The Journal of Immunology

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J Immunol 2004; 173:1779-1786; ;
doi: 10.4049/jimmunol.173.3.1779
http://www.jimmunol.org/content/173/3/1779

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

ICOS Expression by Activated Human Th Cells Is Enhanced by IL-12 and IL-23: Increased ICOS Expression Enhances the Effector Function of Both Th1 and Th2 Cells[1]

Lianne Wassink,* Pedro L. Vieira,* Hermelijn H. Smits,* Gillian A. Kingsbury,† Anthony J. Coyle,† Martien L. Kapsenberg,* and Eddy A. Wierenga[2]*

Previous mouse studies have shown that IL-4 increases the expression of ICOS on activated Th cells, resulting in enhanced ICOS expression on Th2 cells. In this study, we show that ICOS expression on human Th cells is not increased by IL-4, but by IL-12 and by IL-23 instead. Consequently, ICOS expression during IL-12-driven Th1 cell polarization was transiently increased compared with the levels on Th0 cells and IL-4-driven Th2 cells. Addition of IL-12 and/or IL-23 during restimulation increased ICOS expression to the same extent on pre-established Th1, Th2, and Th0 cells, indicating that ICOS levels are not stably imposed by prior polarization. In contrast to the findings in the mouse, IL-4 significantly suppressed the ICOS-enhancing effects of IL-12 and IL-23. The functional consequence of variable ICOS levels was shown in coculture experiments with cells expressing the ICOS-ligand B7-related protein 1 (either transfected Chinese hamster ovary cells or autologous dendritic cells). Ligation of ICOS on 2-day-preactivated effector cells increased their cytokine production to an extent proportional to their ICOS expression levels. As the ICOS-enhancing potentials of IL-12 and IL-23 were maintained for several days after stimulation, both on Th1 and Th2 cells, we propose the concept that local regulation of ICOS expression on activated Th cells by IL-12 and/or IL-23 may provide a powerful means to amplify effector T cell responses in peripheral tissues, independently of the polarized state of the Th cells. ***The Journal of Immunology*****, 2004, 173: 1779–1786.**

Inducible costimulatory molecule (ICOS) belongs to the CD28/CTLA-4 family of T cell costimulatory molecules (1–3). In contrast to the constitutive expression of CD28, ICOS expression requires TCR activation and CD28 costimulation (2, 4, 5). The ICOS ligand B7-related protein 1 (B7RP1)[3] (also known as ICOS-L, B7h, GL-50, or LICOS) is expressed on most types of APCs, including dendritic cells (DC), B cells, and activated monocytes (2, 6, 7), but it can also be expressed in various nonlymphoid organs (8) and by endothelial cells (9). CD28 is important for the initiation of immune responses by mediating the induction of IL-2 production, in turn allowing for proliferation, differentiation, and survival of activated T cells (10). ICOS, in contrast, plays an active costimulatory role during the effector function of activated T cells, supporting proliferation and the secretion of a large array of cytokines (1, 3, 5, 8, 11), typically not including IL-2 (4, 12, 13).

Several studies in the mouse suggest that the role of ICOS is particularly pronounced in Th2 responses. Blocking of the ICOS-B7RP1 interaction in vivo inhibited Th2 cell-mediated allergic airway responses in conjunction with inhibition of the production of Th2 cytokines IL-4, IL-5, and IL-13 (13, 14), while Th1 cell-mediated inflammation of the lung mucosa and IFN-γ secretion in a similar model were not affected (13). Likewise, other studies showed that the majority of ICOS-expressing cells in vivo are tightly associated with the synthesis of Th2-type cytokines (15) and that ICOS-deficient mice have defects in the production of IL-4, but not in the production of IFN-γ (16, 17). However, despite these strong Th2 associations, the role of ICOS in mice is not entirely exclusive to Th2 cells, as allograft rejection and experimental autoimmune encephalomyelitis, both typical Th1-mediated disorders, could also be inhibited by blocking the ICOS-B7RP1 interaction (18, 19). Nevertheless, ICOS expression was shown to be much higher on Th2 cells, as compared with Th1 cells (5, 13, 14, 20), and in a comparative study of different mouse strains, ICOS expression levels appeared to correlate with IL-4 production levels, and IL-4 was shown to enhance ICOS expression in a GATA-3-dependent fashion (21).

So far, comparative data on the regulation and function of ICOS on human Th1 and Th2 cells is scarce. In the present study, we show for the first time that, in sharp contrast to the data obtained in mice, ICOS expression on activated human Th cells is up-regulated by IL-12 and IL-23, and not by IL-4. Consequently, ICOS expression is increased on IL-12- or IL-23-driven Th1 cells, although these cytokines have the same impact on established Th2 cells. We further show that these ICOS-enhancing effects are maintained for several days after TCR activation and that ICOS ligation on preactivated effector Th cells increases cytokine production with a magnitude proportional to their level of ICOS expression. Based on these findings, we propose that the strength of the effector response of Th cells is regulated by the control of ICOS expression by locally produced IL-12 and/or IL-23 in peripheral tissues.

*Academic Medical Center, Department of Cell Biology and Histology, University of Amsterdam, Amsterdam, The Netherlands; and †Millennium Pharmaceuticals Inc., Cambridge, MA 02139

Received for publication January 20, 2004. Accepted for publication May 19, 2004.

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[1] This work was supported by grants from The Netherlands Asthma Foundation (Grant 3.2.00.51, to L.W.) and Stichting de Drie Lichten (Grant 01/46)

[2] Address correspondence and reprint requests to Dr. Eddy A. Wierenga, Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ, Amsterdam, The Netherlands. E-mail address: e.a.wierenga@amc.uva.nl

[3] Abbreviations used in this paper: B7RP1, B7-related protein 1; CHO, Chinese hamster ovary; DC, dendritic cell; iDC, immature DC; MF, maturation factors; ΔMFI, change in mean fluorescence intensity; SEB, staphylococcal enterotoxin B.

0022-1767/04/$02.00

Materials and Methods

Generation of human ICOS-Ig

A fusion protein of human ICOS with human IgG1 (ICOS-Ig) was generated at Millennium Pharmaceuticals (Cambridge, MA). A DNA sequence encoding the extracellular ICOS domain was PCR-amplified and cloned into a vector containing the CD5 signal sequence and a mutated human IgG1 constant region (preventing FcR ligation or complement activation). COS cells were transiently transfected using lipofectamine (Life Technologies-BRL, Gaithersburg, MD) and the recombinant protein was purified over a protein A column. The purity of ICOS-Ig as assessed by Coomassie-stained SDS-PAGE was >90%. The identity of the ICOS-Ig fusion protein was confirmed by peptide mass fingerprinting (MALDI-TOF analysis) based on comparing the trypsin peptides generated from the extracted gel band to a theoretical trypsin digest.

Generation of anti-human ICOS mAb

Female C3H mice (6–8 wk old; Taconic Farms, Germantown, NY) were immunized with plasmids containing the ICOS-Ig-encoding sequence. Briefly, 50 mg of gold microcarriers (Bio-Rad Laboratories, Hercules, CA) were coated with 100 μg of plasmid. Coated particles were delivered to both the chest and abdomen of each animal (0.5 mg of beads and 1 μg of DNA per shot) using the Helios Gene Gun System (Bio-Rad Laboratories). Over a 10-day period, the animals were injected every other day at both sites (10 shots total). Three days before fusion, the animals were boosted i.v. with 25 μg of ICOS-Ig protein in PBS. B cell hybridomas were obtained by fusing the immune spleen cells with nonsecreting murine SP2/0 cells. Hybridoma supernatants were screened for anti-human ICOS production by labeling ICOS-transfected L cells, followed by flow cytometric analysis, and cloned by limiting dilution. Used in the present study is anti-ICOS IgG1 mAb MS9.9C8. This Ab completely blocks the binding of B7RP1-Ig if used at a concentration of 10 μg/ml, but has no agonistic activity. The isotype-matched control IgG1 Ab MOPC-21 was obtained from Sigma-Aldrich (St. Louis, MO).

Generation of human B7RP1-transfected Chinese hamster ovary (CHO) cell line

CHO DG44 cells were transfected by electroporation with linearized full-length human B7RP1 cDNA and selected for successful transfection by culture in the presence of G418 (Life Technologies-BRL). B7RP1-expressing cells were selected by FACS using ICOS-Ig or anti-hB7RP1 Abs and appropriate secondary Abs, and cloned by limiting dilution. The selected cell line was kept in culture in IMDM (Life Technologies, Paisley, U.K.) supplemented with 10% FCS (HyClone, Logan, UT), gentamicin (86 μg/ml; Duchefa, Haarlem, The Netherlands), and G418 (0.8 mg/ml) in 75-cm^2 culture flasks (Corning Glass, Corning, NY). To detach the adherent cell line from the culture bottles, cells were treated with 0.025% trypsin (Life Technologies-BRL), 2 mM EDTA for 10 min at 37°C, suspended, and washed. To prepare confluent layers of cells on the bottom of 96-well culture plates, 20,000 cells were plated 2 days before the start of the experiment. Nontransfected CHO cells, kindly provided by Dr. J. Borst (The Netherlands Cancer Intitute, Amsterdam, The Netherlands) were used as control cells.

Abs, cytokines, and reagents

Mouse mAb to human CD3 (CLB-T3/3 and CLB-T3/4E) and CD28 (CLB-CD28/1) were obtained from Sanquin (Amsterdam, The Netherlands). Neutralizing mouse mAb to human IL-4 (CLB-IL-4/6) was a gift from Dr. L. A. Aarden (Sanquin). Human rIFN-γ (sp. act. 8×10^7 U/mg) and neutralizing rabbit IgG to human IL-12 were gifts from Dr. P.H. van de Meide (U-cytech, Utrecht, The Netherlands). Human rIL-4 (sp. act. 1×10^8 U/mg) was obtained from Pharma Biotechnology (Hannover, Germany). Human rIL-12 (sp. act. 1.7×10^8 U/mg) was a gift from Dr. M.K. Gately (Hoffman-La Roche, Nutley, NJ). Human rGM-CSF (sp. act. 1.11×10^7 U/mg) and rIFN-β (Betaseron) were gifts of Schering-Plough (Uden, The Netherlands). *Escherichia coli*-derived LPS was purchased from Difco (Detroit, MI); rIL-1β, rTNF-α, and rIFN-α from PeproTech (Rocky Hill, NY); rIL-6, rIL-10, and rIL-13 from Strathmann (Hannover, Germany); rIL-15, rIL-18, and rIL-23 from R&D Systems (Minneapolis, MN); PGE_2, PMA, ionomycin, and brefeldin A from Sigma-Aldrich; and PHA from Difco.

Isolation of CD45RA$^+$ Th cells

PBMCs were isolated from buffy coats from healthy donors (Sanquin) by density centrifugation on Lymphoprep (Nycomed, Torshov, Norway). Total CD4$^+$ Th cells were isolated by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. To obtain pure CD4$^+$CD45RA$^+$CD45RO$^-$ Th cells in one round of negative selection, the procedure for total Th cell isolation was modified by adding anti-CD45RO-PE (Dakopatts, Glostrup, Denmark) to the labeling mix and anti-PE-coupled magnetic beads to the CD4$^+$ MACS Beads (Miltenyi Biotec). This procedure normally yielded >98% pure CD45RA$^+$ Th cell populations, as assessed by flow cytometry (data not shown). The experiments were performed in compliance with relevant laws and institutional guidelines.

Generation of polarized Th1 and Th2 cells

Polarized Th1 and Th2 cell lines and unpolarized Th0 cell lines were generated from purified CD45RA$^+$ Th cells, as described before (22). Briefly, purified CD45RA$^+$ Th cells (10^5 cells/200 μl) were stimulated with immobilized anti-CD3 mAb (CLB-T3/3; 1 μg/ml) and anti-CD28 mAb (2 μg/ml) in 96-well flat-bottom enzyme immunoassay/RIA plates (Corning Glass) and cultured for 10 days in the absence (Th0 cell lines) or presence of rIL-4 (1000 U/ml; for Th2 cell lines) or rIL-12 (100 U/ml; for Th1 cell lines). To generate fully polarized Th cells, the cell lines were restimulated twice with PHA (10 μg/ml) and 3000-rad irradiated feeder cells (PBMC of two unrelated donors and EBV-B cells (JY cells)) in the absence (for Th0 cell lines), or presence of either rIL-4 (1000 U/ml) plus neutralizing anti-IL-12 (10 μg/ml) (for Th2 cell lines), or rIL-12 (100 U/ml) plus neutralizing anti-IL-4 mAb (1 μg/ml) (for Th1 cell lines). After three rounds of stimulation, polarization was confirmed by intracellular cytokine staining, as described before (22). All T cell cultures were performed in IMDM supplemented with 10% FCS, gentamicin (86 μg/ml), and rIL-2 (10 U/ml; Chiron, Emeryville, CA). Expansion of the cells was performed in 24-well flat-bottom culture plates (Corning Glass).

Analysis of cell surface ICOS expression

For detection of cell surface expressed ICOS protein, Th cells were labeled with anti-ICOS mAb (5 μg/ml) for 30 min on ice. The isotype-matched Ab MOPC-21 (5 μg/ml) was used as a control. Labeled samples were incubated with FITC-conjugated goat F(ab′)2 anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min on ice and analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

Generation of monocyte-derived mature DC with different IL-12-producing capacities

Monocytes were isolated from PBMC using density centrifugation on a Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient, as described elsewhere (23). Immature DC (iDC) were generated by culturing the monocytes for 6 days in IMDM containing 2.5% FCS and gentamicin, and supplemented with rGM-CSF (500 U/ml) and rIL-4 (250 U/ml), as described before (24). On day 6, the obtained iDC were matured by a 2-day exposure to mixtures consisting of the following factors: 1) IL-1β (10 ng/ml), TNF-α (50 ng/ml), and LPS (100 ng/ml), further referred to as maturation factors (MF), 2) MF plus IFN-γ (1000 U/ml), or 3) MF plus PGE_2 (10^{-6} M). Maturation was confirmed by flow cytometric analysis of CD83 and CD86 expression (data not shown).

ICOS ligation on 2-day-preactivated Th cells with B7RP1-expressing cells

Polarized Th1 and Th2 cell lines were generated from CD45RA$^+$ Th cells, as described above. On day 10 after the last polarizing stimulation, quiescent cells were restimulated with anti-CD3 (CLB-T3/4E; 2 μg/ml) and anti-CD28 (2 μg/ml), in the absence or presence of rIL-12 (100 U/ml) to modulate ICOS expression. After 2 days, the activated cells were harvested and washed to remove thus far secreted cytokines. A small aliquot of the cells was used to confirm differential ICOS expression by flow cytometric analysis. To ligate ICOS, the remaining cells were cultured (10^5 cells/well) for an additional 24 h on a confluent monolayer of B7RP1-transfected CHO cells or in the presence of B7RP1-expressing autologous iDC (25,000 cells/well). Cultures of Th cells alone or with nontransfected CHO cells were included as control conditions. Before coculture, the CHO cell lines or iDC were preincubated for 1 h at 37°C in the absence or presence of ICOS-Ig fusion protein to block the ICOS-B7RP1 pathway. After 24 h, supernatants were harvested and stored at −20°C until cytokine production was measured by ELISA.

Cytokines measurements by ELISA

Measurement of secreted cytokines was performed by specific solid-phase sandwich ELISAs in 24-h culture supernatants. Pairs of specific mAbs and

recombinant cytokine standards were obtained from BioSource International (Camarillo, CA) for the determination of GM-CSF and IL-2, and from BD Pharmingen (San Diego, CA) to measure IL-10. The ELISAs for IFN-γ and IL-4 (25) and for IL-12 p70 (26) were all described previously. IL-13 was determined using a specific ELISA kit (Sanquin) according to manufacturer's instructions. The detection limits of these ELISAs are as follows: GM-CSF, 40 pg/ml; IFN-γ, 100 pg/ml; IL-2, 5 U/ml; IL-4, 60 pg/ml; IL-10, 25 pg/ml; and IL-13, 3 pg/ml.

Statistical analysis

The kinetics of ICOS expression of the different cell lines were analyzed for the area under the curve and compared for statistical significance with the GraphPad InStat software (version 3.00; GraphPad Software, San Diego, CA) using ANOVA. In other experiments ICOS expression was analyzed using ANOVA, followed either by Tukey's or Dunnett's multiple comparison tests, where applicable. A p value of <0.05 was considered as the level of significance.

Results

Increased ICOS expression on Th1 cells

To compare the kinetics of ICOS expression on developing human Th1 and Th2 cells, CD45RA$^+$ Th cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-12 or IL-4, respectively, and ICOS expression was determined by flow cytometric analysis on various days after activation. Because of variability in the absolute expression levels between donors and between independent experiments, ICOS levels shown in Fig. 1*A* were calculated as the mean percentage of ICOS expression (change in mean fluorescence intensity (ΔMFI)) on autologous control Th0 cells that in each experiment were stimulated in parallel, in the absence of exogenous cytokines. As ICOS expression on unstimulated CD45RA$^+$ Th cells was below the detection limit of our assay, expression levels on day 2 after neutral stimulation were set at 100% (this procedure was maintained throughout Figs. 1–4). Th0 (●) and Th2 cells (□) showed a consistent but transient increase in ICOS expression (Fig. 1*A*). Compared with these two populations, however, expression of ICOS was strongly and significantly up-regulated on cells cultured in Th1-promoting conditions (■). ICOS levels peaked on day 3 and had dropped back by day 7 to basal but detectable levels that were similar for Th1, Th0, and Th2 cells. To monitor ICOS expression on pre-established Th1 and Th2 cells, resting cells were restimulated on day 10 under continuing polarizing conditions. Th0 cells, restimulated under neutral conditions, were included as control cells. ICOS expression during secondary responses was more rapid than in primary responses (Fig. 1*B*), not only in Th1 cells (■), but also in Th0 (●) and Th2 cells (□). As during primary responses, ICOS levels during secondary responses were significantly increased on IL-12-driven Th1 cells (Fig. 1*C*). In fact, ICOS expression levels obtained on days 2–3 upon restimulation in the presence of IL-12 proved to be ultimate levels that could not be further enhanced in individual experiments.

IL-12 enhances ICOS expression on activated Th cells irrespective of their polarization state

Cytokine production profiles are relatively stable features of terminally differentiated Th1 and Th2 cells (22). To investigate whether the differences in ICOS expression on Th1 and Th2 cells were stably acquired features, or merely dictated by the presence of the exogenous polarizing cytokines, we further studied the effects of IL-12 and IL-4 on ICOS expression by fully committed Th1 and Th2 cells, obtained by three consecutive rounds of stimulation under polarizing conditions. Th0 cells obtained by three rounds of stimulation under neutral conditions were tested again as a control population. The addition of IL-12 and neutralizing anti-IL-4 mAb during restimulation with anti-CD3 and anti-CD28 resulted in a significant increase in activation-induced ICOS expression in all three subsets, as shown for day 2 in Fig. 2 (▨). Identical results were obtained with freshly isolated CD45RO$^+$ Th cells (data not shown). Th1 cells restimulated in the absence of IL-12 (■) did no longer show increased ICOS expression, as compared with Th0 cells. Th2 cells showed a consistent trend of reduced ICOS expression compared with Th0 cells, but the reduction was never significant (Figs. 1 and 2). The addition of IL-4 during restimulation had no effect on ICOS expression on either subset (□), nor had the addition of neutralizing anti-IL-4 alone (data not shown). Taken together, these data show a dominant role of IL-12 in the control of ICOS expression on activated human Th cells, irrespective of the polarization state of the cells, and indicate that ICOS peak levels on fully polarized Th cells are not imprinted.

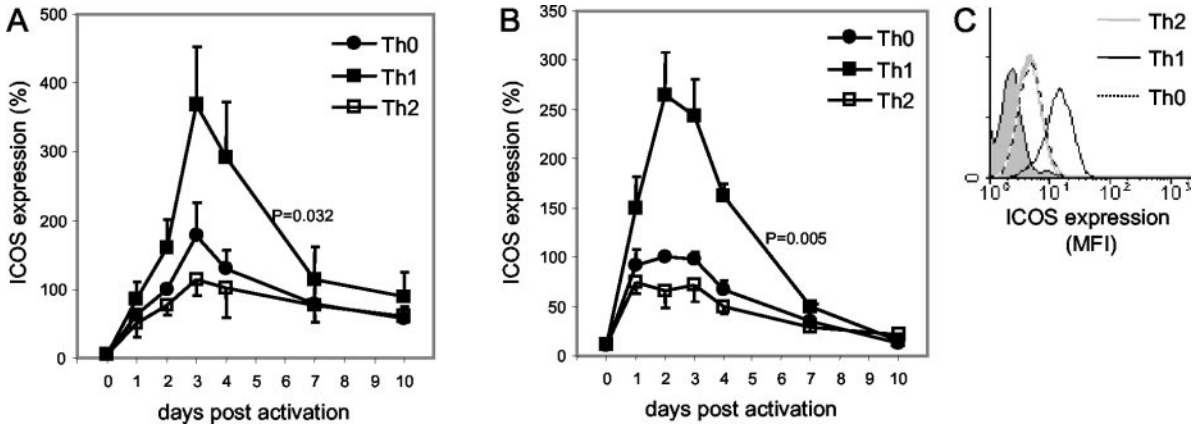

FIGURE 1. Transiently increased ICOS expression by Th1 cells. *A*, CD45RA$^+$ Th cells were stimulated with anti-CD3 and anti-CD28 in the absence (●) or presence of either IL-12 plus anti-IL-4 (■) or IL-4 (□), to generate Th0, Th1, or Th2 cell lines, respectively. On days 0, 1, 2, 3, 4, 7, and 10 after activation, ICOS expression was analyzed by flow cytometry using an ICOS-specific mAb and calculated as ΔMFI, representing the difference between the anti-ICOS signal and the isotype control mAb signal. For each of three different donors, the ΔMFI of Th0 cells on day 2 was set at 100% and the other results were expressed as the mean percentage (± SEM) of this value. For each cell line, the area under the curve was calculated and the different curves were compared for statistical significance using ANOVA. Values of $p < 0.05$ are given in the figure. *B*, On day 10, exactly the same experiment was repeated with the expanded and rested cell lines of all three donors. *C*, Absolute ICOS expression levels on 2-day-restimulated Th1, Th0, and Th2 cells on day 2 are plotted in a histogram for one representative donor. The filled curve represents the signal of the isotype control, the gray line represents the Th2 cells, the dotted line represents the Th0 cells, and the black line represents the Th1 cells.

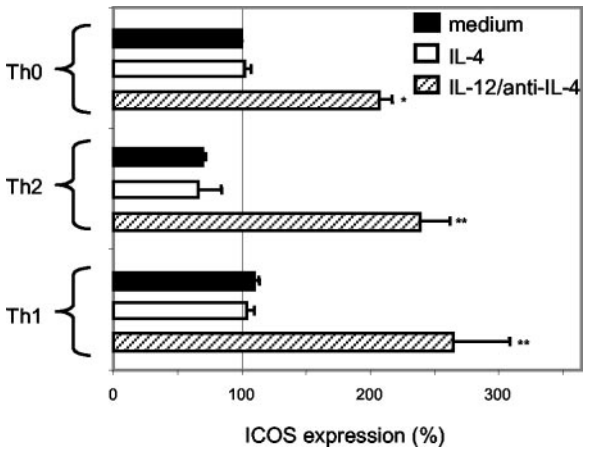

FIGURE 2. IL-12 enhances ICOS expression on activated Th cells irrespective of their polarized state. Quiescent polarized Th1 and Th2 cells and nonpolarized Th0 cells were restimulated with anti-CD3 and anti-CD28 in the absence (■) or presence of either IL-4 (□) or IL-12 plus anti-IL-4 (▨). ICOS expression was measured by flow cytometry during peak levels on day 2, calculated as described in Fig. 1, and expressed as percentage of the ΔMFI of neutrally stimulated Th0 cells (*top bar*). Results are expressed as the mean percentages (± SEM) of three independent experiments. Data were analyzed for statistical significance using ANOVA, followed by Dunnett's multiple comparison test with Th0 cells plus medium as a control. *, $p < 0.05$; **, $p < 0.01$.

Analysis of the effects of other soluble mediators on ICOS expression

As ICOS expression appeared to be dictated by exogenous IL-12, rather than by established polarized phenotypes, the same experimental setting was applied to test the effects of a panel of other factors that may be present during T cell activation in lymphoid organs or in inflamed tissues. These factors were tested at concentrations that are commonly used for in vitro studies with human immune cells. The results presented in Fig. 3*A* are shown for Th0 cells only, but were identical for Th1 and Th2 cells (data not shown). We found that within the analyzed panel of factors at these concentrations, apart from IL-12, only IL-23 significantly enhanced ICOS expression above levels obtained with neutral stimulation, albeit to a lesser extent than IL-12. The combination of IL-12 and IL-23 in optimal concentrations resulted in an intermediate level of ICOS expression between the levels induced by IL-12 or IL-23 alone (Fig. 3*B*), indicating that the effects of IL-12 and IL-23 are clearly not additive or synergistic. Next, we tested the effects of IL-4 on the ICOS-enhancing effects of IL-12 and IL-23. Although IL-4 had no significant effect on anti-CD3/CD28-induced ICOS expression, it significantly reduced the IL-12- and IL-23-mediated up-regulation of ICOS expression (Fig. 4*A*). At a 10-fold lower IL-12 concentration, IL-4 completely blocked the IL-12-mediated increase in ICOS expression in a dose-dependent fashion (Fig. 4*B*).

Role of DC-derived IL-12 and IL-23 in the regulation of ICOS expression

To investigate the combined contribution of IL-12 and IL-23 to the control of ICOS expression during a more physiological setting of DC-T cell interaction, we stimulated $CD45RA^+$ Th cells with super Ag staphylococcal enterotoxin B (SEB), presented by mature DC subsets with different IL-12- and IL-23-producing capacities, and measured ICOS expression by the T cells on day 4. For this purpose, we generated mature DC by culturing monocyte-derived iDC for 2 days with MF alone (LPS DC), with MF plus IFN-γ (IFN-γ DC), or with MF plus PGE_2 (PGE_2 DC). As shown before (27), this procedure yielded mature DC that on average produced moderate (300 pg/ml), high (3000 pg/ml), or low levels (30 pg/ml) of IL-12, respectively, when stimulated with CD40L (data not shown). Real time RT-PCR analysis showed substantial IL-23 p19 mRNA expression in CD40L-stimulated IFN-γ DC, whereas expression was not detectable in LPS DC and PGE_2 DC (data not

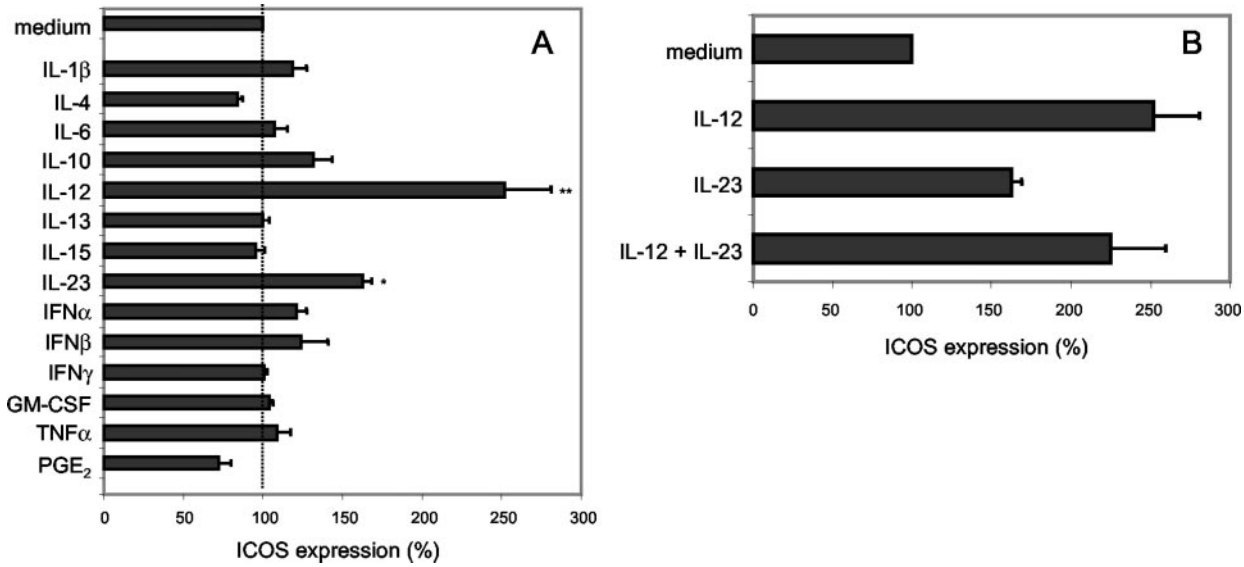

FIGURE 3. Effect of soluble mediators on ICOS expression levels. Quiescent Th0 cells were restimulated with anti-CD3 and anti-CD28 in the absence (medium) or presence of IL-1β (10 ng/ml), IL-4 (1000 U/ml), IL-6 (1000 U/ml), IL-10 (100 U/ml), IL-12 (100 U/ml), IL-13 (25 ng/ml), IL-15 (1 ng/ml), IL-23 (3 ng/ml), IFN-α (1000 U/ml), IFN-β (1000 U/ml), IFN-γ (1000 U/ml), GM-CSF (500 U/ml), TNF-α (50 ng/ml), or PGE_2 (10^{-6} M) (*A*), or in the absence or presence of optimal concentrations of IL-12 and/or IL-23 (*B*). ICOS expression was analyzed on day 2 by flow cytometry and expressed as percentage of the ΔMFI of Th0 cells stimulated in medium alone. Results are expressed as the mean percentages (± SEM) of four independent experiments. Data of Fig. 3*A* were analyzed for statistical significance using ANOVA, followed by Dunnett's multiple comparison test with Th0 cells plus medium as a control. *, $p < 0.05$; **, $p < 0.01$.

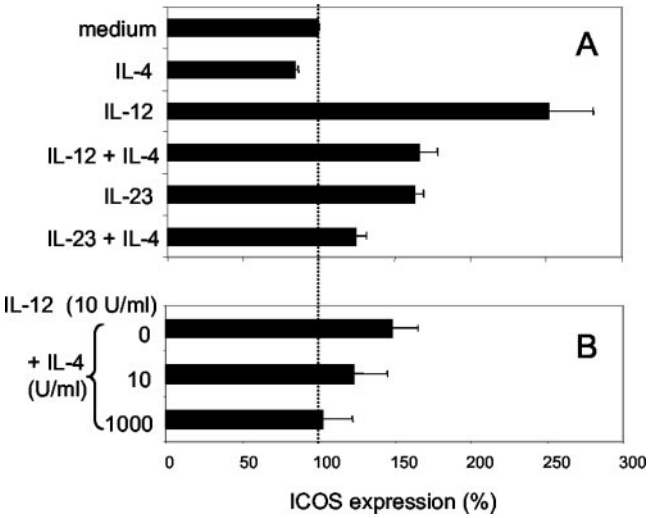

FIGURE 4. IL-4 inhibits the IL-12- and IL-23-mediated up-regulation of ICOS expression. *A*, Th0 cells were restimulated with anti-CD3 plus anti-CD28 in the absence (medium) or presence of IL-12 (100 U/ml) or IL-23 (3 ng/ml), all with or without IL-4 (1000 U/ml). *B*, Alternatively, cells were stimulated in the presence of a 10-fold lower IL-12 concentration (10 U/ml) and an increasing dose of IL-4. ICOS expression was analyzed on day 2 by flow cytometry, processed as described in Fig. 1, and expressed as percentage of the ΔMFI of Th0 cells stimulated in medium alone. Results are expressed as the mean percentages (± SEM) of four independent experiments. Data were analyzed for statistical significance using ANOVA, followed by Tukey's multiple comparison test. $*$, $p < 0.05$.

shown). There was a clear positive correlation between the expression levels of IL-12 p70 protein and IL-23 p19 mRNA by the DC subsets, on the one hand, and the resulting ICOS expression levels on the Th cells activated by these DC, on the other hand, with the strongest ICOS induction obtained with the high IL-12- and p19-expressing IFN-γ DC (Fig. 5). The combined contribution of DC-derived IL-12 and IL-23 to the regulation of ICOS expression was analyzed by comparing ICOS levels obtained in the absence or

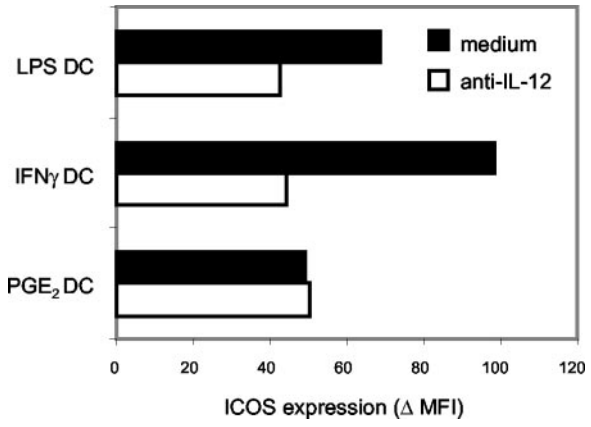

FIGURE 5. DC-derived IL-12 and IL-23 determine ICOS expression levels on SEB-stimulated Th cells. iDC were generated by culturing peripheral blood monocytes for 6 days in IL-4 and GM-CSF. Subsequently, iDC were induced to mature into DC with different IL-12- and IL-23-producing capacities by culturing them for 48 h in the presence of either TNF-α plus IL-1β plus LPS (referred to as MF), MF plus IFN-γ, or MF plus PGE_2. The differentially matured DC were used to stimulate $CD45RA^+$ Th cells with SEB (25 pg/ml) in the absence or presence of anti-IL-12. ICOS expression was analyzed by flow cytometry on day 4 and expressed as ΔMFI. Results of one representative experiment of three are shown.

presence of a polyclonal anti-IL-12 serum, neutralizing the activities of both IL-12 and IL-23. The differences in ICOS expression obtained with the three DC subsets were abolished in the presence of the antiserum (Fig. 5, □), providing no indication that other DC-derived factors have a major role in the modulation of ICOS expression by activated Th cells.

IL-12 maintains its ICOS-regulatory function during the effector phase of activated Th cells

The data so far suggest that levels of ICOS expression on activated Th cells are transient and controlled by cytokines in the local environment, and not by permanently imprinted changes. To test the effects of cytokines on ICOS expression at later time points after activation, we extended our kinetics studies shown in Fig. 1 by analyzing the impact of IL-12, IL-23, and IL-4 whether freshly added on day 3. To this aim, Th1 and Th2 cells were restimulated as before under continued polarizing conditions, but now washed on day 3 and cultured further in IL-2 alone (medium control), or in the additional presence of IL-12, IL-23, or IL-4. As shown in Fig. 6, the addition of IL-12 could still significantly increase ICOS expression on day 4, both on Th1 and Th2 cells. Likewise, addition of IL-23 also enhanced ICOS expression on day 4. The presence of IL-12 or IL-23 did not prolong the time window of increased ICOS expression and the addition of fresh IL-12 or IL-23 on day 6 had no effect anymore (data not shown), suggesting that the control of ICOS expression by IL-12 and IL-23 is restricted to the effector phase of activated Th cells.

Functional consequence of variable ICOS levels on effector Th cells

We next addressed the question of whether the cytokine-mediated regulation of ICOS expression affects the effector function of Th cells. If it does, the consequence of variable ICOS expression is probably most evident when differences are optimal, i.e., at 2–4 days after activation in the absence or presence of IL-12 (Fig. 1). To test this hypothesis, we designed a culture model where Th0 cells were restimulated with anti-CD3 and anti-CD28 for 2 days in the absence or presence of IL-12 to obtain low- or high-ICOS-expressing cells, respectively (Fig. 2). After 48 h, cells were washed and cultured for another 24 h, either alone or, to trigger ICOS, on top of a confluent monolayer of B7RP1-transfected CHO cells. Nontransfected CHO cells were used as a control. The effect of ICOS ligation was determined by measuring cytokine production during these additional 24 h of culture. Cytokine production in the absence CHO cells was set at 100% for individual Th cell lines and for each cytokine (Fig. 7*A*). Exposure of the preactivated Th cells to B7RP1-CHO cells on day 2 significantly increased the production of all cytokines measured. The magnitude of the increase in cytokine production was proportional to the expression level of ICOS, i.e., the percentage of increase was always higher in the high-ICOS-expressing cells, obtained by prestimulation in the presence of IL-12 (Fig. 7*A*, ■), as compared with the low-ICOS-expressing cells (Fig. 7*A*, ●), obtained by prestimulation in the absence of IL-12. Nontransfected CHO cells had no effect on cytokine production (Fig. 7*A*, □ and ○). The contribution of ICOS to the B7RP1-mediated increase in cytokine production was indicated by the abolishment of this increase by blocking the ICOS-B7RP1 interaction by preincubation of the CHO cells with ICOS-Ig. In a similar experimental set up, polarized Th1 and Th2 cells were restimulated under continued polarizing conditions, yielding cells expressing high or low levels of ICOS on day 2 (Fig. 1). The polarized cytokine profiles in these start populations were confirmed by ELISA in supernatants collected 24 h after onset of this preactivation (data not shown). Exposure of these preactivated Th

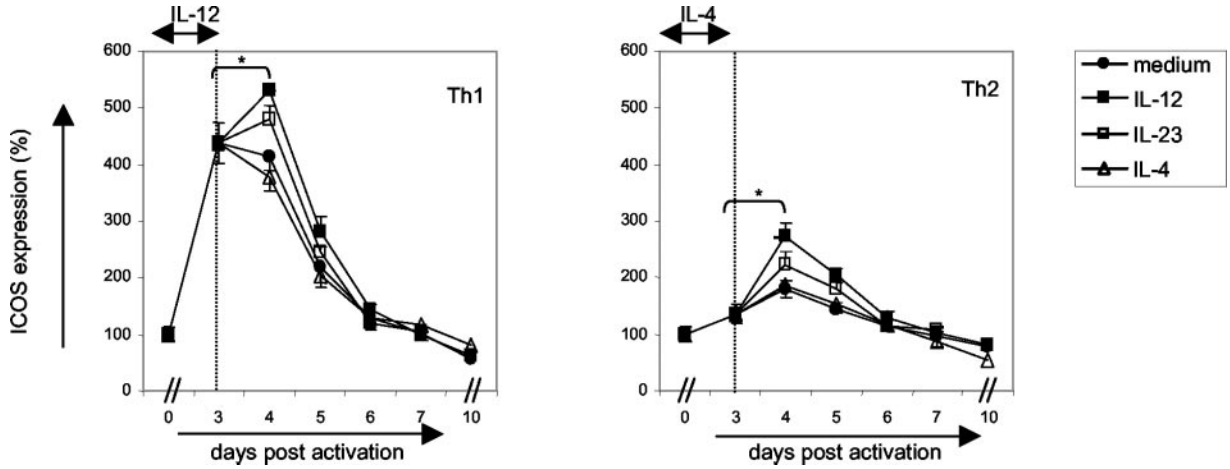

FIGURE 6. IL-12 and IL-23 enhance ICOS expression throughout the effector phase. Polarized Th1 and Th2 cells were restimulated with anti-CD3 and anti-CD28 in the presence of IL-12 plus anti-IL-4 (Th1 cells) or in the presence of IL-4 (Th2 cells). On day 3, cells were extensively washed to remove residual cytokines and Abs, and cultured further in the absence of cytokines (medium control) or in the presence of IL-12, IL-23, or IL-4. ICOS expression was analyzed by flow cytometry on days 0, 3–7, and 10. ΔMFI on day 0 was set at 100% for resting cells and the other results were expressed as the percentage of this value. Results are expressed as the mean percentages (± SEM) of three independent experiments. Data were analyzed for statistical significance using ANOVA followed by Dunnett's multiple comparison test with Th1 or Th2 cells on day 3 as controls. *, $p < 0.05$.

cells to B7RP1-CHO cells on day 2 clearly induced a greater increase in cytokine production in the Th1 cells expressing high levels of ICOS (Fig. 7*B*, ▲), as compared with the Th2 cells expressing low levels of ICOS (△). Notably, the increased secretion levels did not essentially change the polarized Th1/Th2 profiles. For example, although the percentage of increase of IL-13 secretion by the high-ICOS-expressing Th1 cells was higher than in the low-ICOS-expressing Th2 cells, absolute quantities of IL-13 production were still higher in the Th2 cells. Possibly due to early kinetics and/or autoconsumption, IL-2 and IL-4 produced on day 3 was not detectable in any of the cultures. To test the relative contribution of ICOS in the context of a more physiological cross-talk between effector Th cells and DC, the experiments with Th0 cells preactivated for 2 days in the absence or presence of IL-12 were extended by replacing the B7RP1-tranfected CHO cells by autologous B7RP1-expressing iDC, a cell type likely to interact with effector Th cells in inflamed peripheral tissues. Similar to B7RP1-transfected CHO cells, iDC increased the cytokine production levels of the T cells and the increase was higher in the high-ICOS-expressing Th0 cells (Fig. 7*C*, ■), as compared with the low-ICOS-expressing cells (●). However, blocking experiments with ICOS-Ig indicated that ICOS has a major, but not an exclusive role in these cytokine enhancing effects, suggesting a contribution of additional costimulatory pathways. It has been shown, for example, that IL-12 can restore CD28 expression on human $CD4^+CD28$ null T cell clones and lines, which will most likely influence costimulatory events (28). Collectively, our results indicate that triggering of ICOS on effector Th cells by B7RP1-expressing cells amplifies the effector response by increasing the cytokine production levels. The magnitude of this increase is proportional to the ICOS expression level, which in turn is subject to prolonged regulation by IL-12 and IL-23.

Discussion

Our present data show for the first time that ICOS expression by activated human Th cells is up-regulated by IL-12 and IL-23 and, in contrast to data shown in the mouse, not by IL-4. Instead, IL-4 reduced the ICOS up-regulatory effects of IL-12 and IL-23. These results were obtained, without exception, with cells of over 20 nonrelated healthy individuals. As a consequence of the effect of IL-12, ICOS expression is strongly increased on in vitro generated Th1 cells. However, unlike expression of signature Th1 and Th2 cytokines, ICOS expression levels are not stable features of polarized Th cells, but depend on local environmental cytokine concentrations after each restimulation. Blocking experiments in cocultures with Ag-presenting DC and the analysis of a panel of inflammatory mediators suggest that IL-12 and IL-23 are of major relevance in this respect. Their similar effects may not be unexpected, as IL-12 and IL-23 are closely related cytokines that share several structural and functional properties. IL-23 is a heterodimeric molecule composed of the IL-23-specific p19 subunit and the p40 subunit, which it shares with IL-12 (29), and the IL-23R is a heterodimer composed of the IL-12Rβ1 subunit of the IL-12R and a subunit referred to as IL-23R (30). The similarity in the intracellular signaling cascades induced by ligation of these receptors (30) probably explains the overlapping biological activities of both cytokines on activated Th cells (29). However, the effects of IL-12 and IL-23 are not identical and with respect to the enhancing effect on ICOS expression IL-23 is less potent. The finding that the simultaneous addition of IL-12 and IL-23 in optimal concentrations resulted in an intermediate ICOS expression level between the IL-12- and IL-23-induced levels may reflect competition for ligation of the IL-12Rβ1 subunit of their respective receptors. IL-4 had no effect on TCR-induced ICOS expression, but it significantly inhibited the ICOS-enhancing capacities of IL-12 and IL-23, suggesting interference in the expression or signaling of the IL-12R and IL-23R. Indeed, IL-4 is known to suppress expression of the IL-12-specific signaling β2 subunit of the IL-12R (31), and not of the IL-12Rβ1 subunit. An effect of IL-4 on the expression of the IL-23R has not been reported, so far.

The evidence indicating that ICOS expression in the mouse is up-regulated by IL-4 is quite solid. For example, ICOS expression is strongly increased on mouse Th2 cells upon restimulation in the presence of IL-4 (5, 13), and activated Th cells of the Th2-prone BALB/c mice strain show a 6-fold higher expression of ICOS, as compared with Th cells of C57BL/6 mice (20). Furthermore, both exogenously administered IL-4 and ectopically expressed GATA-3 increased ICOS expression, irrespective of the strain of mice (21). The apparent reciprocal regulation of ICOS expression on mouse and human Th cells by T cell-polarizing cytokines is

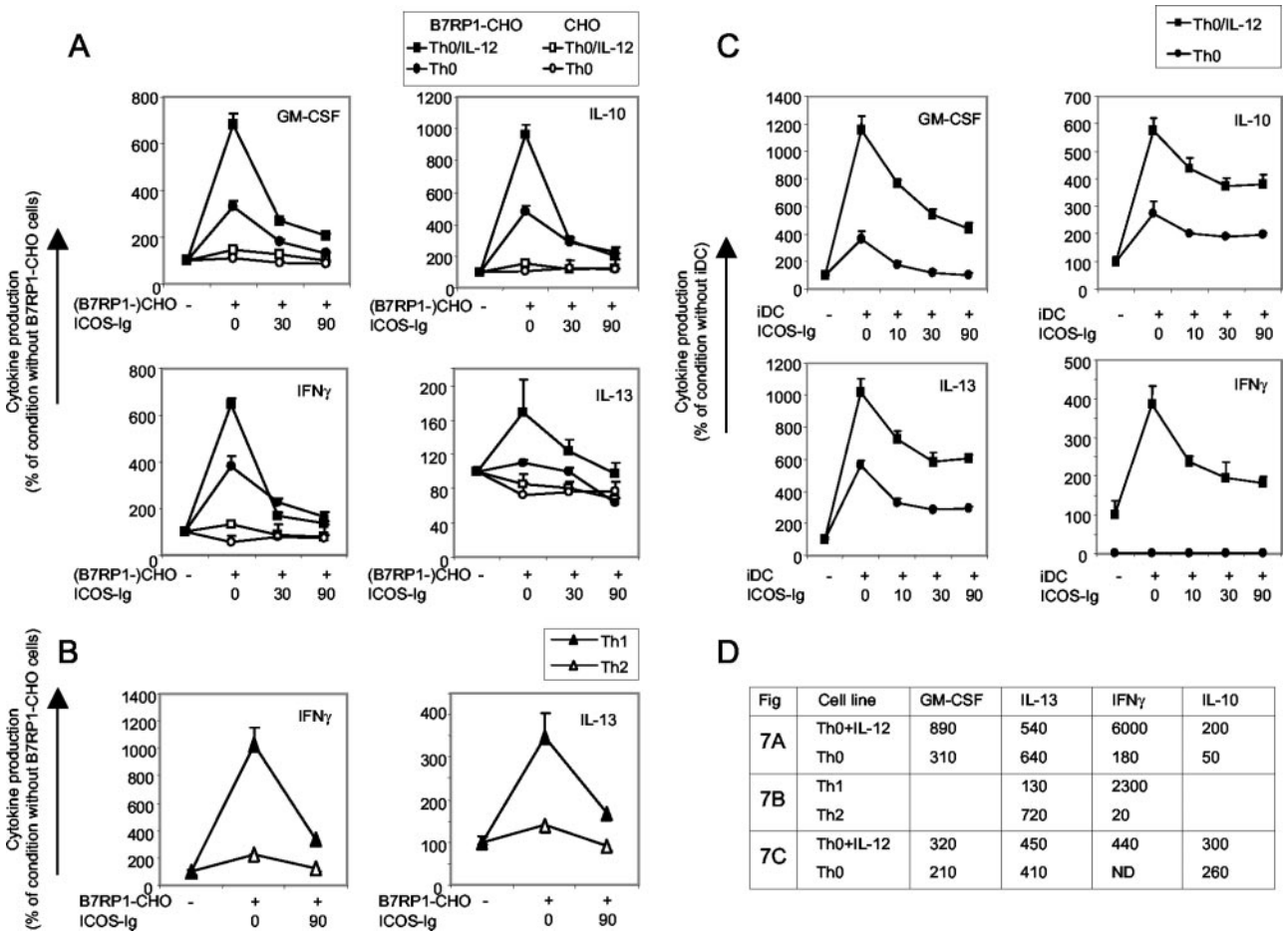

Fig	Cell line	GM-CSF	IL-13	IFNγ	IL-10
7A	Th0+IL-12	890	540	6000	200
	Th0	310	640	180	50
7B	Th1		130	2300	
	Th2		720	20	
7C	Th0+IL-12	320	450	440	300
	Th0	210	410	ND	260

FIGURE 7. Functional consequence of variable ICOS expression levels. Th0 cell lines were restimulated with anti-CD3/CD28 for 2 days in the absence (Th0) or presence of IL-12 plus anti-IL-4 (Th0/IL-12), to obtain cells expressing different ICOS levels. On day 2, cells were washed and seeded onto confluent layers of B7RP1-transfected CHO cells or nontransfected CHO cells, and after 24 h, T cell cytokines secreted during day 3 were measured by ELISA. *A*, To block ICOS ligation, the CHO cells were preincubated with an increasing dose of ICOS-Ig. *B*, In a similar experiment, Th1 and Th2 cells were restimulated for 2 days under continuing polarizing conditions, washed, and exposed to B7RP1-transfected CHO cells. *C*, Alternatively, 2-day-preactivated Th0 and Th0/IL-12 cells (obtained as in *A*) were cocultured with autologous B7RP1-expressing iDC and further analyzed as described in *A*. *D*, Cytokine levels obtained in the absence of CHO cells or iDC were determined by ELISA and expressed in picograms per milliliter. These neutral production levels were set at 100% for each Th cell line and for each cytokine, and production levels obtained in the presence of CHO cells or iDC were expressed as the percentage of these values. Results are expressed as the mean ± SD of triplicate cultures from one representative experiment of three.

striking and urges for caution when extrapolating mouse data to the human situation.

Several studies in the human system have indicated the importance of the costimulatory function of ICOS for the effector function of activated Th cells and suggest an active contribution of this molecule in inflamed peripheral tissues (1, 9, 12, 32). Indeed, a previous study showed that memory, but not naive, Th cells could be induced to secrete cytokines by B7RP1-expressing endothelial cells in the presence of super Ag SEB, but in the absence of CD80 and CD86, and that this cytokine release could be reduced considerably by blocking the ICOS-B7RP1 interaction (9). This study suggests that ICOS-ligation in the absence of CD28 costimulation is important for cytokine release by activated T cells in peripheral tissues. In this study, we show that ICOS ligation up to several days after onset of T cell activation significantly increases cytokine production levels of both Th1 and Th2 cells. There is some evidence to suggest that this ICOS-mediated mechanism of amplification of cytokine production might operate in peripheral tissues in vivo. For example, during both Th1- and Th2-inducing infections, $ICOS^+$ Th cells are localized to peripheral tissues, rather than to the lymph nodes (32), and B7RP1 expression is widely distributed over various lymphoid and nonlymphoid cell types in peripheral tissues, not only including professional APC, but also fibroblasts, endothelial cells, epithelial cells, and maybe even organ-specific tissue cells (2, 6–9, 12, 33–35), implicating ample opportunity for ICOS to meet its ligand.

Our present data show that the amplification of cytokine production by effector T cells is proportional to their ICOS expression level, and that this ICOS expression level varies with a changing cytokine environment. Our further observation that exposure of activated T cells to IL-12 and/or IL-23 could still enhance ICOS expression at least up to 4 days after onset of T cell activation allows for speculation that local regulation of ICOS expression on effector Th cells provides a mechanism to continuously control the strength of effector T cell responses in peripheral tissues. We found no indication that IL-12 and IL-23 affected pre-established cytokine profiles of polarized Th1 and Th2 cells, suggesting that this putative, peripheral, ICOS-mediated control mechanism supports both Th1 and Th2 responses. This could explain, for example, the paradoxical expression of IL-12 in the lesional skin of patients suffering from atopic dermatitis (36), a typical Th2 disease, and is in line with in vivo data in the mouse, showing that,

depending on the pathogen and the affected organ, ICOS expression can be positively correlated both with Th1- and Th2-type responses (32).

In conclusion, our data confirm the importance of ICOS for the effector function of human Th1 and Th2 cells and suggest the existence of a novel peripheral control mechanism focusing much of the cytokine production potential of tissue-infiltrating effector cells to the site of infection. The local cytokine milieu, IL-12 and IL-23 in particular, will regulate the expression level of ICOS, in turn determining the strength but not the type of effector T cell response. Based on data obtained in the mouse, it has been suggested that blockade of ICOS may be beneficial for patients suffering from allergic disease. However, the striking difference in the cytokine-mediated regulation of ICOS expression between human and mouse Th cells, as shown in this study, implies that extrapolation of mouse data to the human system will be complicated.

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