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ICOS Contributes to T Cell Expansion in CTLA-4 Deficient Mice[1]

Miranda E. A. T. van Berkel,* Elise H. R. Schrijver,* Frans M. A. Hofhuis,* Arlene H. Sharpe,† Anthony J. Coyle,‡ Chris P. Broeren,§ Kiki Tesselaar,* and Mariëtte A. Oosterwegel[2]*

Both CD28 and ICOS are important costimulatory molecules that promote Ag-specific cellular and humoral immune reactions. Whereas CD28 is generally thought to be the most important molecule in the initiation of a T cell response, ICOS is considered to act during the effector phase. We have investigated the contribution of ICOS to T cell responses in the absence of CTLA-4-mediated inhibition. Mice lacking CTLA-4, which show spontaneous CD28-mediated CD4$^+$ T cell activation, expansion and differentiation, were treated with antagonistic αICOS antibodies. Blocking the interaction between ICOS and its ligand B7RP-1 significantly reduced this aberrant T cell activation and caused a reduction in T cell numbers. In vitro analysis of CD4$^+$ T cells from treated mice revealed that ICOS blockade significantly reduced Th1 differentiation, while Th2 differentiation was only moderately inhibited. Further in vitro stimulation experiments demonstrated that ICOS is able to induce proliferation of murine CD4$^+$ and CD8$^+$ T cells but only in the presence of IL-2. These results indicate that ICOS is not only important for T cell effector function but also contributes to the expansion phase of a T cell response in the presence of CD28 signaling. ***The Journal of Immunology,*** **2005, 175: 182–188.**

For an optimal T cell response, a combination of signals generated by the Ag-specific TCR and costimulatory molecules is required. One of these is CD28, which is considered to be one of the most important costimulatory molecules during the onset of a T cell response. Ligation of CD28 with its ligands B7.1 and B7.2 can initiate T cell activation and production of IL-2 (reviewed in Refs. 1 and 2). Mice deficient for CD28 have disrupted primary T cell responses, which is reflected by reduced proliferative capacity, and a higher sensitivity to cell death (3–5). In addition, CD28 is important for CD4$^+$ T cell differentiation toward Th2, as shown in several disease models (reviewed in Refs. 1, 2, and 6). Although the requirement for CD28 in CD8$^+$ T cell responses seems to be less profound, defects in antiviral responses have been observed in the absence of CD28 (3, 7–9).

Similar functions have been described for the CD28 homologue ICOS (10–15). However, CD28 and ICOS differ in kinetics of expression, such that CD28 is constantly expressed on all T lymphocytes, whereas ICOS is enhanced after T cell activation (10, 11, 16). Like CD28, ICOS is required for both Th1 and Th2 development, being most indispensable in ongoing immune responses (17–21). This, together with the fact that ICOS triggering does not induce IL-2 production (11, 22), has led to the idea that ICOS is mainly important during the effector phase of the T cell response. Yet, there is evidence that ICOS can also support expansion of CD4$^+$ and CD8$^+$ T cells. In vitro, co-crosslinking the TCR and ICOS initiates CD4$^+$ T cell activation, as monitored by the induction of T cell proliferation (10, 11). Furthermore, primary CD4$^+$ T cell responses are diminished in the absence of ICOS signaling (14, 23). In addition, continuous stimulation of ICOS causes an enhanced cellular response in vivo, in terms of activation and cell numbers (10, 24). Although the function of ICOS is most studied on Th cells, the importance of ICOS on CTLs is also starting to become clear. Antitumor responses mediated by CD8$^+$ T cells could be enhanced by ICOS (25, 26). Moreover, expansion of CD8$^+$ T cells in an allograft rejection model was decreased after blocking ICOS (27, 28, 29). In contrast, ICOS is not required for a prominent primary antiviral response (18, 19).

CTLA-4 (CD152), the inhibitory receptor that opposes CD28, also binds B7.1 and B7.2 and serves to down-regulate T cell activation (30, 31). This is most clearly seen in mice that lack CTLA-4. These mice display severe T cell activation and expansion of the CD4$^+$ and CD8$^+$ T cell pool, which is considered to be driven by autoantigens (32). The CD4$^+$ T cells present in these mice are differentiated, mostly into Th2-type CD4$^+$ T cells. The lymphoproliferative phenotype is dependent on signals generated by CD28, because abrogating CD28 and B7.1/B7.2 interaction by gene targeting or blocking agents completely abolishes this aberrant T cell activation (33–35).

Because CD28 and ICOS function similarly in many ways, we questioned which part of the elicited T cell activation and differentiation in CTLA-4$^{-/-}$ mice could be attributed to signals generated by ICOS. Therefore, the ICOS-B7RP-1 interaction was blocked using an antagonistic Ab in mice deficient for CTLA-4. The aberrant T cell activation and high T cell numbers normally observed in mice lacking CTLA-4 were reduced by abrogating ICOS signaling, indicating a role for ICOS in T cell expansion. This observation is supported by in vitro studies where ICOS can initiate T cell expansion by induction of cell cycle progression in the presence of exogenous IL-2. So, in the presence of a CD28 signal, ICOS contributes to expansion of primed T cells.

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Materials and Methods

Mice and treatment

Mice deficient for B7.1, B7.2, and CTLA-4 (CTLA-4$^{-/-}$ B7.1/B7.2 $^{-/-}$) (34) and CTLA-4$^{-/-}$ (32) on a 129/SvS4Jae background, were kindly provided by A. Sharpe (Brigham and Women's Hospital, Boston, MA). C57BL/6 wild-type mice were purchased at Harlan. All mice were bred under specific pathogen-free conditions, housed in accordance with institutional guidelines of American Association of Accreditation of Laboratory Animal Care. In experiments, neonatal CTLA-4$^{-/-}$ mice were used. These mice were treated from birth with 10 μg per gram body weight antagonistic αICOS Ab (clone 12A8) or 4 μg per gram body weight murine CTLA-4-Ig (kind gift from A. Coyle, Millennium Pharmaceuticals, Cambridge), which were injected i.p. on alternating days for a period of 14 days. CTLA-4$^{-/-}$ B7.1/B7.2 $^{-/-}$ mice were used in experiments at 6–8 wk of age and treated with 100 μg of agonistic αCD28 Ab PV-1, kindly provided by C. Broeren (University of Utrecht) (36). αCD28 Ab was injected i.v. on days 0, 7, and 14. Furthermore, these mice received 100 μg of either 12A8 or rat IgGs (own production) i.p. on alternating days, starting at day 0. Mice treated with CD4$^+$ depleting Ab (GK1.5) received 50 μg i.p. on alternating days, starting 3 days before αCD28 treatment. Mice were sacrificed and lymph nodes were collected for analysis on day 17.

Flow cytometry

Cell suspensions were prepared from pooled isolated lymph nodes, counted and stained with a panel of fluorochrome-conjugated Abs, all purchased at BD Pharmingen, except for αICOS Ab (C398.4A; eBioscience). The Ab stainings were performed in PBS/0.5% BSA and 0.1% azide supplemented with 2% goat serum. Nonspecific FcR binding was prevented by preincubating cells on ice with FcR-blocking Ab, clone 2.4G2 (BD Pharmingen) for 10 min. Subsequently, dilutions of the staining Abs were added and cells were incubated at 4°C for 30 min. CD4$^+$ and CD8$^+$ T cells were analyzed for CD25, CD69, CD62L, CD44, CD45RB, and CD43 surface expression. Stained cells were washed twice before analysis on a flow cytometer (BD Biosciences).

T cell purification and T cell culture

CD4$^+$ T cells or CD8$^+$ T cells were purified from peripheral lymph nodes by positive selection with either αCD4 or αCD8 magnetic MACS beads (Miltenyi Biotec). Populations were reproducibly >98% pure. T cell populations were stimulated with magnetic beads (Dynal) covalently linked according to the manufacturer's instructions with either αCD3 (145-2C11)-hamster Ig (BD Pharmingen), αCD3-αCD28 (PV-1), or αCD3-αICOS (C398.4A; eBioscience) in 1:10 molar ratio. T cells were mixed in a 1:1 ratio with the Ab-coated beads at 1×10^5 cells per well and incubated at 37°C for several days. Exogenous IL-2 was added at a concentration of 360 U/ml. After the indicated numbers of days, supernatants were harvested for cytokine measurement.

Cell cycle analysis

Purified CD4$^+$ and CD8$^+$ T cells were labeled with the membrane dye CFSE for analysis of T cell proliferation. CD4$^+$ and CD8$^+$ T cells were harvested after 4 and 3 days of culture, respectively, counted, and the reduction of signal of the CFSE dye per cell was analyzed using flow cytometry. Cell viability was assessed by resuspending cell pellets in Viaprobe dye (BD Pharmingen) followed by incubation at room temperature for 5 min before analysis. The total number of viable/nonviable cells after each round of division was calculated as follows. Cell populations that displayed similar dilution of the CFSE dye were gated, so that each gate represented cells that underwent an equal number of cell divisions. Within each gated population the percentage of Viaprobe negative and positive cells were distinguished. The absolute numbers of cells in the acquired gates were calculated by taking the respective percentage of the counted total T cell numbers. Cell debris and beads were excluded from the analysis.

ELISA

To detect cytokine production from in vitro stimulated CD4$^+$ T cells, supernatants were collected at the indicated time points. Cytokines were quantified by sandwich ELISA purchased from BD Pharmingen (IL-2, IL-4, IL-10) or eBiosource (IFN-γ).

Statistical analysis

Results were analyzed using a Student's t test (two-tailed). Differences between groups were considered statistically significant at the $p < 0.05$ level. Data is expressed as mean ± SE of mean (SEM) unless indicated.

Results

ICOS expression is up-regulated on CD4$^+$ and CD8$^+$ T cells by CD28 costimulation

First, the effect of CD28 costimulation on the augmentation of ICOS expression was tested in vitro by coculturing T cells with Ab-coated beads. ICOS was readily detectable on the surface of both resting CD4$^+$ and CD8$^+$ T cells (Fig. 1). Stimulation with αCD3 in the presence of exogenous IL-2 enhanced the expression of ICOS to some degree on CD4$^+$ T cells, whereas on CD8$^+$ T cells IL-2 did not elevate the level of ICOS compared with αCD3 alone. Additional stimulation with αCD28 greatly enhanced the expression levels of ICOS on both CD4$^+$ and CD8$^+$ T cells, independent of the addition of IL-2. So, expression of ICOS can be up-regulated by signals generated by CD28 in conjunction with TCR signaling. These results prompted us to study the role of ICOS in CTLA-4 deficient mice, where a TCR-driven immune response was enhanced by a strong CD28 signal.

Blocking ICOS inhibits expansion of T cells in mice lacking CTLA-4

The contribution of ICOS to an in vivo immune response in the presence of a CD28 signal was determined in mice lacking CTLA-4. As expected, T cells from these mice had a 5- to 6-fold enhanced expression of ICOS when analyzed ex vivo (data not shown). Mice lacking CTLA-4 were treated from birth with the antagonistic αICOS Ab 12A8. As previously reported, this Ab functionally blocks the interaction between ICOS and its natural ligand B7RP-1 in vivo (21, 28, 37). Interestingly, administration of blocking αICOS Ab caused a 4-fold reduction in the normally observed expanded T cell pool in peripheral lymph nodes of CTLA-4$^{-/-}$ mice (Fig. 2). As shown previously, treatment with CTLA-4-Ig, preventing CD28 and B7.1/B7.2 interaction, completely reduced T cell numbers back to levels found in wild-type animals (33). Treatment with either CTLA-4-Ig or blocking αICOS Ab alone did not affect T cell numbers in wild-type mice (data not shown). So, the expansion of the T cell pool in mice

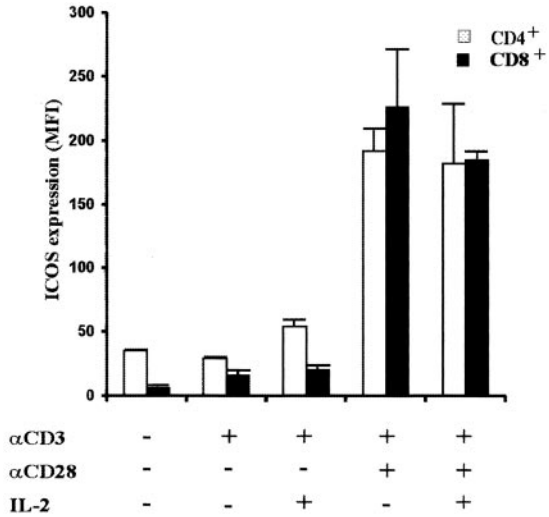

FIGURE 1. ICOS expression is enhanced by αCD28 stimulation. CD4$^+$ and CD8$^+$ T cells were purified from peripheral lymph nodes and were either directly stained with αICOS Ab or stained after stimulation with αCD3 or αCD3-αCD28-coated beads with or without exogenous IL-2 for 24 h. Expression levels of ICOS were analyzed by flow cytometry. Indicated are means of duplicates ± SD. Shown is a representative of two independent experiments.

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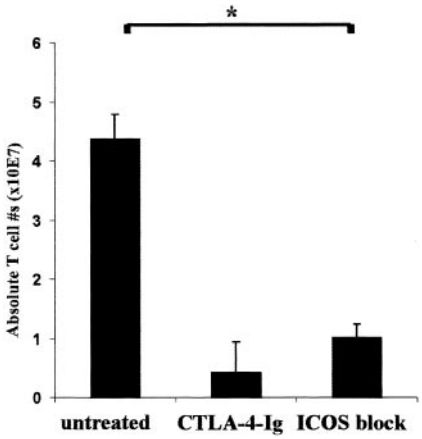

FIGURE 2. Blocking ICOS partly prevents lymphoproliferation in CTLA-4$^{-/-}$ mice. Mice deficient for CTLA-4 were treated from birth with CTLA-4-Ig or blocking αICOS Ab by injection i.p. on alternating days and were sacrificed on day 14. Peripheral lymph nodes were isolated, and single cell suspensions were prepared. The percentage of CD3$^+$ T cells was analyzed by cell surface staining and flow cytometry. Absolute numbers were determined by calculating the percentages of CD3$^+$ cells of the total number of cells isolated from peripheral lymph nodes. Indicated are means ± SEM (*, $p < 0.05$). Three animals were analyzed per treatment.

lacking CTLA-4 is partly dependent on signals generated by ICOS-B7RP-1 interaction.

ICOS and CD28 both contribute to CD4$^+$ and CD8$^+$ T cells expansion in the absence of CTLA-4

To look in more detail at the contribution of ICOS to CD28-mediated T cell expansion in vivo, we used mice lacking CTLA-4 and B7.1/2 (CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$). The advantage of using this strain over mice solely deficient for CTLA-4 is that in adult CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice a strong CD28 signal can be generated by administration of agonistic αCD28 Ab. This way any disorders by having CD28 signals present in the absence of CTLA-4 during development can be circumvented, and the T cell responses can be studied in adult mice. As shown previously, injection of agonistic αCD28 Ab in CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice causes vigorous T cell expansion, reflected in an increase in CD69 expression as a marker for T cell activation and a 10- and 20-fold increase in CD8$^+$ and CD4$^+$ T cell numbers, respectively, in peripheral lymph nodes (34) (Fig. 3, *A* and *B*). The peripheral T cell pool in these treated mice was also affected by antagonistic αICOS Ab treatment, like that observed in the CTLA-4$^{-/-}$ mice, in terms of T cell numbers and T cell activation. Both CD4$^+$ and CD8$^+$ T cell numbers in the lymph nodes were 2-fold reduced when stimulating αCD28 Ab was coinjected with blocking αICOS Ab, compared with αCD28 Ab alone (Fig. 3*A*). But T cell numbers of mice that received both agonistic αCD28 Ab and antagonistic αICOS Ab were still higher than T cell numbers of mice that did not receive treatment, indicating that CD28-mediated CD4$^+$ and CD8$^+$ T cell expansion was partially independent of ICOS. T cells from mice treated with blocking αICOS Ab alone remained naive and were similar in absolute numbers compared with untreated animals (data not shown).

To strengthen the observation that ICOS plays a role in T cell expansion, we looked at the activation state of CD4$^+$ and CD8$^+$ T cells phenotypically. The percentage of CD69 expressing CD4$^+$ as well as CD8$^+$ T cells was decreased when ICOS was blocked (Fig. 3*B*, *top* and *bottom panel 3*) but did not decline to expression levels found on T cells from untreated mice (Fig. 3*B*, *top* and *bottom panel 1*). The lower activation status following ICOS blockade was confirmed by analysis of other markers of activation, like up-regulation of CD25 and down-regulation of CD62L (data not shown). So, blocking ICOS in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice results in reduced activation and expansion of CD4$^+$ and CD8$^+$ T cells.

Previously it was shown that CD8$^+$ T cell expansion in CTLA-4$^{-/-}$ mice is dependent on the presence of CD4$^+$ T cells (38). We therefore questioned whether the effect of ICOS blockade on CD8$^+$ T cell numbers was caused by direct inhibition of CD8$^+$ T cell proliferation or indirectly due to blocking of CD4$^+$ T cell activation and expansion in our model. First, we investigated CD8$^+$ T cell expansion in CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice after αCD28 treatment in the presence of CD4$^+$ depletion Abs. Absence of CD4$^+$ T cells in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice resulted in complete normalization of the CD8$^+$ T cell pool, leaving CD8$^+$ T cell numbers similar to untreated animals (Fig. 3*C*). These results show that for CD8$^+$ T cells to proliferate the presence of CD4$^+$ T cells is required. However, this excludes the possibility of testing the direct effect of ICOS on CD8$^+$ T cell expansion in αCD28-treated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice. Taken together, ICOS contributes directly to CD28-mediated CD4$^+$ T cell activation and expansion in the absence of CTLA-4.

ICOS partly drives Th1 and Th2 differentiation in mice lacking CTLA-4

Next, the contribution of ICOS to CD28-induced CD4$^+$ Th1 and Th2 cell differentiation was assessed in CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice. Cytokines produced by CD4$^+$ T cells from these mice injected with αCD28 Ab in the absence or presence of blocking αICOS Ab were determined in vitro. Treatment of CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice with agonistic αCD28 Ab caused CD4$^+$ T cell differentiation toward Th1- and Th2-type cells, producing IFN-γ and IL-4/10, respectively (Fig. 4). When treated simultaneously with antagonistic αICOS Ab there was a noticeable (but not statistically different) decrease in IL-4 and IL-10 cytokine production. Although the Th1 cytokine levels were much lower compared with the Th2 cytokine levels, there was a significant reduction in IFN-γ production after blocking ICOS. IL-2 production was similar between the two groups. CD4$^+$ T cells from untreated CTLA-4$^{-/-}$ B7.1/B7.2$^{-/-}$ mice failed to produce any cytokines after in vitro stimulation (data not shown). Overall, there is a reduction in cytokine production from both Th1- and Th2-type T cells, indicating that cells from mice treated with blocking αICOS Ab were less differentiated. Thus, ICOS is involved in both Th1 and Th2 differentiation driven by CD28 signaling.

ICOS-induced division of CD4$^+$ and CD8$^+$ T cells is dependent on IL-2 in vitro

The observed decrease in expansion of the CD4$^+$ T cell pool after abrogation of ICOS function could either be due to less cell division or increased cell death. To distinguish between these two putative consequences of ICOS blocking, the contribution of ICOS to both cell cycle progression and cell survival was further investigated in vitro. Purified wild-type CD4$^+$ T cells were labeled with CFSE to be able to track numbers of cells that go through cell cycle by flow cytometry. Viability of cells going through rounds of division was analyzed simultaneously by using Viaprobe as a fluorescent dye, which only enters the cell when the membrane integrity is compromised. Results are shown in Fig. 5*A*, where the total numbers of viable and nonviable CD4$^+$ T cells that have undergone the indicated number of cell divisions are displayed. Most of the T cells stimulated with αCD3 alone underwent only

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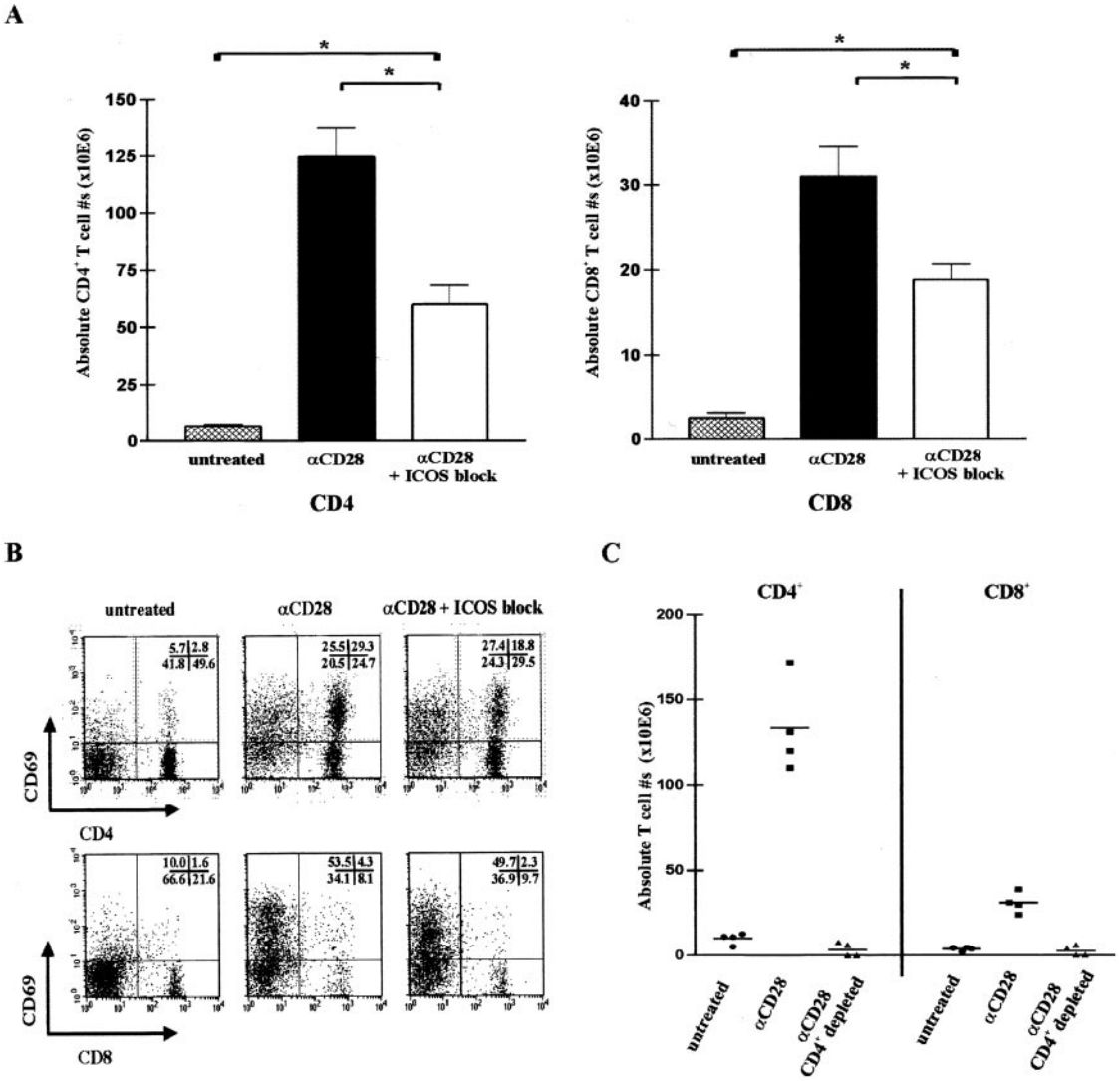

FIGURE 3. Blocking ICOS inhibits expansion of $CD4^+$ and $CD8^+$ T cells in mice deficient for CTLA-4/B7.1/B7.2. CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice were injected i.v. with agonistic αCD28 Ab (PV-1) on days 0, 7, and 14, with additional antagonistic αICOS Ab or isotype control Ab i.p. on alternating days, starting on day 0. Mice were sacrificed on day 17, and cell suspensions were prepared from pooled peripheral lymph nodes. *A*, Absolute numbers of $CD4^+$ and $CD8^+$ T cells in peripheral lymph nodes. Cells were stained with αCD4 and αCD8 Ab, and the percentages of each subset were determined by flow cytometry. Absolute numbers of $CD4^+$ and $CD8^+$ T cells were calculated by the percentage of positive cells of the total number of lymph node cells. Values represent means ± SEM (*, $p < 0.05$). *B*, Comparison of the expression of activation markers on $CD4^+$ and $CD8^+$ T cells. Cells were stained with αCD4, αCD8, and αCD69 Ab and analyzed by flow cytometry. Percentages of cells in each quadrant are indicated. FACS plots show a representative of three independent experiments. A minimum of seven mice was analyzed for each treatment. *C*, Absolute T cell numbers after $CD4^+$ T cell depletion. In addition to αCD28 treatment, CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice received $CD4^+$ depletion Ab on alternating days, starting at day −3. Mice were sacrificed on day 17 and absolute T cell numbers were calculated as in *A*. Four mice were analyzed per group.

one or two cell divisions (Fig. 5*A*, *panel 1*). However, after simultaneous co-crosslinking with CD28 (Fig. 5*A*, *middle panel*) cells went through four to six rounds of division. In both of these stimulation conditions, exogenous IL-2 did not significantly influence cell division. In contrast, addition of IL-2 considerably changed the outcome of the response after αCD3-αICOS stimulation. As depicted in the third top/bottom panels, cell cycle progression of $CD4^+$ T cells could only be induced by ICOS in the presence of IL-2 (Fig. 5*A*). Because the majority of the cells progressed through cell cycle, it is difficult to draw conclusions about an effect on cell survival by ICOS. Still, the total amount of nonviable αCD3-αICOS-stimulated cells that have gone through one cell division was unchanged compared with T cells that were stimulated by αCD3 alone, indicating that ICOS may not promote cell survival in cells that were refractory to further cell division (Fig. 5*A*, *left* and *right lower panels*, black bars).

To address the question whether there can be a direct effect of ICOS on $CD8^+$ T cell expansion, we performed similar proliferation assays using purified wild-type $CD8^+$ T cells. As shown in Fig. 5*B*, $CD8^+$ T cells are able to progress through cell cycle in response to αCD3-αICOS stimulation, but only in the presence of exogenous IL-2. Taken together, ICOS induced cell cycle progression of both $CD4^+$ and $CD8^+$ T cells in the presence of IL-2. Moreover, ICOS did not rescue cells that had not progressed through cell cycle from cell death.

Discussion

We here report a study in which the contribution of ICOS to an in vivo immune response was investigated in CTLA-4 deficient mice. When ICOS was blocked in CTLA-$4^{-/-}$ mice, the aberrant activation of $CD4^+$ T cells and expansion of both $CD4^+$ and $CD8^+$ T

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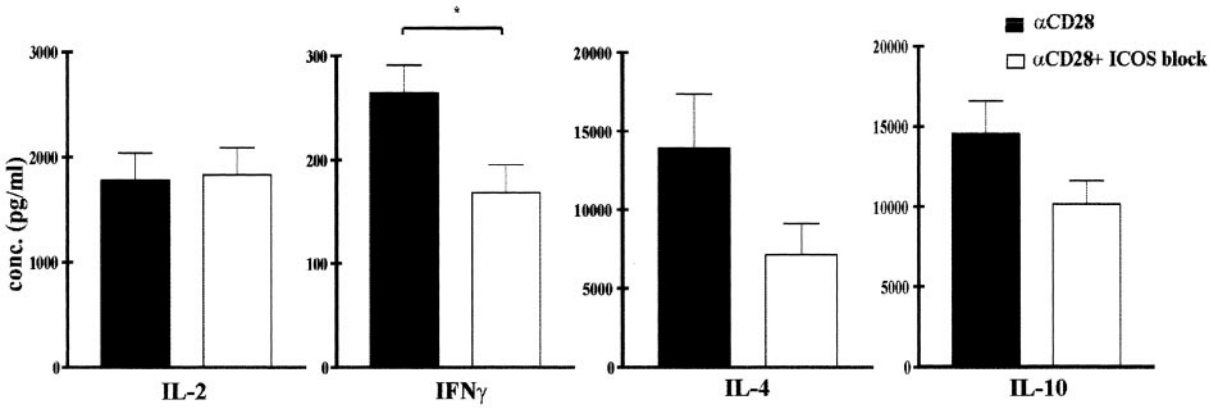

FIGURE 4. ICOS and CD28 both contribute to Th1 and Th2 development. Purified $CD4^+$ T cells were isolated from peripheral lymph nodes of CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice treated with αCD28 with or without blocking αICOS Ab. Similar numbers of $CD4^+$ T cells were stimulated in vitro with αCD3-αCD28 coated beads for a period of three days. Supernatants were collected, and cytokines were measured by sandwich ELISA. Indicated are IL-2 levels on day 1, IFN-γ on day 2, and IL-4/10 on day 3. A minimum of seven mice was analyzed for each treatment. Values represent means ± SEM (*, $p < 0.05$).

cells was reduced. Moreover, CD28-mediated differentiation toward Th1- and Th2-type $CD4^+$ T cells was partly dependent on ICOS. Further in vitro studies showed that ICOS induced cell cycle progression of both $CD4^+$ and $CD8^+$ T cells, which was dependent on exogenous IL-2. Taken together, in the presence of CD28 signaling, ICOS functions early in the cellular response, contributing to expansion of T cells and differentiation of $CD4^+$ T cells.

The results presented here shed new light on the role of ICOS in the expansion phase of a T cell response. These data are in accordance with other studies, which also show that ICOS enhances T cell activation and expansion both in vitro and in vivo (11, 24, 37). In contrast, several blocking studies during different phases of an immune response showed the most pronounced effect when ICOS signaling is abrogated later in the immune response (21, 27). It is possible that a delicate balance between TCR signal and CD28 determines the requirement for ICOS early in the response. When comparing the contribution of ICOS and CD28 to the initial phase of the immune response, we hypothesize that CD28 is the most important molecule for initial activation and expansion of T cells. This can be explained not only by low expression of ICOS on naive T cells, but might also lie in the fact that ICOS, unlike CD28, cannot induce IL-2 production in murine T cells (11, 22). The importance of CD28 as initiator molecule is also seen in CTLA-$4^{-/-}$ mice where blocking of CD28 completely abolishes T cell activation whereas ICOS blocking only partly does so. Still, we have shown that CD28 does not induce a complete T cell response in the absence of ICOS in αCD28-treated CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice. The contribution of ICOS to T cell expansion might be more pronounced when T cell activation occurs after signals generated by CD28, as suggested by the strong ICOS up-regulation after TCR-CD28 coligation and the necessity of IL-2 for ICOS-induced proliferation in vitro. In conclusion, in the absence of CTLA-4, although CD28 functions as an initiator molecule, sequential engagement of ICOS has an additive effect on T cell expansion.

The observed reduction in peripheral T cells could result from less division or increased cell death. We conclude from our in vitro T cell stimulation assay that ICOS, in the presence of IL-2, is able to induce cell cycle progression. Therefore, it is likely that the observed inhibition of T cell expansion after in vivo blockade of B7RP-1-ICOS interaction in CTLA-$4^{-/-}$ is caused by reduced cell proliferation. We cannot exclude that abrogation of ICOS signaling could have caused increased cell death in vivo. Whether ICOS induces an intrinsic cell survival mechanism independent from proliferation like CD28 does by up-regulation of Bcl-x_L (39, 40) is currently under investigation.

One way to explain the results obtained here is by using the term "cell fitness," first proposed by Lanzavecchia and Sallusto (41). When optimal signals from the TCR, costimulatory molecules, and cytokine receptors are integrated, cells go through cell division acquiring resistance to apoptosis and susceptibility to cytokines like IL-2, IL-15, and IL-7. In the present study, cells stimulated with αCD3 alone or αCD3-αICOS cells can be termed as "unfit" cells. The signals provided are suboptimal; therefore no cell cycle progression occurs. When IL-2 is provided in the presence of a TCR signal and ICOS, these cells obtain a higher level of "fitness" and proceed through cell cycle remaining viable. The importance of IL-2 is also supported by studies in human $CD4^+$ T cells, where neutralizing IL-2 Ab can inhibit ICOS-induced proliferation (42). We and others (43) have observed that IL-2 increases ICOS expression on murine T cells in vitro. However, it is unclear whether this up-regulation is mandatory for the induction of proliferation via ICOS. Other possible mechanisms by which IL-2 can enhance ICOS-induced proliferation in TCR-triggered cells remain to be determined.

Next to the effect on $CD4^+$ T cell expansion, we have found a clear effect of ICOS on $CD8^+$ T cell expansion in the absence of CTLA-4. In agreement with these results, ligation of ICOS on $CD8^+$ T cells induced proliferation in the presence of IL-2 in vitro. However, depletion of $CD4^+$ T cells in αCD28-treated CTLA-$4^{-/-}$ B7.1/B7.$2^{-/-}$ mice showed that the expansion of the $CD8^+$ T cells is dependent on the presence of $CD4^+$ T cells. Therefore, we cannot conclude from our model that ICOS has a direct effect on $CD8^+$ T cell expansion in vivo.

The assumption that therapeutic interventions in the ICOS/B7RP-1 pathway is restricted to the effector phase may have to be revised, because our data show that ICOS plays a role in the expansion phase of a T cell response. CD28 is believed to be one of the most important initiator molecules of an immune response discovered to date; so, blocking CD28 could have major implications when used in a therapeutic setting. Future therapies targeting ICOS rather than CD28 may have to take into account that abrogation of ICOS signaling may not only affect secondary responses but may also inhibit earlier in cellular responses.

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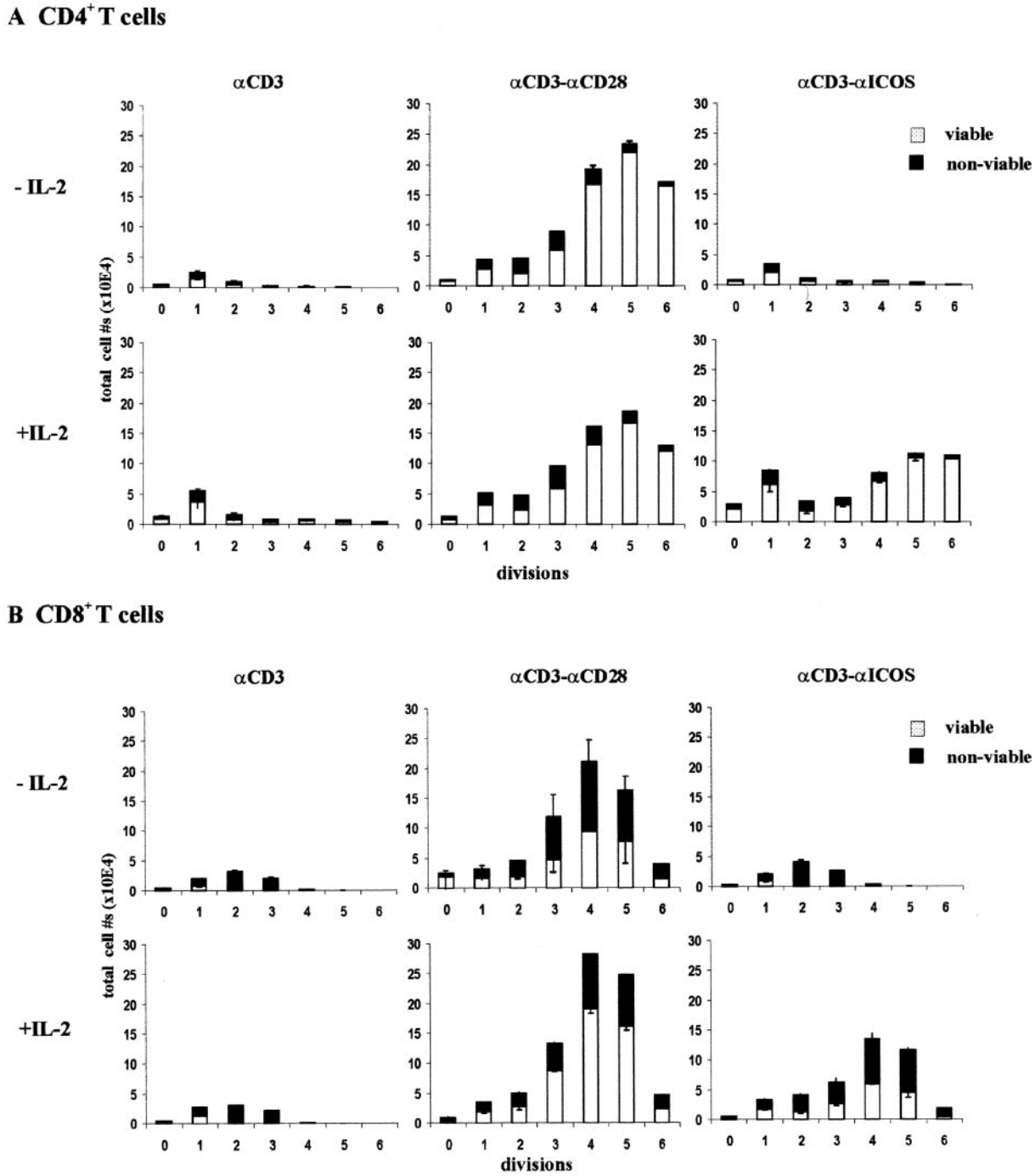

FIGURE 5. ICOS induces cell cycle progression of $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells in the presence of IL-2. Peripheral lymph node T cells were purified from wild-type mice, labeled with CFSE and activated with Ab-coated beads as indicated, with or without addition of exogenous IL-2. $CD8^+$ T cells were harvested after 3 days and $CD4^+$ T cells after 4 days. The percentage of cells that went through the indicated number of divisions was determined by dilution of the CFSE label, whereas cell viability was simultaneously analyzed with Viaprobe as a dye for nonviable cells. Cultured cells were counted and total viable (white bars) and non-viable (black bars) T cell numbers, which had undergone the indicated number of cell divisions, were calculated using the percentages of each population of the total number of T cells. Indicated are means of duplicates ± SD.

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Disclosures

The authors have no financial conflict of interest.

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