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Mouse Inducible Costimulatory Molecule (ICOS) Expression Is Enhanced by CD28 Costimulation and Regulates Differentiation of CD4⁺ T Cells¹

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The inducible costimulatory (ICOS) molecule is expressed by activated T cells and has homology to CD28 and CD152. ICOS binds B7h, a molecule expressed by APC with homology to CD80 and CD86. To investigate regulation of ICOS expression and its role in Th responses we developed anti-mouse ICOS mAbs and ICOS-Ig fusion protein. Little ICOS is expressed by freshly isolated mouse T cells, but ICOS is rapidly up-regulated on most CD4⁺ and CD8⁺ T cells following stimulation of the TCR. Strikingly, ICOS up-regulation is significantly reduced in the absence of CD80 and CD86 and can be restored by CD28 stimulation, suggesting that CD28-CD80/CD86 interactions may optimize ICOS expression. Interestingly, TCR-transgenic T cells differentiated into Th2 expressed significantly more ICOS than cells differentiated into Th1. We used two methods to investigate the role of ICOS in activation of CD4⁺ T cells. First, CD4⁺ cells were stimulated with beads coated with anti-CD3 and either B7h-Ig fusion protein or control Ig fusion protein. ICOS stimulation enhanced proliferation of CD4⁺ cells and production of IFN- γ , IL-4, and IL-10, but not IL-2. Second, TCR-transgenic CD4⁺ T cells were stimulated with peptide and APC in the presence of ICOS-Ig or control Ig. When the ICOS:B7h interaction was blocked by ICOS-Ig, CD4⁺ T cells produced more IFN- γ and less IL-4 and IL-10 than CD4⁺ cells differentiated with control Ig. These results demonstrate that ICOS stimulation is important in T cell activation and that ICOS may have a particularly important role in development of Th2 cells. *The Journal of Immunology*, 2000, 165: 5035–5040.

The recent discovery of the inducible costimulatory (ICOS)⁴ molecule (1) has revealed a new means by which T cell responses may be regulated and raised questions about the functional significance of ICOS and its relationship with the CD28 pathway. ICOS is related to CD28 and CD152 (CTLA-4); mouse ICOS has 38% and 31% similarity with mouse CD28 and CD152, respectively (2, 3). The limited number of functional studies suggest that stimulation of ICOS increases proliferation of mouse and human T cells in response to TCR stimulation (1, 2, 4, 5). cross-linking of ICOS on human T cells also increases cytokine production and help for Ig class switching (1). Stimulation of

ICOS on human T cells preferentially promotes IL-10 production, although production of IL-4, IL-5, IFN- γ , TNF- α , and GM-CSF are also increased (1).

The inducible expression of ICOS is especially interesting because it suggests that ICOS may be particularly important in costimulation of activated T cells. CD28 is expressed by most resting human T cells, and virtually all resting mouse T cells, whereas human ICOS is expressed after T cell activation (1, 2, 6, 7). Costimulation of CD28 appears to be key in the activation and differentiation of naive T cells early in the immune response (6, 8). The inducible expression of ICOS, together with preferential induction of IL-10 by ICOS stimulation, suggests that ICOS may amplify and/or regulate T cell responses.

Recently, a novel B7 family member (originally called B7h (4) but also B7RP-1 (2), GL50 (9), and LICOS (10)), has been identified as the ligand for ICOS (2, 3, 9, 10). B7h has 20% amino acid identity with CD80 (B7-1) and CD86 (B7-2), which is similar to the identity between CD80 and CD86. B7h has the necessary cysteines to form extracellular IgV- and IgC-like domains, like CD80 and CD86 (4). As predicted from the lack of conservation of the MYPPPY binding motif for CD80 and CD86, ICOS did not have detectable binding to CD80 or CD86 (2, 9). B7h mRNA is expressed by a variety of tissues, including spleen and unstimulated B cells, as well as nonlymphoid tissues, and expression is up-regulated by TNF- α and LPS (4). ICOS-Ig fusion protein binds to freshly isolated B cells and macrophages, suggesting that some APC constitutively express B7h (2). However, ICOS ligand expression on macrophages is increased by activation, and this induction is protein kinase dependent (5). Dendritic cells from peripheral blood do not bind to ICOS-Ig, but monocyte-derived dendritic cells do express an ICOS ligand (2, 5).

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⁴ Abbreviations used in this paper: ICOS, inducible costimulatory; OVA peptide, amino acids 323–329 of OVA; B7-1/2^{-/-} mice, mice lacking CD80 and CD86; HA, hemagglutinin; CHO, Chinese hamster ovary; CD40L, CD40 ligand.

The discovery of the ICOS-B7h pathway raises a number of questions about the function of this pathway. To further characterize ICOS expression and function, we have made anti-mouse ICOS mAb and mouse ICOS-Ig to assess the expression and function of ICOS during activation and differentiation of mouse T cells. Our studies show that mouse T cells express very little ICOS, ICOS expression is induced on the surface of T cells after 24 h of stimulation with anti-CD3, and CD28 stimulation is an important inducer of ICOS expression. Further, we found that Th2 cell lines express significantly higher levels of ICOS than do Th1 cell lines. To investigate the function of ICOS in activation and differentiation of CD4⁺ T cells, we have used ICOS-Ig and B7h-Ig. Stimulation of CD4⁺ T cells with beads coated with anti-CD3 and B7h-Ig fusion protein increased proliferation and production of IFN- γ , IL-4, and IL-10, but not IL-2. When differentiated in the presence of ICOS-Ig, DO11.10 T cells produce substantially more IFN- γ and less IL-4 and IL-10 than T cells differentiated in the presence of control IgG2a.

Taken together, our studies demonstrate that ICOS can stimulate both Th1 and Th2 cytokine production, but may have a preferential role in the generation of Th2 cells. Our data also indicate that B7 costimulation may be needed to optimally induce ICOS expression, suggesting that some of the functions ascribed to the CD28 pathway may be mediated through ICOS.

Materials and Methods

Cloning and sequencing of mouse ICOS

A search of the mouse expressed sequence tag database for sequences with homology to CD28 identified AI006009. This expressed sequence tag was sequenced and found to be the mouse orthologue of human ICOS with 69% amino acid identity. The extracellular domain contains a single IgV-like domain. Like CD28 and CTLA-4, ICOS contains an extra pair of cysteines in the IgV domain, a membrane-proximal cysteine that mediates dimerization of the protein, and a phosphatidylinositol-3 kinase signaling motif in the cytoplasmic tail. The mouse ICOS protein sequence is identical with that recently described by Yoshinaga et al. (2). The mouse ICOS cDNA was excised by *EcoRI*/*NotI* digestion and subcloned into the pCDNA3.1 (Invitrogen, San Diego, CA) mammalian expression vector. A hemagglutinin (HA)-epitope-tagged mouse ICOS was generated by cloning a PCR fragment consisting of the IgV transmembrane and cytoplasmic domains downstream of the IgK signal and HA epitope domains in the pDisplay vector (Invitrogen). Jurkat cells were transfected with mouse HA-ICOS in pDisplay, selected with G418, sorted twice for expression using the anti-HA mAb, 12CA5 (Boehringer Mannheim, Indianapolis, IN), and subcloned. A soluble, hexahistidine-tagged form of mouse ICOS was generated by cloning the IgV domain into the pPICZ α B vector, followed by transformation into *Pichia pastoris*. Protein was induced according to the manufacturer's instructions (Invitrogen) and purified to homogeneity on Nickel-NTA and Mono-Q columns. Chinese hamster ovary (CHO) cells were transfected with mouse ICOS cDNA in pCDNA3.1, selected with G418, sorted twice using flow cytometry for mouse ICOS expression using anti-ICOS polyclonal anti-serum, and subcloned.

Preparation of mAbs to ICOS

Rats were immunized i.m. three times with 500 μ g ICOS cDNA in the pAXEF mammalian expression vector and boosted twice with 200 μ g ICOS hexahistidine fusion protein. Spleen cells were fused with SP2/0 myeloma cells and cloned, and the hybridomas were screened by ELISA for reactivity with ICOS hexahistidine fusion protein and by flow cytometry with HA-ICOS-Jurkat cell transfectants. Positive hybridomas were further screened on ICOS-transfected COS or CHO cells. Ten anti-ICOS-specific hybridomas were made. mAb from clone 7E.17G9 (rat IgG2b) was purified and conjugated to FITC using standard techniques.

Animals

BALB/c mice were bought from Taconic Farms (Germantown, NY). BALB/c mice expressing a transgene for the DO11.10 TCR (DO11 mice; Ref. 11), specific for amino acids 323–329 of OVA (OVA peptide) and I-A^d, wild-type 129/SvS4Jae and 129/SvS4Jae mice lacking CD80 and CD86 (B7-1/2^{-/-}; Ref. 12) were bred in our facility. All mice were 6–8 wk old. Brigham and Women's Hospital is accredited by the American

Association of Accreditation of Laboratory Animal Care and mice were cared for in accordance with institutional guidelines in a pathogen-free facility.

Analysis of ICOS expression

For measurement of ICOS expression on freshly isolated cells the thymus, spleen, Peyer's patches, peripheral lymph nodes (pooled axillary and inguinal), and mesenteric lymph nodes were obtained from BALB/c mice. Thymocytes were triple stained for ICOS (FITC), CD4 (Cy-Chrome), and CD8 (PE). Lymphocytes of the other tissues were double stained for ICOS (FITC) and either CD4 or CD8 (PE). Except for anti-ICOS, all Abs used for flow cytometry were from PharMingen (San Diego, CA). Staining and analysis were completed as previously described (13).

To determine ICOS expression on Th1 and Th2 cells, CD4⁺ cells from DO11 mice were purified by magnetic cell separation system (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. A total of 2×10^5 CD4⁺ cells were stimulated with 3×10^6 mitomycin-C-inactivated APC and 1 μ g/ml OVA peptide. Cultures of Th1 cells were initiated by adding 1 μ g/ml anti-IL-4 (11B11, from American Type Culture Collection, Manassas, VA) and 10 ng/ml IL-12 (prepared at Genetics Institute, Cambridge, MA), and Th2 cells were initiated by adding 1000 U/ml IL-4 (from PharMingen) to DO11.10 CD4⁺ cells stimulated with peptide and APC. T cells were restimulated every 7 days with the same level of APC and peptide as in the primary stimulation. Differentiating factors (IL-4, IL-12, and anti-IL-4) were used at half the initial concentration for each stimulation after the primary. Four days after each restimulation, Th lines were stained for ICOS expression with anti-ICOS-FITC (or with control rIg-FITC) and the transgenic TCR (biotin-conjugated KJ126 Ab followed by avidin-PE). The data were analyzed by measuring fluorescence of ICOS (or rIg control) after gating on forward scatter and side scatter for activated lymphocytes and on DO11.10 TCR expression. ICOS-specific fluorescence was calculated by subtracting the fluorescence with rIg-FITC from the fluorescence of anti-ICOS-FITC. The fluorescence with rIg-FITC was always low (range 2.7–5.7). To confirm differentiation of Th1 and Th2, the cell lines were also stimulated each week with APC and peptide without differentiating factors; 2 days after stimulation, the supernatants of these cultures were collected for cytokine analysis by ELISA.

The effect of CD3 and CD28 stimulation on ICOS expression was determined by incubating splenocytes from 129/SvS4Jae mice (wild type or B7-1/2^{-/-}) with no Ab (unstimulated control), anti-CD3 (1 μ g/ml 145-2C11, prepared by Bioexpress, West Lebanon, NH), anti-CD28 (a 1/1000 dilution of a concentrated preparation of 37.51), or both anti-CD3 and anti-CD28. Twenty-four hours later, cells were stained with anti-ICOS-FITC and CD4-PE or CD8-PE.

The role of ICOS in Th differentiation

Two systems were used to investigate the role of ICOS in proliferation and differentiation of CD4⁺ cells. First, purified CD4⁺ cells were stimulated with beads coated with anti-CD3 and either B7h-Ig or control fusion protein. Second, purified CD4⁺ cells from TCR-transgenic mice were stimulated with cognate peptide and APC, in the presence of either ICOS-Ig (9) or control IgG2a.

Latex beads (Interfacial Dynamics Corporation, Portland, OR) were resuspended in PBS at 10^7 beads/ml. Purified anti-CD3 (1 μ g/ml) and either B7h-Ig (prepared at Genetics Institute) or control fusion protein (each at 3 μ g/ml) were added and conjugated to the beads for 2 h at 37°C. The B7h-Ig was purified from CHO cells and consists of the extracellular domains of mouse B7h fused to the Fc domain of mouse IgG2a. B7h-Ig binds to COS cells transfected with mouse ICOS, but not to control COS transfectants. The beads were extensively washed and then incubated with RPMI 1640 with 10% FCS for 1 h at 37°C. CD4⁺ cells were purified from BALB/c mice by magnetic cell sorting system (Miltenyi Biotec). A total of 10^5 purified CD4⁺ cells were stimulated in 96-well round-bottom plates with beads ranging from 690 to 5×10^5 /well (or no beads as a control). [³H]Thymidine was added for the last 6 h of a 3-day culture to determine proliferation. To determine the effect of ICOS stimulation on cytokine production, supernatants were collected from CD4⁺ cells stimulated as above with 1.7×10^5 beads. Supernatants were collected on day 3 for measurement of IL-2, IL-4, and IFN- γ , and day 4 for IL-10. Cytokines were measured by ELISA as previously described (14).

To evaluate the effects of ICOS on the differentiation of naive T cells during stimulation with splenic APC, CD4⁺ cells were purified from naive DO11 mice. A total of 2×10^5 CD4⁺ cells were stimulated with 3×10^6 mitomycin-c-inactivated APC and 1 μ g/ml OVA peptide, with 10 μ g/ml of either ICOS-IgG2a fusion protein or control mouse IgG2a. ICOS-Ig fusion protein has previously been shown to bind to B7h/GL50 (9). Five days later, the cells were harvested and restimulated with APC and OVA peptide

as described above. Supernatants were collected from the first 3 days of the primary and secondary cultures and tested for IL-2, IL-4, IL-10, and IFN- γ by ELISA as previously described (14).

Results

ICOS expression is rapidly up-regulated and enhanced by CD28 stimulation

We made anti-mouse ICOS-specific mAbs to characterize mouse ICOS expression. Anti-mouse ICOS specificity was confirmed by reactivity with ICOS-transfected CHO and COS cells and lack of reactivity with vector-transfected cells (Fig. 1A and data not shown). The 7E.17G9 Ab blocks binding between ICOS and B7h (data not shown).

We first used the anti-ICOS mAb to determine the expression of ICOS by normal lymphocyte populations. When freshly isolated cells from the thymus were stained using anti-ICOS mAb, ICOS expression was seen on a subpopulation of CD4⁺8⁺ and CD4⁺8⁺ thymocytes (9.3 and 7.4%, respectively; Fig. 1B). This level of expression is significantly higher than that of resting peripheral lymphocytes (compare with Fig. 1C). Thymic CD4⁺8⁺ and CD4⁺8⁺ cells do not show appreciable staining with anti-ICOS. Expression of ICOS on thymocyte subpopulations raises the possibility that ICOS may be involved in thymic selection.

Freshly isolated cells from peripheral lymphoid tissues were also analyzed for ICOS expression. The T cells from the spleen, peripheral lymph nodes (pooled axillary and inguinal nodes), and mesenteric lymph nodes express a low level of ICOS (Fig. 1C). A total of 17% of CD4⁺ cells in the Peyer's patches express a higher level of ICOS, although Peyer's patches CD8⁺ cells express only

a low level. Expression of ICOS by the Peyer's patch CD4⁺ cells may reflect activation of these T cells.

To investigate the regulation of ICOS expression, we stimulated whole splenocytes from wild-type or B7-1/2^{-/-} mice with anti-CD3, anti-CD28, both, or neither for 24 h. Expression of ICOS on unstimulated T lymphocytes was similar to the very low level on freshly isolated splenocytes. Stimulation of wild-type splenocytes with anti-CD3 for 24 h induced expression of a high level of ICOS on nearly all of the CD4⁺ and CD8⁺ cells (Fig. 2). After 48 or 72 h, levels of ICOS were lower than at 24 h, although still higher than on resting cells (data not shown). Interestingly, anti-CD3 stimulation was less effective at inducing ICOS expression in cultures lacking CD80 and CD86 (Fig. 2). The level of ICOS induced in the wild-type culture is ~8-fold higher than that in the culture lacking CD80 and CD86. This suggested that expression of ICOS might be partially dependent on CD28 costimulation. Indeed, addition of anti-CD28 to anti-CD3 stimulated B7-1/2^{-/-} splenocytes increased ICOS expression on CD4⁺ and CD8⁺ cells to levels similar to that on anti-CD3 stimulated cells from wild-type splenocytes (Fig. 2). Addition of anti-CD28 alone (without anti-CD3) did not up-regulate ICOS expression by T cells in wild-type or B7-1/2^{-/-} cultures (data not shown).

Th2 express more ICOS than do Th1

Because human ICOS has been implicated in cytokine production (1), we investigated the ICOS expression during differentiation of Th1 and Th2 cell lines. CD4⁺ DO11-transgenic T cells were stimulated with OVA peptide under Th1 or Th2 differentiating conditions every 7 days for 9 wk. Four days after each stimulation, cells were stained and analyzed for ICOS expression. In addition, each week the Th1 and Th2 cell lines were stimulated with OVA peptide and APC without differentiating agents to determine the extent of the differentiation to Th1 or Th2. Supernatant from T cells deviated toward Th1 contained IFN- γ (mean, >25 ng/ml) and IL-2 (mean, 0.93 ng/ml), but no detectable IL-4 or IL-10 (<0.1 ng/ml). Supernatants from Th2-deviated cells contained IL-4 (mean, 4.1 ng/ml) and IL-10 (mean, 5.1 ng/ml), but little IFN- γ (mean, 0.23 ng/ml) and no detectable IL-2 (<0.1 ng/ml), indicating that our differentiation protocol was effective.

Expression of ICOS was very low on the freshly isolated TCR-transgenic T cells (Fig. 3A). After primary stimulation, Th1 and Th2 cells expressed comparably high levels of ICOS. At each subsequent time tested, the Th2 DO11.10⁺ cells continued to express high levels of ICOS (ICOS-specific fluorescence of Th2 ranged from 81 to 135, which is less than 2-fold variation). In contrast, the

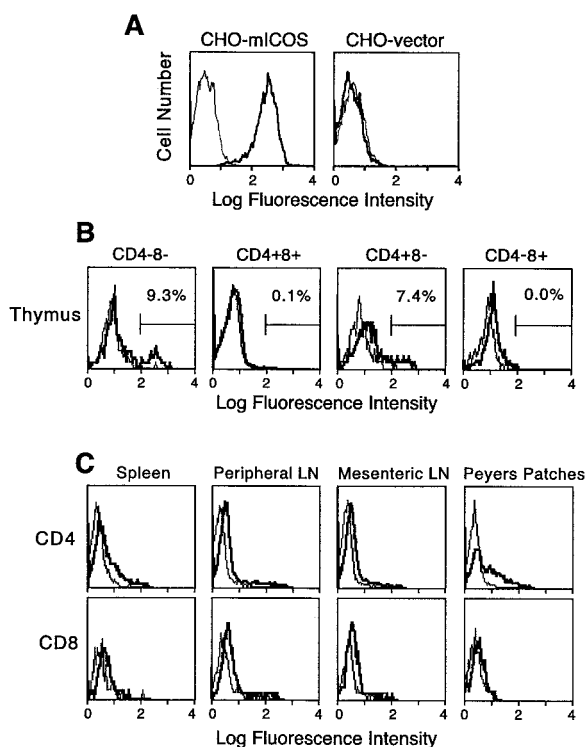


FIGURE 1. Specificity of anti-ICOS mAb and expression of ICOS on freshly isolated T cells. *A*, CHO cells transfected with ICOS or vector alone. *B*, BALB/c thymus. The percent of cells expressing ICOS is shown for the indicated thymic populations. *C*, BALB/c peripheral lymphoid tissues. Cells were stained for ICOS and CD4 or CD8 as described in *Materials and Methods* and analyzed by flow cytometry. Histograms show reactivity with anti-ICOS-FITC mAb (thick line) or a negative control (rat IgG-FITC, thin line).

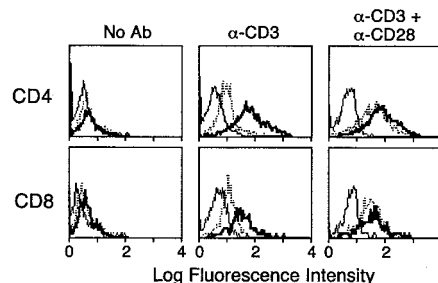


FIGURE 2. Expression of ICOS on activated T cells. Dissociated splenocytes from wild-type or B7-1/2^{-/-} 129/SvS4Jae mice were incubated with anti-CD3, anti-CD3 and CD28, or no Ab. The thick line shows ICOS expression on T cells from wild-type splenocyte cultures, the dotted line shows ICOS expression on T cells from B7-1/2^{-/-} splenocyte cultures, and the thin line represents a negative staining control (rat IgG-FITC).

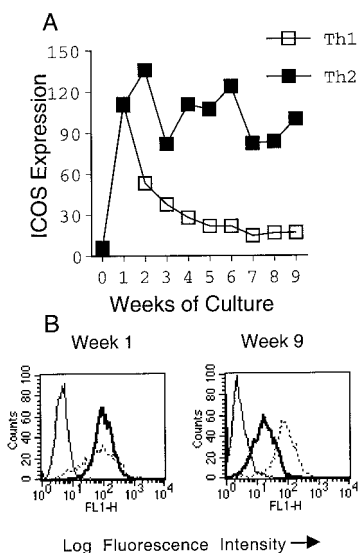


FIGURE 3. Th2 cells express higher ICOS levels than do Th1 cells. Purified DO11.10 CD4⁺ cells were stimulated weekly with APC and peptide. The Th1 line was stimulated in the presence of IL-12 and neutralizing Ab to IL-4. The Th2 line was stimulated in the presence of IL-4. Four days after each stimulation, cells were stained for ICOS and for the transgenic TCR. ICOS expression was determined after gating for cells expressing the transgenic TCR, and gating on live cells by forward and side scatter. **A**, ICOS expression was calculated by subtracting the mean log fluorescence intensity with control rat-IgG from anti-ICOS staining. Zero weeks of culture designates the staining of purified DO11.10 cells before stimulation. **B**, The staining for ICOS of Th1 (thick solid line) and Th2 (dotted line) after 1 or 9 wk of stimulation is shown. The thin solid line is a representative negative control stain (rat-IgG-FITC on the Th1 line).

ICOS expression on the Th1 cell line decreased over time. Th1 cells consistently expressed a low level of ICOS during weeks 5–9. Despite being at a significantly lower level than on the Th2, ICOS was detectable on the Th1 throughout the experiment (Fig. 3B). To determine whether the level of ICOS on differentiated Th1 and Th2 varied with the time after restimulation, we tested ICOS expression 1, 2, 4, and 6 days after stimulation during the fifth week of culture. On all days, ICOS was lower on Th1 than Th2.

Stimulation of ICOS by B7h increases T cell proliferation and cytokine production

To evaluate the role of ICOS in T cell stimulation, we first used a simplified system, stimulating purified CD4⁺ cells with latex beads coated with anti-CD3 and either B7h-Ig or control fusion protein. This allows determination of the effects of ICOS costimulation in the absence of costimulation or other signals from APC.

Stimulation of CD4⁺ T cells with anti-CD3 and B7h-Ig increased both proliferation and cytokine production by CD4⁺ T cells compared with anti-CD3 and control Ig (Fig. 4). Between 6.2×10^3 and 1.7×10^5 beads per well, anti-CD3/B7h-coated beads elicited 1.5- to 1.9-fold more proliferation than did anti-CD3/control Ig-coated beads (Fig. 4A). At the highest concentrations of beads, the effect of anti-CD3 reached a plateau, so that at 5×10^5 beads per well, proliferation was similar in cells stimulated with the two types of beads. B7h on the beads did not significantly affect production of IL-2 (Fig. 4B). This was true over a range of bead concentrations (as in Fig. 4A) at days 1–4 (Fig. 4B and data not shown). However, B7h costimulation increased production of IFN- γ , IL-4, and IL-10 by 7.5-, 3.2-, and 3.7-fold, respectively (Fig. 4B). These data demonstrate that ICOS costimu-

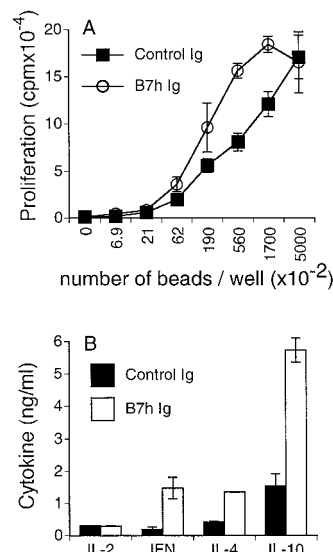


FIGURE 4. Stimulation of ICOS enhances proliferation and cytokine production by purified CD4⁺ cells. Purified CD4⁺ cells from BALB/c mice were stimulated with beads conjugated to anti-CD3 and either B7h-Ig or a control Ig fusion protein. **A**, The indicated number of beads was used to stimulate CD4⁺ cells and proliferation was measured on day 3 by incorporation of [³H]thymidine. **B**, A total of 1.7×10^5 beads were used to stimulate CD4⁺ cells and cytokines were collected for measurement of cytokines by ELISA. IL-2, IL-4, and IFN- γ were measured on day 3, and IL-10 was measured on day 4.

lation can enhance proliferation of CD4⁺ T cells. Further, both Th1 and Th2 cytokines are enhanced by ICOS costimulation.

Blocking the ICOS pathway inhibits Th2 differentiation

ICOS ligand is expressed by a variety of cells, including most B cells and macrophages in freshly isolated splenocytes (2). Because B7h is expressed by resting APC, we decided to manipulate the ICOS pathway by blocking the interaction of B7h and ICOS during primary T cell stimulation using an ICOS-Ig fusion protein. This protein binds to B7h (9), and so would be expected to block the interaction of ICOS with ICOS ligand.

We used CD4⁺ T cells from DO11 TCR-transgenic mice (11) to evaluate the role of ICOS costimulation in CD4⁺ T cell activation and differentiation. The use of TCR-transgenic T cells has several advantages for the study of CD4⁺ T cell differentiation. First, the majority of the T cells are naive. Second, T cells are of known antigenic specificity, so they can be stimulated with physiologic signals (peptide and MHC on APC).

We have previously identified conditions under which naive DO11 TCR-transgenic T cells produce IL-2, IFN- γ , and IL-4 upon restimulation with APCs and OVA peptide (14, 15). To determine whether ICOS influences T cell differentiation, we used these conditions to prime naive DO11 T cells with APCs and OVA peptide in the presence of ICOS-Ig or control mouse IgG2a. After 5 days, the T cells were restimulated with OVA peptide and APC for 3 days. Proliferation and cytokine production were measured during the first 3 days of each culture.

ICOS-Ig did not significantly alter proliferation in the primary or secondary stimulations (Fig. 5). However, the addition of ICOS-Ig during primary stimulation dramatically skewed Th differentiation. There was increased production of the Th1 cytokine IFN- γ and decreased production of the Th2 cytokines IL-4 and IL-10 upon secondary stimulation (Fig. 6). In the primary culture, the presence of ICOS-Ig increased production of IFN- γ on day 3

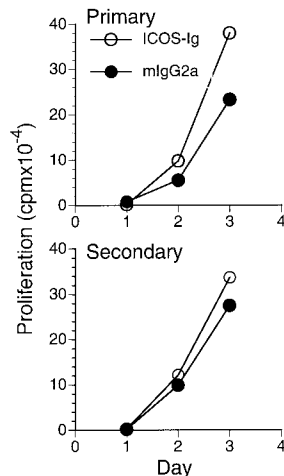


FIGURE 5. Effect of ICOS-Ig on T cell proliferation with splenic APC. Purified CD4⁺ DO11 T cells were stimulated with OVA peptide and APC in the presence of either ICOS-Ig or mouse IgG2a. ICOS-Ig or IgG2a was included only during the primary stimulation. Five days later the T cells were washed and restimulated with OVA peptide and APC. Proliferation during the primary and secondary stimulations were determined by incorporation of [³H]thymidine.

by 3-fold. ICOS-Ig did not significantly alter production of IL-2 in the primary culture. IL-4 and IL-10 were not detectable in primary culture supernatants. Production of cytokines in secondary culture was profoundly affected by ICOS-Ig in the primary culture. Production of IFN- γ was increased by the addition of ICOS-Ig by 7-fold on days 2 and 3, respectively. IL-2 was not detectable in supernatants from secondary cultures. The addition of ICOS-Ig decreased production of IL-4 to 25 and 4% of control levels on days 2 and 3, respectively. IL-10 production was decreased to 20 and 7% of control levels on days 2 and 3 of the secondary culture.

Discussion

The discovery of the ICOS-B7h costimulatory pathway has given impetus to studies directed at understanding its functional role during T cell activation and its relationship with the CD28/CD152-CD80/CD86 pathway. To dissect the function of this newly defined costimulatory pathway, we have developed anti-mouse ICOS mAb, ICOS-Ig, and B7h-Ig. Our anti-ICOS mAbs also provide a means to characterize ICOS expression on mouse T cells and investigate stimuli that regulate ICOS expression. Our studies indicate that ICOS expression is rapidly induced on both CD4⁺ and CD8⁺ T cells; ICOS up-regulation is augmented by CD28 costimulation; a high level of ICOS is expressed initially on Th1 and Th2 cells, but expression on Th1 cells decreases over time; ICOS stimulation leads to production of IFN- γ , IL-4, and IL-10, but not IL-2; and ICOS blockade decreases Th2 differentiation. Taken together, these studies demonstrate that ICOS stimulates both Th1 and Th2 cytokine production but may have a preferential role in Th2 cell development. Our studies also suggest a functionally significant interaction between CD28 and ICOS pathways. In particular, our data suggest that CD80/CD86 costimulation may be needed to optimally up-regulate ICOS expression and that the CD28 pathway may exert some of its effects (such as Th2 differentiation) via ICOS.

In the two major costimulatory pathways for the initial activation of lymphocytes, CD28-CD80/CD86 and CD40-CD40 ligand (CD40L), the signaling molecule (CD28 or CD40) is constitutively expressed and the counterreceptor is inducible (CD80, CD86, and CD40L; Ref. 6). In contrast, in the B7h/ICOS pathway, the putative signaling molecule, ICOS, is induced after activation (Ref. 1

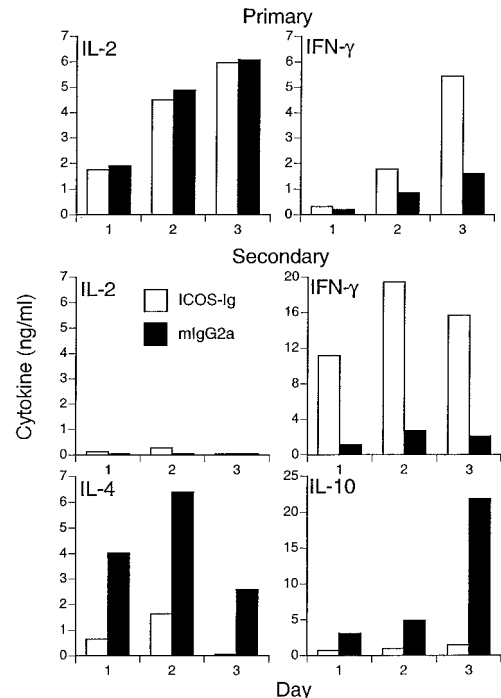


FIGURE 6. Effect of ICOS-Ig on T cell cytokine production with splenic APC. Purified CD4⁺ DO11 T cells were stimulated with OVA peptide and APC in the presence of either ICOS-Ig or mouse IgG2a. ICOS-Ig or IgG2a was included only during the primary stimulation. Five days later, the T cells were washed and restimulated with OVA peptide and APC. Supernatants were collected at the indicated times during primary and secondary stimulations and analyzed for cytokine production by ELISA. No IL-4 or IL-10 was detectable in the primary supernatants.

and this report). The ICOS counter receptor is constitutively expressed by B cells and macrophages (2, 5), although it can be further induced by activation of macrophages (5). Therefore, stimulation of T cells through ICOS will depend primarily on induction of ICOS, which can then interact with the constitutively expressed ligand. Determining the factors that regulate ICOS induction may be key to understanding when this pathway is important.

We have found that costimulation of CD28 by CD80 or CD86 enhanced expression of ICOS, although ICOS could be induced in the absence of CD80 and CD86 (Fig. 2). This enhancement of ICOS expression by CD28 is similar to CD28 mediated enhancement of IL-2R and CD40L expression; a CD3 signal alone can induce expression, but CD28 costimulation increases and prolongs expression (16, 17). However, the ICOS pathway appears to preferentially promote Th2 development, whereas CD40L-CD40 interaction can enhance generation of a Th1 response by increasing IL-12 production (18). Human CD4⁺ cells express higher levels of ICOS than do human CD8⁺ cells (1). Similarly, after activation with anti-CD3, mouse CD8⁺ cells expressed slightly less ICOS than do CD4⁺ cells (Fig. 2). The level and percent of mouse T cells expressing ICOS appears to be higher than that on human T cells (Fig. 3 and Ref. 1). This may reflect actual differences in the level of ICOS expressed by human and mouse cells, or differences in the staining reagents or activation conditions.

Our results indicate that ICOS stimulation has a modest effect on proliferation. Consistent with this, there was no detectable effect of ICOS blockade or ICOS stimulation by B7h on IL-2 production (Figs. 4 and 6, and Ref. 1). ICOS-Ig did not block proliferation of CD4⁺ cells stimulated with peptide and APC (Figs. 5 and 6). Conversely, B7h-Ig costimulation modestly increased proliferation of

CD4⁺ cells stimulated with anti-CD3-coated beads (1.5- to 1.9-fold, Fig. 4). Stimulation of the purified CD4⁺ cells with anti-CD3-conjugated beads is an APC-free system in which the B7h is the primary source of costimulation. In contrast, when APC are present during the stimulation of the DO11.10 TCR-transgenic T cells, the modest effect of ICOS stimulation on proliferation may be obscured by the presence of other costimulatory molecules. Consistent with the modest effect of ICOS stimulation on proliferation of CD4⁺ cells, it has been demonstrated that allo-reactive or secondary proliferation of human T cells are better inhibited by blocking the CD28 pathway than by blocking the ICOS pathway (5).

ICOS stimulation had marked effects on cytokine production. When purified CD4⁺ cells were stimulated with anti-CD3 and B7h-Ig-coated beads, production of IL-4, IL-10, and IFN- γ were enhanced, whereas production of IL-2 was not affected (Fig. 4). Moreover, blocking the ICOS pathway with ICOS-Ig skewed differentiation of DO11.10 TCR-transgenic cells from Th2 and toward Th1 with high IFN- γ production (Fig. 6). How can the apparent discrepancy in IFN- γ production be reconciled? Multiple pathways, including IL-12, CD80/CD86, signaling lymphocytic activation molecule, and DNAX accessory molecule-1 can enhance production of IFN- γ (6, 19–21). These may stimulate IFN- γ production whether ICOS is blocked with ICOS-Ig. Because IL-4 and IL-10 antagonize IFN- γ production, the strong inhibition of IL-4 and IL-10 production by ICOS-Ig may allow these other pathways to more strongly stimulate IFN- γ production. IL-4 production is largely dependent on costimulation through CD28 (6, 15, 22, 23), and we have shown here that ICOS costimulation can also enhance IL-4 production. Because induction of ICOS is partially dependent on CD28 costimulation (Fig. 2), it may be that the ability of CD28 stimulation to enhance IL-4 production is mediated through up-regulation of ICOS. Together, these results show that, whereas ICOS stimulation can enhance both Th1 and Th2 cytokines, ICOS may be particularly important in Th2 generation. It is also possible that the changes in cytokine production may be due to ICOS stimulation of other T cell subsets, such as T-regulatory 1 cells, which produce high levels of IL-10 (24). However, T-regulatory 1 cells do not produce IL-4, so we favor the hypothesis that ICOS stimulation contributes to Th2 generation (25).

The effects of ICOS blockade correlate with the higher level of ICOS expression on Th2 compared with Th1 cells. Immediately after activation of CD4⁺ cells under conditions to generate Th1 or Th2, both populations express high levels of ICOS. When the supernatants from cells restimulated after this first cycle of stimulation were tested for cytokines, it was evident that they had already differentiated into Th1 and Th2, so differentiation into Th1 and Th2 precedes the difference in ICOS expression. After secondary stimulation, ICOS expression declines on Th1 cells but remains high on Th2 cells. The pattern of ICOS expression is in contrast to SLAM expression. SLAM is expressed at higher levels by Th1 than Th2 (20). It will be interesting to determine what signals or cytokines regulate ICOS expression in these populations.

The demonstration that ICOS stimulation may enhance Th2 development suggests that the ICOS pathway may be a good target for immunotherapy in a variety of immune-mediated diseases. Blocking reagents, such as the ICOS-Ig used in this report, have significant potential to reduce the development of harmful Th2 responses, such as those seen in allergic responses (26). Alternatively, stimulation of the ICOS pathway might shift undesirable Th1 responses in graft rejection and autoimmunity toward Th2 responses (26). Manipulation of ICOS may have the potential to affect responses of naive or effector T cells. Our results give impetus to further studies to determine whether manipulation of the ICOS pathway in vivo can alter the outcome of the immune response.

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