this condition might be found in alterations of the regulation of the co-stimulatory molecules associated with this process. We therefore studied the expression of CD80 and CD86, as well as human leucocyte antigen (HLA)-DR, on purified peripheral B cells from normal and atopic donors. Furthermore, we examined the effect of these molecules on proliferation and immunoglobulin synthesis during interaction with CD28 and CTLA-4 monoclonal antibodies in stimulated peripheral blood mononuclear cells (PBMC) co-stimulated with anti-CD40 and IL-4. We show that CD80 and CD86 are expressed at increased levels in both groups on peripheral B cells after stimulation with anti-CD40 and IL-4 and that, in the presence of anti-CD28 and anti-CTLA-4, proliferation and IgE synthesis of PBMC are particularly enhanced in the AD group. This suggests that interaction of the co-stimulatory molecules with their receptors CD28 and CTLA-4 promotes cell activation in CD40 + IL-4-stimulated PBMC from donors with AD.

MATERIALS AND METHODS

Cells

PBMC were isolated from peripheral heparinized blood of 6 non-allergic healthy donors (controls with a negative history of allergic diseases, negative skin prick test against the most common inhalant allergens and total serum IgE level <100 KU/ml) and 6 allergic donors suffering from severe AD proven by the criteria of Hanifin & Raika (14) (Severity Scoring of Atopic Dermatitis [SCORAD] score >60 points and total IgE serum levels >2000 KU/ml) after informed consent, using Ficoll-hypaque separation (1400 rpm, 30 min, room temperature). As previously described (15), B cells were purified using anti-CD19-coupled magnetic beads (Dianova, Hamburg, Germany). Briefly, cells and antibody-conjugated beads (1:5 ratio) were incubated for 1 h on ice, CD19-positive B cells were then selected by magnetic separation and, after several washes, the cells were resuspended in medium and cultured overnight at 37°C. On the following morning, cells were separated from the beads after several washes and counted. The purified cell population contained >95% B cells, as assessed by immunofluorescence with anti-CD20 antibody. In previous studies, this procedure has been shown not to activate B cells (16).

PBMC (10^5 per well) were cultured for 3 days for proliferation and 10 days for IgE assays. The culture medium RPMI 1640 was supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% heat-inactivated fetal calf serum (all from Biochrom KG, Berlin, Germany). All cell cultures were performed at 37°C in humidified air and 5% CO₂.

Immunofluorescence staining

Expression of the costimulatory molecules by purified human B cells was monitored by measuring immunofluorescence on a flow cytometer using phycoerythrin-conjugated monoclonal antibodies directed against human CD80 and CD86 (Pharmingen, Hamburg, Germany). As time-course analysis showed maximal expression of CD80 and CD86 after 48 h this timepoint was applied for further analysis. For fluorescent staining, 5×10^5 cells were suspended in 90 µl staining buffer (2% bovine serum albumin [BSA] in phosphate-buffered saline [PBS], pH 7.4, 0.1% sodium azide) and 10 µl of phycoerythrin-conjugated monoclonal antibodies was added, followed by incubation for 45 min on ice. After washing the cells in PBS, they were fixed in 2% paraformaldehyde and analysed within 24 h with a Coulter counter. The appropriate isotype controls were done for each staining in parallel (purchased from Pharmingen). For double staining, PBMC were additionally stained with anti-CD20 FITC (Dianova).

Reagents

IL-4 was purchased from Pharmingen and purified anti-CD40 monoclonal antibody (clone 626.1) was a kind gift from Raif Geha (Children's Hospital, Boston, MA, USA). The anti-CTLA-4 and anti-CD28 antibodies were purchased from Pharmingen. For control experiments the isotype-matched IgG1 mouse antibody (MOPC21 from Sigma, Dreieich, Germany) was used.

Proliferation assay

For assessment of proliferation, radioactive thymidine (0.5 µCi per well) was added during the last 16 h of culture. Cells were harvested on filter paper and thymidine incorporation was measured using liquid scintillation spectroscopy. All experiments were done in triplicate and data expressed as arithmetic means.

Immunoglobulin production

Cells were cultured for 10 days and immunoglobulins were measured in the supernatants of stimulated cells by enzyme-linked immuno-

sorbent assay (ELISA). The monoclonal antibodies for IgE detection (clones HP 6061 and HP 6029) were kindly provided by Robert Hamilton (Johns Hopkins University, Baltimore, MD, USA). The antibodies for IgA, IgG and IgM ELISA (anti-IgA, anti-IgG and anti-IgM) were purchased from Dianova.

Briefly, for the immunoglobulin assays, immuno plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with anti-human Ig-Fc antibodies and diluted in 0.1 M bicarbonate buffer. The antibody-coated wells were blocked for 1 h with 2% BSA buffer. After several washes, supernatants and internal standards were incubated in duplicate overnight. On the next morning, after several washes, the second alkaline phosphatase-conjugated anti-Ig (A, G, M) antibody was added. Because the second anti-IgE monoclonal antibody was biotinylated, cells were incubated for another hour with alkaline phosphatase-conjugated streptavidin. Following the final reaction with phosphatase substrate (Sigma), plates were read in a microplate ELISA reader at 410 nm and the amount of Ig was calculated according to the standard curve. The variations of readings in duplicate cultures never exceeded 15%.

Statistical analysis

Statistical significance was assessed using the Wilcoxon signed rank test for paired data to compare the results from 1 group and the Mann-Whitney test for unpaired data to compare the different donor groups.

RESULTS

Increased expression of CD86 by human B cells from donors with AD after CD40+IL-4 stimulation

It has been shown recently that expression of CD86 is enhanced in patients with atopic diseases such as AD or allergic asthma (17, 18). We first tried to confirm these findings and studied the expression of CD86, and in addition of CD80, on purified B cells after stimulation with anti-CD40 and IL-4 in controls and donors with AD.

The results show that baseline expression of CD80 and CD86 (Fig. 1) on human peripheral B cells is slightly enhanced in the AD group (mean baseline after subtraction of the isotype control values for CD80 were 10% vs. 4% and for CD86 were 7% vs. 3% in normal donors). CD80 expression of unstimulated B cells ranged from 2–7% in the normal donor group with a modest upregulation (7–13%) after stimulation with IL-4 and/or anti-CD40. In the AD group upregulation of CD80 ranged from 15–21% after stimulation with IL-4 and/or anti-CD40.

The expression of CD86 was also upregulated on B cells in both groups. After stimulation with IL-4 and/or anti-CD40, a significantly higher upregulation ($p < 0.05$), compared with unstimulated cells, was observed after CD40+IL-4 stimulation in patients with AD, as is more readily evident when control values from both groups are set at 100% (Fig. 2).

To rule out the possibility that low baseline expression of the co-stimulatory molecules CD80 and CD86 was due to the purification procedure, resulting in a loss of surface molecule expression, HLA-DR expression by B cells was also studied in both groups. The data show strong HLA-DR expression in both groups (Fig. 1), with a higher baseline expression on purified B cells in the AD group (86%) compared with the control group (74%) ($p < 0.05$). After anti-CD40+IL-4 stimulation, HLA-DR expression was practically unchanged in the AD group (91%) and increased up to 88% in the

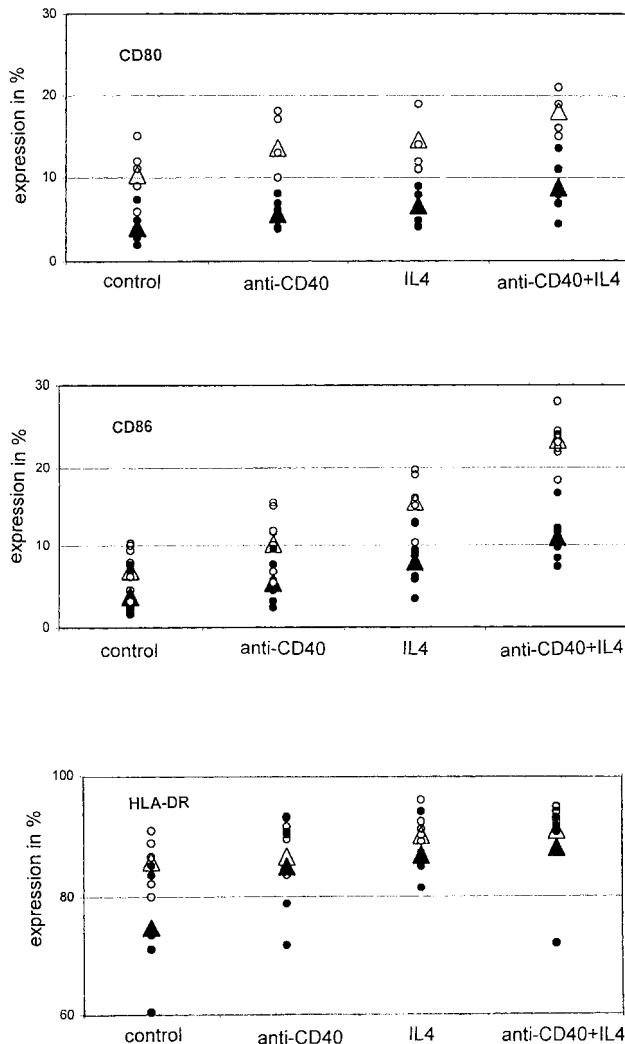


Fig. 1. Expression of CD80, CD86 and HLA-DR on human peripheral B cells from controls and patients with AD. Purified B cells from controls ($n=6$; ●) and patients with AD ($n=6$, ○) were cultured in the presence of anti-CD40 (1 $\mu\text{g/ml}$) and IL-4 (5 ng/ml) for 2 days. CD80, CD86 and HLA-DR expression was assessed by flow cytometric analysis and is expressed as percent positive cells (\blacktriangle , \triangle = mean expression) after subtracting the percentage of the appropriate isotype control.

control group ($p < 0.05$). Furthermore, examination of B cells in freshly isolated PBMC by double staining revealed comparable results of CD80, CD86 and HLA-DR expression for controls and donors with AD (data not shown).

Increased proliferation and IgE production of PBMC from donors with AD after CD40+IL-4 stimulation in the presence of anti-CD28

Binding of the co-stimulatory molecules CD80 and CD86 via their co-receptor CD28 results in T cell activation, including enhanced IL-4 production and CD40L expression, which in turn enhances B-cell activation (11). Therefore, we examined next whether addition of anti-CD28 to CD40+IL-4-stimulated PBMC resulted in different effects on proliferation and IgE synthesis of PBMC from controls and donors with AD. The data show (Fig. 3A) that addition of anti-CD28 resulted

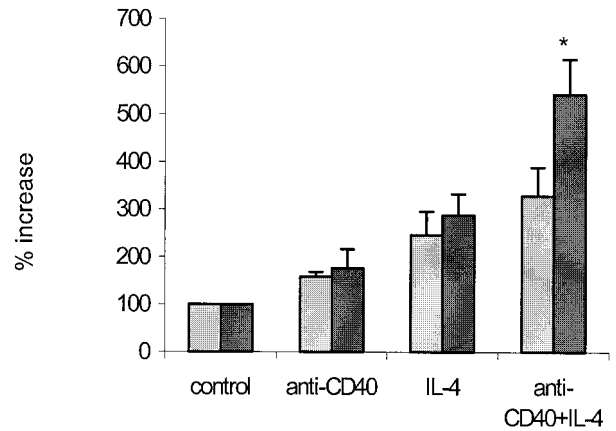


Fig. 2. Comparison of induction of CD86 expression from controls and patients with AD ($n=6$) on human peripheral B cells after stimulation with anti-CD40 and IL-4 alone or in combination. Control values are set at 100% in both groups. Increased expression of CD86 in patients with AD (dark bars) was statistically significant ($p < 0.05$) compared with controls (light bars).

in enhanced, dose-dependent proliferation of CD40+IL-4-stimulated PBMC in both groups (132% for controls, 148% for AD). The proliferative response of PBMC after CD40+IL-4 stimulation in the presence of anti-CD28 was, however, higher in the AD group ($p < 0.05$).

Because stimulation of PBMC with anti-CD40+IL-4 results not only in B-cell proliferation, but also in IgE synthesis, we examined next whether IgE production of CD40+IL-4-stimulated PBMC was also enhanced in the presence of anti-CD28. The results (Fig. 3B) show that IgE production of CD40+IL-4-stimulated PBMC was increased in the presence of anti-CD28 only in the AD group (140%) and not in normal donors (104%). This indicates that the presence of anti-CD28 enhances not only proliferation, but also IgE-synthesis, in CD40+IL-4-stimulated PBMC in patients with AD. In contrast, the production of other immunoglobulins, including IgA, IgG and IgM, was not altered in the presence of anti-CD28 in CD40+IL-4-stimulated PBMC (data not shown). Using an isotype-matched control antibody at the same concentrations affected neither proliferation nor IgE production of anti-CD40+IL-4-stimulated PBMC.

Increased PBMC proliferation and IgE production in the presence of anti-CTLA-4

The co-stimulatory molecules CD80 and CD86 exert their effects not only by binding to their common counter-receptor CD28, but also by binding to CTLA-4. We therefore studied the role of CTLA-4 engagement in CD40+IL-4-stimulated PBMC from controls and donors with AD by determining proliferation and IgE synthesis of CD40+IL-4-stimulated cells in the presence of anti-CTLA-4. The data (Fig. 4A) show that, in the presence of the blocking antibody anti-CTLA-4, CD40+IL-4-mediated proliferation was enhanced in both groups. As observed with anti-CD28, an increased proliferative response was determined in the AD (173%) compared to the control group (129%) ($p < 0.05$). This increase in PBMC proliferation was accompanied by an enhanced IgE synthesis only in the AD group and not in the control group (Fig. 4B).

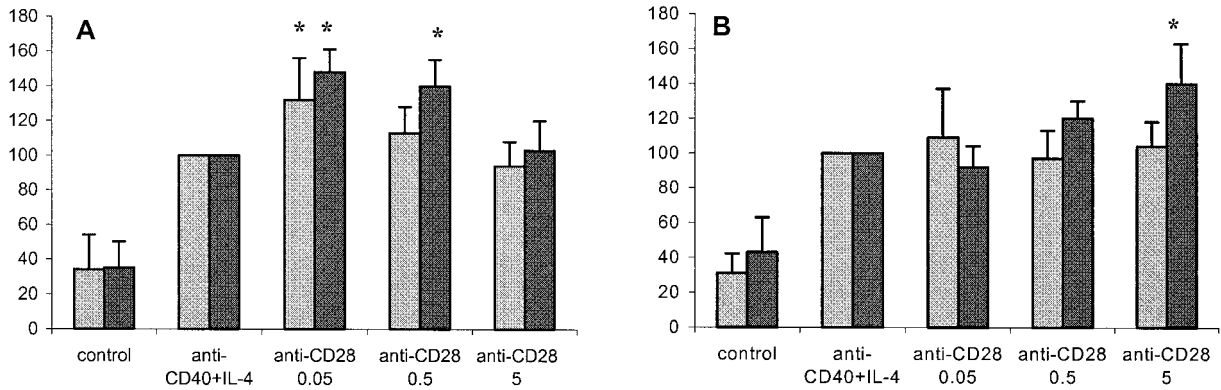


Fig. 3. Anti-CD28 enhances CD40+IL-4-mediated proliferation (A) and IgE production (B) of PBMC from controls ($n=6$; light bars) and patients with AD ($n=6$; dark bars). PBMC ($10^6/\text{ml}$) were incubated in the presence of anti-CD40 (1 $\mu\text{g}/\text{ml}$) and IL-4 (5 ng/ml) alone or in combination with anti-CD28 (0.05–5 $\mu\text{g}/\text{ml}$). Results are expressed as percentage change, with the anti-CD40+IL-4-stimulated data being set at 100%. CPM/well in unstimulated PBMC ranged in both groups from 813 to 4560. IgE production (ng/ml) ranged in the control group from 0.15 to 0.3 ng/ml and in the AD group from 0.3 to 22 ng/ml. Significant values are indicated by an asterisk.

The production of other immunoglobulins in CD40+IL-4-stimulated PBMC, including IgA, IgG and IgM, also remained unchanged in the presence of anti-CTLA-4 (data not shown). An isotype-matched control antibody at the same concentrations did not affect proliferation or IgE production of anti-CD40+IL-4-stimulated PBMC.

DISCUSSION

In the present study, we found enhanced baseline expression of CD80 and CD86 on purified human peripheral B cells from patients with AD compared with controls; CD80 and CD86 are upregulated after stimulation with anti-CD40+IL-4 in both groups, but significantly more so in patients with AD. In a previous study (17) a high baseline expression of CD86 was determined in B cells from freshly isolated peripheral blood from controls and patients with AD by double staining. However, these authors found a generally higher expression of CD86 on peripheral B cells: 53% mean expression of CD86 in the AD group; and 38% mean CD86 expression in the control group. To rule out the possibility that these differences may be due to the methodology employed, namely that baseline expression of CD80 and CD86 was determined after 48 h of culture rather than immediately after isolation, we also

performed double staining of freshly isolated PBMC, which revealed basically the same results for CD86 expression. However, we could also show an increased upregulation of CD86 in B cells from patients with AD, suggesting that this surface molecule is differentially expressed in patients suffering from allergic diseases. This has also been demonstrated recently in other studies, by showing that CD86 expression is increased on B cells in patients suffering from other atopic diseases, such as allergic asthma (18). The possible clinical significance of these findings is suggested by data showing that, in these patients, CD86-positive B cells produce increased amounts of IgE *in vitro* after CD40+IL-4 stimulation (17).

Expression of CD80 was, however, low in the control group and slightly enhanced in the AD group in unstimulated peripheral B cells and only modestly upregulated after CD40+IL-4 stimulation, in accordance with previous data (19), suggesting that CD86 rather than CD80 is of importance in the allergic immunological environment. These findings are also in agreement with data showing increased CD86 expression on dendritic cells in atopic skin and an upregulation of CD86 on B cells after antigen-specific stimulation in patients with AD (20).

In the present study, we also proved the interaction of

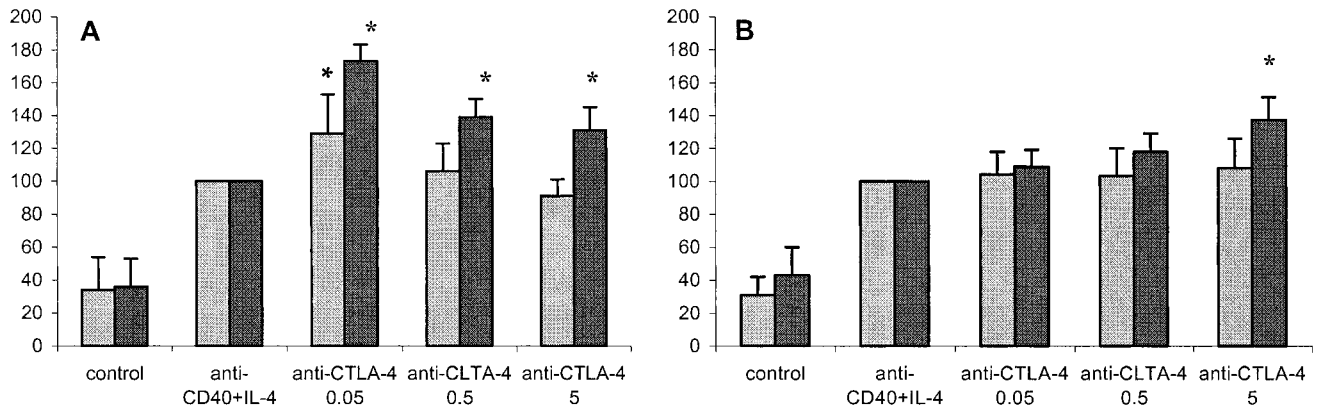


Fig. 4. Anti-CTLA-4-induced enhancement of CD40+IL-4-mediated proliferation (A) and IgE production (B) PBMC from controls ($n=6$; light bars) and patients with AD ($n=6$; dark bars). All assay details are as indicated in Fig. 3. Significant values are indicated by an asterisk.

CD80 and CD86 with their receptors CD28 and CTLA-4. Using monoclonal antibodies against these receptors, we could show that their interaction with CD80 and CD86 results in an enhanced proliferative response of CD40+IL-4-stimulated PBMC in controls and patients with AD. A statistically significant increase in IgE production after CD40+IL-4 stimulation in the presence of anti-CD28 or anti-CTLA-4 was, however, observed only in the AD group. This suggests that stimulation of this activation pathway results in an enhanced TH₂-dominated humoral immune response in allergic donors only. Furthermore, this effect was IgE isotype-specific as the production of other immunoglobulins was not affected in this system. These results are in agreement with a study by Life et al. (21), who demonstrated a stimulatory role of CD28 on IgE production as blocking anti-CD28 monoclonal antibodies caused a dose-dependent inhibition of *in vitro* IgE synthesis in the presence of IL-4 and stimulated, allergen-specific T cells. The results confirm the evidence suggesting that interactions of CD80 and CD86 with their receptors CD28 and CTLA-4 are involved in the regulation of immunoglobulin synthesis. Defective production of immunoglobulin production has been shown in CD28 knockout mice or transgenic CTLA-4 mice (22, 23).

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