

Importance of the inducible costimulator molecule for the induction of allergic immune responses and its decreased expression on T helper cells after venom immunotherapy

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SUMMARY

The inducible costimulator (ICOS), a newly identified member of the CD28 receptor family that is induced after T-cell activation, and its ligand (ICOSL), being expressed on activated monocytes and dendritic cells play a key role in T-cell-mediated immune responses. As ICOS costimulation also seems to regulate T helper 2 effector cells, the aim of this study was to analyse the function of this molecule in allergic immune responses and their specific therapy, mainly venom immunotherapy (VIT). CD4⁺ T cells from grass pollen-, or bee or wasp venom-allergic donors were stimulated in the presence of autologous mature dendritic cells, which were pulsed with different allergen doses. In this system, costimulation of ICOS strongly enhanced the production of the T helper 2 cytokines interleukin (IL)-4, IL-5 and IL-10 and, to a lesser extent, secretion of the T helper 1 cytokine, interferon- γ . Expression of ICOS on CD4⁺ T cells was induced, in a dose-dependent manner, after a few days of stimulation with allergen-pulsed dendritic cells, reaching a peak on day 6. The upregulation of ICOS after stimulation with venom allergens was significantly reduced after VIT. Addition of exogenous IL-10 (which is induced during VIT) to the co-cultures before VIT also led to an inhibition of ICOS expression, while blocking of IL-10 in co-cultures after VIT partially restored the expression of ICOS. These data indicate that the inhibition of T cells after immunotherapy also involves decreased induction of the costimulatory molecule ICOS, which, in turn, seems to be dependent on the presence of IL-10, also associated with the inhibited status of T cells after VIT. This makes the ICOS–ICOSL pathway a potential target for therapeutic intervention in T helper 2-mediated diseases, such as allergic diseases.

Keywords costimulation; immunotherapy; allergy; dendritic cells

INTRODUCTION

A recently identified T-cell marker related to CD28 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, CD152), namely, the inducible costimulator (ICOS), is

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Abbreviations: DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; HLA, human leucocyte antigen; ICOS, inducible costimulator; ICOSL, ICOS ligand; IL, interleukin; IFN- γ , interferon- γ ; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate-buffered saline; TCR, T-cell receptor; Th1, T helper 1; Th2, T helper 2; TNF- α , tumour necrosis factor- α ; VIT, venom immunotherapy.

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expressed on activated T cells and has been characterized as an important regulator of T-cell activation.^{1–3} Several groups have shown that the stimulation of ICOS promotes T-cell proliferation of murine and human T cells but, in contrast to CD28, without the effect of increased production of interleukin (IL)-2.^{3,4} In contrast, ICOS has been described to preferentially upregulate the production of IL-10, and, to a lesser extent, the production of IL-4, IL-5, interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in T-cell receptor (TCR)-activated CD4⁺ T cells.^{3–5} Unlike CD28 and CD152, ICOS does not bind to CD80/CD86, but to a novel member of the B7 family called B7h, B7RP-1, GL50, ICOS ligand (ICOSL) or LICOS.^{2,6–10} In mice, B7h is expressed in peripheral lymphoid tissues, including spleen and unstimulated B cells, but also in non-lymphoid tissues, and expression is induced by TNF- α and upregulated by lipopolysaccharide (LPS).⁶ In humans,

ICOSL is expressed on CD14⁺ monocytes after contact with plastic surfaces or after stimulation with IFN- γ .⁹ Monocytes that were further differentiated into dendritic cells (DCs) by culture with GM-CSF and IL-4 showed no alteration of ICOSL expression, in contrast to the increased expression of CD80 and CD86 observed after stimulation of CD83⁺ immature DCs with known potent inducers of DC maturation, such as TNF- α , anti-CD40 or LPS.^{11–14} Furthermore, ICOSL is not expressed on resting human T or B lymphocytes, but is expressed at low levels by a subset of tonsillar B cells, indicating a special function of the ICOS pathway for terminal B-cell differentiation.⁹ The addition of ICOS-Ig fusion protein inhibits allogeneic and antigen-specific T-cell proliferation in DC T-cell cultures, but is less effective than CD152-Ig.⁹ Recently, analysis of ICOS-deficient mice revealed defective T-cell activation and proliferation, a failure to produce IL-4, deficits in immunoglobulin isotype class switching, and enhanced susceptibility to inflammatory autoimmune diseases.^{15–17} Thus, ICOS seems to play an important role in the regulation of T helper 2 (Th2) effector cells.¹⁸ However, the precise role of the ICOS-ICOSL interaction in allergic immune responses is not yet clear. This study was carried out to analyse the function of ICOS in human allergic immune responses and during specific immunotherapy, which not only induces a shift from Th2 to T helper 1 (Th1), but also energy and production of IL-10 from CD4⁺ T cells.^{19,20} Mature allergen-pulsed monocyte DCs were used as antigen-presenting cells (APCs) to stimulate autologous CD4⁺ T cells from bee or wasp venom-allergic individuals before and after venom immunotherapy (VIT) *in vitro*. In this allergen-specific setting we demonstrated that expression of ICOS is induced by allergens and can be inhibited by VIT.

MATERIALS AND METHODS

Patients and immunotherapy

Heparinized blood was obtained from atopic donors suffering from allergic rhinoconjunctivitis and asthma to grass pollen or from severe systemic reactions to bee or wasp stings (i.e. generalized urticaria, angioedema, hypotension or shock). Specific sensitization was documented by a positive skin-prick test to the respective allergen and detection of allergen-specific immunoglobulin E (IgE) in the sera [radioallergosorbent test (RAST) class ≥ 2].

VIT was carried out according to a rush protocol, reaching a maintenance dose of 100 μ g of venom (ALK-lyophilized SQ; ALK-Scherax, Hamburg, Germany) injected subcutaneously within 1 week. Blood samples were taken on the day of admission to hospital (before VIT) and 1 week later on the day of discharge, when the maintenance dose of 100 μ g was reached.

Generation of monocyte-derived DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Paque 1-077 (Biochrom, Berlin, Germany) density-gradient centrifuga-

tion. To enrich CD14⁺ monocytes, 1×10^7 PBMC/well were incubated for 45 min at 37° in a six-well plate (Costar, Bodenheim, Germany) in Iscove's modified Dulbecco's medium containing L-glutamine and 25 mm Hepes (IMDM; Invitrogen GmbH, Karlsruhe, Germany) supplemented with 3024 mg/l sodium bicarbonate, 100 μ g/ml streptomycin, 100 U/ml penicillin and 3% autologous plasma. After washing the non-adherent cells with pre-warmed phosphate-buffered saline (PBS), the remaining monocytes (purity > 90%) were incubated in 3 ml/well IMDM supplemented with 1% heat-inactivated autologous plasma, 1000 U/ml IL-4 (Strathmann Biotech GmbH, Hannover, Germany) and 800 U/ml GM-CSF (Leucomax®; Sandoz AG, Nürnberg, Germany). Cells were fed with fresh medium every other day. On day 7, the resulting immature DCs were pulsed with 10 μ g/ml grass pollen or 0.05–5 μ g/ml bee or wasp venom allergen extracts (ALK-Scherax), or 1 μ g/ml tetanus toxoid (Behring-Werke, Marburg, Germany), and further stimulated with 1000 U/ml TNF- α , 2000 U/ml IL-1 β (Strathmann Biotech GmbH) and 1 μ g/ml prostaglandin E₂ (PGE₂) (Minprostin® E₂; Pharmacia & Upjohn GmbH, Erlangen, Germany) to induce their full maturation. Mature DCs were harvested 48 hr after stimulation, washed twice and used for T-cell stimulation assays.

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMC using antibody-coated paramagnetic MicroBeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), according to the protocol of the manufacturer. Separation was controlled by flow cytometry (purity > 98%).

Co-culture of T cells and autologous allergen-pulsed DC

CD4⁺ T cells (5×10^5) were cultured in 48-well plates, in the presence of 5×10^4 autologous allergen-pulsed DCs, in 1 ml of IMDM supplemented with 5% heat-inactivated autologous plasma. After 1 week of culture, T cells were restimulated with newly generated allergen-pulsed DCs, and supernatants were collected 24 hr later. In some experiments, 0.2 μ g/ml stimulatory anti-ICOS F44 monoclonal antibody (mAb) or immunoglobulin G1 (IgG1) isotype-control 2A11 (kindly provided by Dr R. Kroczeck, Robert Koch Institute, Berlin, Germany), 20 ng/ml IL-10 (DNAX, Palo Alto, CA) or 1 μ g/ml rat anti-human IL-10 (JES3-19F1; BD Biosciences Pharmingen, San Diego, CA) were added from the beginning of the co-culture.

Flow cytometric analysis

CD4⁺ T cells (5×10^5) were cultured in the presence of 5×10^4 autologous allergen-, venom- or tetanus toxoid-pulsed DCs in 1 ml of IMDM supplemented with 5% heat-inactivated autologous plasma, with or without the addition of 1 μ g/ml neutralizing mouse anti-human leucocyte antigen (HLA)-DR mAb (G46-6; BD Biosciences Pharmingen). After 2–8 days of culture, cells were harvested, stained with phycoerythrin (PE)-conjugated anti-ICOS (F44) mAb for 30 min on ice, and analysed in a

FACSCalibur (Becton Dickinson, Mountain View, CA) equipped with cellquest software.

For the two-colour immunofluorescence of mature allergen-pulsed DCs on day 9, 5×10^4 cells were stained with rat anti-human HLA-DR (YE2/36HLK; Camon, Wiesbaden, Germany) and mouse IgG1 anti-human CD80 (MAB104; Immunotech, Hamburg, Germany), mouse IgG2b anti-human CD83 (HB15a; Immunotech), mouse IgG1 anti-human CD86 (BU63; Camon), mouse IgG1 anti-human ICOSL (HIL-131; kindly provided by Dr R. Kroczyk, Robert Koch Institute), or mouse IgG1 and IgG2b isotype controls, for 20 min on ice. After washing with PBS containing 2% fetal calf serum (FCS), the cells were stained with fluorescein (DTAF)-conjugated goat anti-rat IgG (Dianova, Hamburg, Germany) and PE-conjugated donkey anti-mouse IgG (Dianova), for 20 min on ice and then analysed as described above.

Quantification of cytokine production by enzyme-linked immunosorbent assay (ELISA)

The levels of human IL-4, IL-5, IL-10 and IFN- γ were measured by ELISA, according to the instructions of the distributors (BD Biosciences PharMingen) of the antibody pairs employed, as described previously.²¹ The detection limit was 8 pg/ml for IL-4 and 32 pg/ml for all other cytokines.

Statistics

The Student's *t*-test was employed to test the statistical significance of the results; a *P*-value of ≤ 0.05 was considered significant.

RESULTS

Stimulation of ICOS enhances Th2 cytokine and IL-10 secretion of human Th cells stimulated by allergen-pulsed DC

In the murine system, ICOS signalling is generally important for the regulation of Th2 effector cells.^{17,18} To analyse whether costimulation of human allergen-specific T cells through ICOS may influence the secretion of Th1 or Th2 cytokines, we added the stimulatory ICOS mAb, F44, to co-cultures of CD4⁺ T cells from atopic individuals and autologous mature allergen-pulsed DC. Production of IL-4, IL-5, IL-10 and IFN- γ were measured 24 hr after restimulation with allergen-pulsed autologous DCs. As depicted in Fig. 1, secretion of the Th2 cytokines IL-4, IL-5 and IL-10 was strongly and significantly enhanced by costimulation through ICOS, while the production of IFN- γ was not significantly increased. Addition of isotype-control Ab had no effect on Th1 or Th2 cytokine production.

ICOS expression on Th cells is induced by the cognate interaction of T-cell receptor and MHC class II molecules on autologous DCs loaded with peptides

Recently, it has been shown that the ICOS is up-regulated after T-cell activation with the anti-CD3 mAb, OKT3.³ To determine whether the stimulation of T cells with allergens (peptides of allergens in MHC class II molecules on DC)

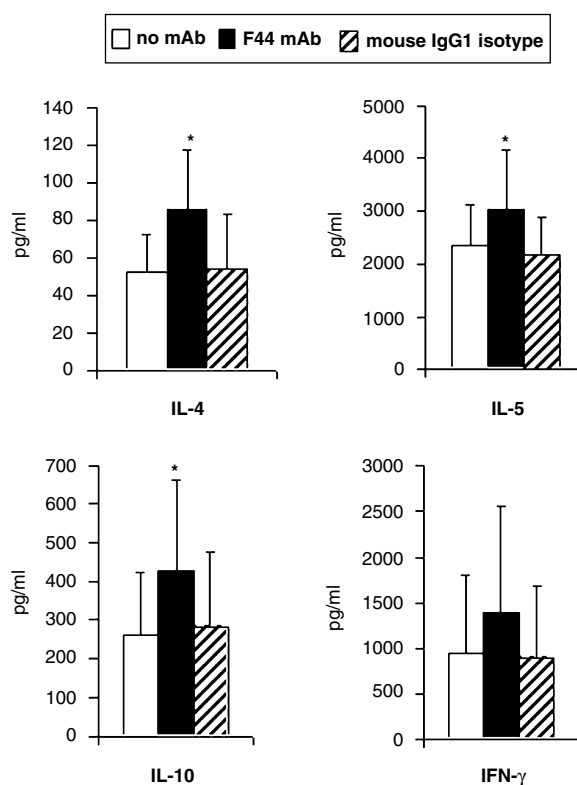


Figure 1. Induction of T helper 2 (Th2), but also of T helper 1 (Th1), cytokine secretion of human T helper cells activated by allergen-pulsed autologous dendritic cells (DCs) and additional costimulation through the inducible costimulator molecule (ICOS). A total of 5×10^5 CD4⁺ T cells were cultured in the presence of 5×10^4 allergen-pulsed autologous DCs, with or without 0.2 μ g/ml of the stimulatory anti-ICOS monoclonal antibody (mAb), F44. After 1 week of culture, T cells were restimulated with newly generated allergen-pulsed autologous DCs. Supernatants were collected 24 hr later and analysed for interleukin (IL)-4, IL-5, IL-10 and interferon- γ (IFN- γ) content by enzyme-linked immunosorbent assay (ELISA). The data represent the mean $\pm$ standard deviation (SD) of nine independent experiments, involving nine different donors. *Statistically significant differences (*P* = 0.05), with or without the addition of anti-ICOS mAb.

also results in ICOS induction, CD4⁺ T cells from atopic donors were co-cultured with autologous DCs that were pulsed with different amounts of allergens. Flow cytometric analysis revealed that mature MHC class II⁺ DCs expressed high levels of CD80, CD86, CD83 and ICOSL (Fig. 2). Unstimulated ICOS expression of T cells was low (3–4%), but ICOS was strongly and dose-dependently upregulated by allergen-pulsed, but not unpulsed, DCs from day 2 onwards, reaching a maximum (up to 50%) after 6 days of culture (Fig. 3a). Using high allergen doses (10–20 μ g/ml), ICOS expression was already significantly increased after 2–4 days of stimulation, while with low allergen doses (≤ 1 μ g/ml) ICOS expression was only increased after 4–6 days of culture (Fig. 3a). Nearly all ICOS⁺ T cells also expressed CD25 and CD45RO (data not shown). Blocking of MHC class II molecules inhibited

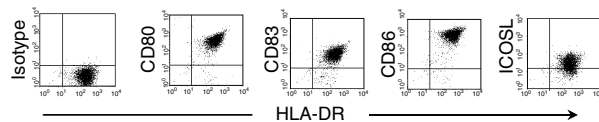


Figure 2. Expression of the inducible costimulator molecule ligand (ICOSL) on allergen-pulsed human dendritic cells (DCs). Monocyte-derived DCs from atopic donors were pulsed with the respective allergen on day 7 of culture and matured with interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and prostaglandin E₂ (PGE₂), as described in the Materials and methods. After 48 hr, cells were double stained for human leucocyte antigen (HLA)-DR and an additional marker, as indicated (the isotype control comprises mouse IgG1 and IgG2b). The figure shows one representative example of 12 independent experiments, involving 12 different donors.

the induction of ICOS expression on T cells, while mouse IgG2a isotype-control Ab had no effect, indicating that this induction did not result from non-specific stimulation via pathways other than the cognate interaction of T-cell receptor and MHC class II loaded with peptides. This does not rule out, however, that additional costimulatory factors, abundantly provided by DCs, are also necessary for the induction of ICOS (Fig. 3b).

Rush VIT inhibits the induction of ICOS expression by CD4⁺ T cells

Specific immunotherapy is one effective tool for the inhibition of allergen-specific Th2 responses by inducing T-cell anergy (inhibited allergen-specific proliferation), a Th2 to Th1 shift and IL-10-producing T-regulatory 1 (Tr1) cells.¹⁹ To analyse whether expression of ICOS is influenced during VIT, co-cultures of CD4⁺ T cells and allergen-pulsed DCs were investigated before VIT and 1 week later, after completion of the induction phase of VIT. After the optimal stimulation time of 6 days, expression of ICOS was strongly decreased in the co-cultures after initiation of VIT compared to the co-cultures before VIT. The inhibition of ICOS was observed using different allergen doses and the inhibition seemed to be antigen-specific, as ICOS expression was not reduced using tetanus toxoid-pulsed DCs (Fig. 4a,b). A slower kinetic of ICOS expression in co-cultures after VIT could be ruled out as ICOS expression was still reduced at later time-points (data not shown).

Neutralization of IL-10 in co-cultures of CD4⁺ T cells and allergen-pulsed DC partially restores the expression of ICOS after VIT, while addition of IL-10 inhibits ICOS expression by CD4⁺ T cells before VIT

To determine the role of IL-10 in the reduced expression of ICOS after VIT (when IL-10 is induced), we added exogenous IL-10 to co-cultures of CD4⁺ T cells and allergen-pulsed DC before VIT, or added neutralizing anti-human IL-10 mAb after VIT. The addition of IL-10 before VIT led to a decreased expression of ICOS induced by allergen-pulsed DC after 6 days of co-culture (Fig. 5). Furthermore,

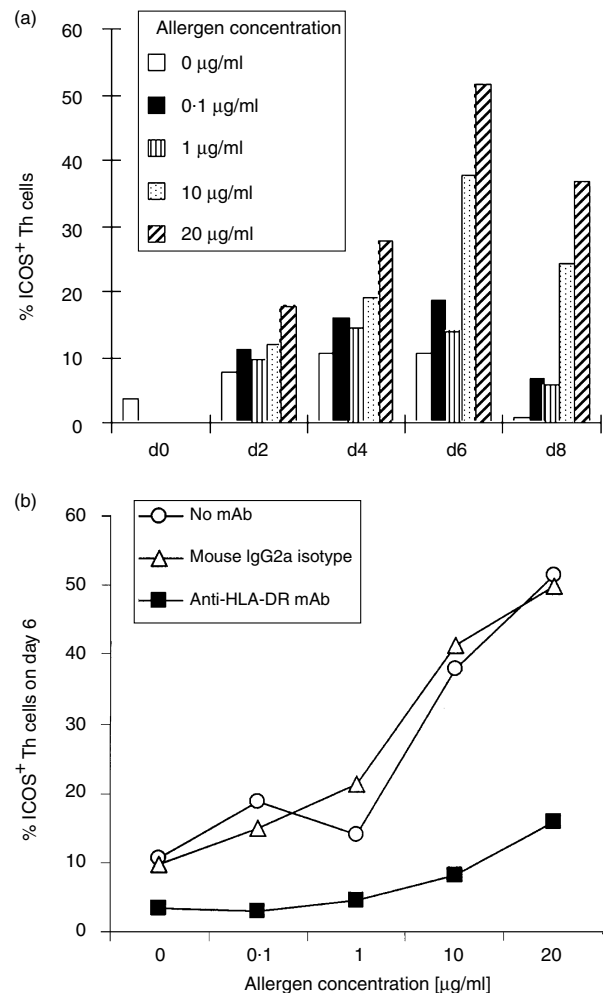


Figure 3. Induction of inducible costimulator molecule (ICOS) expression on human T helper (Th) cells activated by allergen-pulsed autologous dendritic cells (DC). A total of 5×10^5 CD4⁺ T cells were cultured in the presence of 5×10^4 autologous DCs, pulsed with different amounts of allergen. After 0–8 days of culture, cells were stained with the phycoerythrin (PE)-conjugated anti-ICOS monoclonal antibody (mAb), F44. Shown is one representative example of five independent experiments involving five different donors (a). In separate sets of experiments, 1 µg/ml neutralizing anti-human leucocyte antigen (HLA)-DR mAb was added to the co-culture (b).

ICOS expression was also inhibited by the addition of IL-10 in co-cultures of CD4⁺ T cells from patients with rhinitis and grass pollen-pulsed DC as well as after mitogenic stimulation of T cells with phytohaemagglutinin (PHA) (data not shown). Conversely, neutralization of IL-10 production after VIT partially restored the expression of ICOS, while addition of isotype-control antibodies had no effect (Fig. 5).

DISCUSSION

The data presented indicate that the recently discovered costimulatory pathway, ICOS-B7h, plays an important

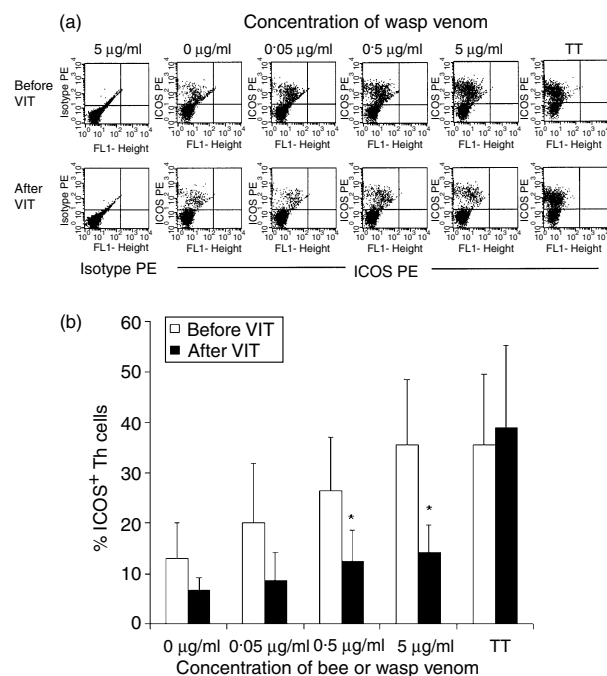


Figure 4. Reduced induction of inducible costimulator molecule (ICOS) expression after completion of the induction phase of venom immunotherapy (VIT). A total of 5×10^5 CD4⁺ T cells, before or after initiation of VIT, were cultured in the presence of 5×10^4 autologous dendritic cells (DCs) pulsed with different amounts of bee or wasp venom or 1 µg/ml tetanus toxoid (TT). After 6 days of culture, cells were stained with phycoerythrin (PE)-conjugated anti-ICOS monoclonal antibody (mAb), F44. The data represent the dot-plot of one representative experiment (a) and the mean $\pm$ standard deviation (SD) from 11 independent experiments involving 11 different donors (b). *Statistically significant differences ($P = 0.01$) between ICOS expression before and after VIT.

role during the allergen-specific activation of human Th2 effector cells. Mature, allergen-pulsed DCs, expressing high levels of ICOSL, are able to upregulate ICOS expression by allergen-specific T cells from atopic individuals. In this allergen-specific setting, additional stimulation of ICOS strongly increases Th2 and, to a lesser extent, Th1 cytokine production. Furthermore, we demonstrate that induction of ICOS expression is decreased by specific immunotherapy in bee and wasp venom-allergic patients as a result of the induction of IL-10.

The finding, that upregulation of ICOS expression on T cells after stimulation with allergen-pulsed DC could be inhibited by the addition of anti-MHC class II mAb, indicates that the specific cognate interaction of allergen peptide in the MHC class II molecule on the DCs and the T-cell receptor is essential for the induction of ICOS expression. Other factors, however, may contribute to the upregulation of ICOS, but are not sufficient to accomplish this in the absence of stimulation of the T-cell receptor. In this context, it is noteworthy that in the murine system, costimulation via CD28 additionally enhances ICOS expression on T cells.⁴

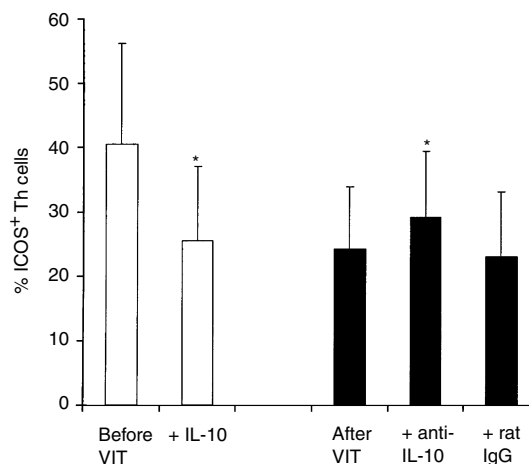


Figure 5. Inhibition of inducible costimulator molecule (ICOS) expression before venom immunotherapy (VIT) by addition of exogenous interleukin-10 (IL-10) and restoration of ICOS expression after VIT by addition of neutralizing anti-IL-10 monoclonal antibody (mAb). A total of 5×10^5 CD4⁺ T cells, before or after VIT, were cultured in the presence of 5×10^4 autologous dendritic cells (DCs) pulsed with 5 µg/ml allergen, with or without the addition of 20 ng/ml IL-10 or 1 µg/ml anti-IL-10. After 6 days of culture, cells were stained with phycoerythrin (PE)-conjugated anti-ICOS (F44) mAb. The data represent the mean $\pm$ standard deviation (SD) from 13 independent experiments involving 13 different donors. *Statistically significant differences ($P = 0.01$) of ICOS expression, with or without the addition of IL-10 or anti-IL-10.

Concerning the effects of costimulation through ICOS, our results are in line with studies also showing an increase in cytokine production after stimulation with anti-ICOS mAb F44 or B7h-Ig.^{3,4} In the study by McAdam *et al.*, inhibition of ICOS by ICOS-Ig in the murine system enhances the production of IFN- γ by ovalbumin (OVA)-transgenic T cells, while it decreases IL-4 and IL-10 production. McAdam *et al.* further found that transgenic Th2 cell lines express more ICOS than cells differentiated into Th1.⁴ Recently, two groups have reported that ICOS plays an important role in promoting Th2 responses, as mice lacking ICOS are unable to produce IL-4 but still produce IFN- γ ^{15,17} and, using human endothelial cells for the stimulation of resting memory CD4⁺ T cells in the presence of superantigen, Khayyamian *et al.* described that the production of IL-2, IL-4, IL-10, IL-13 and IFN- γ was reduced after addition of the inhibitory mAb, HIL-131.²² Unlike CD28, which is constitutively expressed on resting T cells and required for the development of Th2 cells,^{23–25} ICOS is induced on activated CD45RO⁺ and CD25⁺ T cells and might be necessary for the continuation of an already established Th2 response. In this respect, reduced Th2- but not Th1-dependent lung inflammation and airway hyper-responsiveness has been described after inhibition of ICOS in an adoptive transfer model of *in vitro*-polarized Th2 or Th1 cells,²⁶ while both Th-cell subsets were inhibited after *de novo*-induced virus and parasite infection in the presence of ICOS-Ig.²⁷ More recently, two different groups

have shown that blockade of ICOS signalling reduces Th2-mediated airway inflammation without affecting Th2 differentiation. Thus, in contrast to CD28, which plays a key role in T-cell priming, the ICOS pathway seems to be more important for the effector function of Th2 cells than for Th2 differentiation.^{18,28}

Specific immunotherapy, including VIT, is associated with immunological changes from a Th2-dominated immune response towards a Th1 response, leading to a decline in allergen-specific IgE and an increase in allergen-specific IgG production.²⁹ Most importantly, VIT also induces IL-10-producing Tr1 cells and T-cell anergy, resulting in reduced allergen-specific proliferative responses, especially of Th2 cells.^{19,20} This state of hyporesponsiveness of allergen-specific T cells also relates to the induction of ICOS, as demonstrated in this study, an effect that can also be observed after the addition of exogenous IL-10. Although costimulation of ICOS mainly induces the production of IL-10,⁵ IL-10 seems to be a negative regulator of ICOS expression, as the addition of IL-10 in our experimental setting strongly reduces the expression of ICOS. However, the finding that the reduced ICOS expression after VIT is only partially abolished by neutralizing anti-IL-10 mAb indicates that factors other than IL-10 might also be involved in the regulation of ICOS.

Taken together, exposure to allergens leads to induction of ICOS, and ICOS stimulation enhances Th1 and, especially, Th2 cytokine production of human Th cells. Therefore, blocking the ICOS pathway may represent a novel strategy for therapy of Th2-mediated diseases, such as atopic/allergic diseases.

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