A distinct role for ICOS-mediated co-stimulatory signaling in CD4$^+$ and CD8$^+$ T cell subsets

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Abstract

While the ligand of inducible co-stimulator (ICOS), B7 homologous protein, is widely expressed in somatic cells, B7-1 and B7-2 expression is mainly limited to lymphoid organs. Thus, the activation of T cells through ICOS without a CD28-mediated signal may occur in physiological situations. In order to gain a better understanding of the role of the ICOS co-stimulatory signal in immune responses, we studied the cellular response of T cells to beads coated with anti-ICOS or anti-CD28, plus sub-optimal anti-CD3 mAb. We demonstrate that while CD28 ligation induced expansion of both CD4$^+$ and CD8$^+$ populations, ICOS ligation only resulted in the expansion of CD8$^+$ T cells, and induced apoptosis in the CD4$^+$ T cell population. It was found that IL-2 is critically required for CD8$^+$ T cell expansion triggered by ICOS ligation, whereas it had only a limited effect on the expansion of CD4$^+$ T cells. This distinct reactivity of CD4$^+$ and CD8$^+$ T cell populations to exogenous IL-2 strongly correlates with the expression level of IL-2 receptor $\beta$-chain, CD122, on T cells. Furthermore, we defined a small but distinct population of memory phenotype CD4$^+$ T cells that constitutively express ICOS. Interestingly, while naive CD4$^+$ T cells were unable to produce IL-2, ICOS-expressing T cells produced a substantial amount of IL-2 by stimulation with anti-ICOS/CD3 beads, suggesting that IL-2, which is indispensable for CD8$^+$ T cell expansion, is produced by this ICOS-expressing T cell population. These results provide evidence indicating that the ICOS co-stimulatory signal plays a distinct role in the development of CD4$^+$ and CD8$^+$ T cell-mediated immune responses.

Introduction

Although engagement of the T cell antigen-specific receptor by antigen/MHC products is essential for the initial stages of T cell activation, a second signal termed a co-stimulatory signal is necessary for clonal expansion and functional differentiation of antigen-specific T cells (1). It has been well accepted that CD28 is a major positive co-stimulatory molecule for T cell activation and functional differentiation, and that cytotoxic T lymphocyte antigen-4 (CTLA-4) is a negative co-stimulatory signal which provides a signal for the termination of activation and cellular function of T cells (2). Because of the strong biological effects of co-stimulatory signals mediated by these two CD28 family molecules on various immune responses, these pathways have been attractive targets for new therapeutic strategies for the treatment of tumors, autoimmune diseases and graft rejection (3, 4).

A third CD28 family receptor has recently been identified by several groups and designated as inducible co-stimulator (ICOS, also reported as activation-inducible lymphocyte immunomediatory molecules [AILIM] and H4) (5–8). The physiological ICOS ligand has subsequently been identified as a B7 homologous protein (B7h, also known as B7RP-1, ICOSL, GL-50 or LICOS) (6, 9–12). The potency of ICOS as a co-stimulatory receptor has been demonstrated by the induction of significantly enhanced T cell proliferation and cytokine production following ICOS ligation (5, 6). However, several unique features of the ICOS system that differ from CD28 have been reported, for example while CD28-mediated co-stimulatory signaling facilitates the production of IL-2, ICOS ligation results in a negligible enhancement of IL-2 production. Furthermore, CD28 is constitutively expressed by naive T cells, whereas ICOS expression is inducible by activation (5, 6). These observations led to the hypothesis that ICOS serves to enhance T cell effector function. This hypothesis has recently been supported by functional studies.
and analysis of ICOS-deficient mice (13–15). In particular, much attention has been drawn to the role of ICOS in helper T cell differentiation. Defective ICOS-mediated co-stimulation by blocking with antibodies or an ICOS-Ig fusion protein (16, 17), as well as with gene targeting of ICOS (13–15) and B7h (18), resulted in the preferential inhibition of Tp-2-mediated immune responses, although some Tp-1 responses were also found to be affected (19, 20). These defined features indicate that ICOS is a very attractive molecule as a regulator of various immune responses, but its ability to regulate CD8+ responses remains unclear. Kopf et al. showed minimal involvement of ICOS in anti-virus CTL responses by ICOS-Ig administration in a virus infection system, suggesting that ICOS may not be important for CD8+ T cell function (21). However, recently, Wallin et al. demonstrated that ICOS–B7h interaction enhanced in vivo primary and secondary responses of CD8+ T cells (22), indicating a different conclusion.

In this report, we studied cellular responses of peripheral T cells to ICOS-mediated co-stimulatory signals by the use of beads coated with anti-ICOS mAb or anti-CD28 mAb plus sub-optimal anti-CD3 mAb. We found that while the stimulation of splenic T cells by anti-CD28/CD3 mAb beads induced a massive expansion of both CD4+ and CD8+ populations, anti-ICOS/CD3 mAb beads led to a dominant expansion of the CD8+ T cell population and apoptosis of CD4+ T cells. For expansion of CD8+ T cells, the presence of IL-2 in the culture was found to be critical, since anti-IL-2 mAb completely inhibited ICOS-stimulated CD8+ T cell expansion. In contrast, administration of exogenous IL-2 had only a limited effect on proliferative response of CD4+ T cells. We defined a small but distinct population of memory phenotype CD4+ T cells that constitutively express ICOS, and found that this population produced substantial amounts of IL-2, suggesting that the IL-2 that is required for CD8+ expansion is produced by ICOS-positive T cells. These results indicate that ICOS-mediated co-stimulatory signals lead to different outcomes in each T cell subset. Since B7h is constitutively expressed on antigen-presenting cells (APCs), as well as many other somatic cells (9), the ICOS-mediated co-stimulatory signal may play a potent co-stimulatory effect on the activation of CD8+ T cells in potentially inflammatory sites.

**Methods**

**Mice**

BALB/c mice were obtained from Sankyo Laboratory (Hamamatsu, Japan). Mice were maintained in our animal facility in a specific pathogen-free condition. For the entire experiment, 8 to 10-week old mice were used. The experiment herein was conducted according to the principles set forth by the Institute of Laboratory Animal Resources (23).

**Antibodies and reagents**

Anti-mouse-ICOS mAb (B10.5) was produced as previously described (17) and provided by JT Pharmaceutical Frontier Research Laboratory (Yokohama, Japan). Purified anti-CD3 (2C11), anti-CD28 (PV-1) and rat anti-nitrophenal(NP)-IgG2a antibodies, non-purified anti-CD4 (H12-2C19), CD8 (53-6-7), B220 (RA36-B2) and MHC class II antibodies were prepared in our laboratory from hybridoma culture media. FITC-labeled anti-CD3 and CD4 antibodies, PE-labeled anti-CD8, CD25, CD69, CD44, CD62L, CD45RB and IL-2Rβ (CD122) antibodies, allophycocyanin(APC)-labeled anti-CD4 and CD8 antibodies, biotin-labeled anti-IL-2 antibody and purified anti-IL-2 and CD132 (γc) antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Anti-Bcl-XL antibody was purchased from Transduction Laboratories (Lexington, KY, USA). Anti-Stat5 and phospho-Stat5 antibodies were purchased from Cell Signaling (Beverly, MA, USA).

**Preparation of antibody-coated beads**

Latex beads were purchased from Interfacial Dynamics Corporation (Portland, OR, USA). Briefly, Latex beads (5 × 10^7) were washed twice with PBS and were incubated with anti-CD3 mAb (2C11, 1 μg ml^-1^) plus one of the following antibodies: anti-CD28 (PV-1, 9 μg ml^-1^), anti-ICOS (B10.5, 9 μg ml^-1^) or anti-NP-IgG2a (isotype control, 9 μg ml^-1^), for 1.5 h at 37°C. Beads were then washed with PBS, and blocked with 10% FCS RPMI 1640 medium.

**Cell purification and stimulation**

T cells were purified by negative selection. Briefly, a single-cell suspension was prepared from spleen and erythrocytes were eliminated. The cells were treated with anti-B220 and anti-MHC class II antibody, and incubated on an anti-mouse Ig-coated dish to eliminate B cell and MHC class II-expressing cells. In order to enrich CD4+ T cells, the enriched T cells were further treated with anti-CD8 antibody, and incubated in an anti-lg-coated dish to eliminate CD8+ T cells. Naive (CD44low) and memory (CD44high) phenotype CD4+ and CD8+ T cells were purified by cell sorting. The enriched T cells, as described above, were stained with anti-CD44-PE and anti-CD4-APC or anti-CD8-APC. Each cell population was collected with FACS Vantage™ (BD Pharmingen). Purity of sorted population was constantly >95%. T cells (1 × 10^5^ or 5 × 10^5^) were stimulated with equal numbers of antibody-coated beads in a 96- or 48-well culture plate, respectively. For some experiments, recombinant human IL-2 (30 U/ml) was added to culture media and was used for phospho-Stat5 analysis. For cell culture, RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FCS, 2-mercaptoethanol (2ME), L-glutamine, penicillin/streptomycin and 0.1 mM HEPES was used.

**Flow cytometry**

Briefly, cells were washed with wash buffer (PBS containing 1% BSA and 0.05% NaN3), treated with anti-FCR (2.4G2) and stained with fluorescent conjugated antibodies. For cell division analysis, the cytosol dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) was utilized. Briefly, T cells were washed with PBS containing 0.1% BSA and incubated in staining solution (2.5 μM CFSE) for 10 min at 37°C. Cells were then washed twice with 10% FCS/RPMI 1640 medium. Electrical data were acquired on a FACS Calibur™ and analyzed by CELLQuest™ software (BD Pharmingen).
Proliferation assay

T cells (1 × 10^5) were co-cultured with antibody-coated beads (1 × 10^5) in a 96-well culture plate. [3H]Thymidine ([3H]TdR, 1 μCi ml⁻¹) incorporation was measured during the last 8 h of the culture period by liquid scintillation counter, Wallac MicroBeta 1450 (Perkin Elmer, Wellesley, MA, USA).

ELISA

Primary antibody was coated on a 96-well plate at 37°C for 1 h, and the plate was blocked with PBS containing 1% BSA. Culture supernatants were then applied on the plate and incubated for 2 h at 37°C. The plate was washed and reacted with biotinylated secondary antibody for 1 h, followed by reacting with HRP-coupled streptavidin (Sigma–Aldrich) for 30 min. Substrate [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate acid) plus H_2O_2] was applied and optical density at 405 nm was measured by Microplate Reader Model 3550 (Bio-Rad, Hercules, CA, USA).

Western blot analysis

To prepare total cell lysate, T cells were treated with lysis buffer (500 mM NaCl, 5 mM EDTA, pH 8.0, 100 mM Tris-HCl, pH 8.0, 1.25% NP-40) for 30 min on ice, and centrifuged at 15 000 rpm for 10 min. The supernatants were used for experiments. Protein concentration of each sample was determined by Bicinchoninic acid (BCA) protein assay reagent (PIERCE, Rockford, IL, USA). Equal protein levels of each sample were resolved by SDS-PAGE with 12% acrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane, probed with specific primary antibody, followed by reacting with HRP-conjugated secondary antibody, and developed by enhanced chemiluminescence detection system (Perkin Elmer).

Results

The ICOS-mediated co-stimulatory signal dominantly induces CD8⁺ T cell expansion but not CD4⁺ T cells

While the ligand of ICOS, B7h, is widely expressed in somatic cells, B7-1 and B7-2 expression is mainly limited to lymphoid organs (9). Thus, activation of T cells through ICOS without a CD28-mediated signal may occur in physiological situations. In order to define cellular responses of T cells to ICOS and CD28 ligation, splenic T cells were cultured in the presence of beads coated with anti-ICOS mAb, anti-CD28 mAb or control mAb, plus sub-optimal anti-CD3 mAb. To simplify nomenclature, these immunobeads are called ICOS/CD3, CD28/CD3 or CD3 beads, respectively. Cultured cells were harvested at designated time points, and live cells were isolated and stained with anti-CD4 or anti-CD8 mAbs. As shown in Fig. 1, while both CD4⁺ and CD8⁺ T cells were expanded in the culture stimulated with CD28/CD3 beads, CD8⁺ T cells were predominantly expanded in the presence of ICOS/CD3 beads compared with CD4⁺ T cells.

In order to verify the mechanism underlying a selective expansion of CD8⁺ T cells in ICOS-stimulated cultures, splenic T cells were first stained with CSFE, and then stimulated with ICOS/CD3, CD28/CD3 or CD3 beads. As shown in Fig. 2(A), stimulation with CD28/CD3 beads induced cell division and proliferation of both CD4⁺ and CD8⁺ T cells. In contrast, while ICOS cross-linking led to cell division in both CD4⁺ and CD8⁺ T cells to a level similar to CD28 cross-linking, expansion of cells at each division was substantially reduced in the CD4⁺ T cell population. In order to define the mechanism of the low expansion of ICOS-stimulated CD4⁺ T cells, we evaluated the percentage of dead cells in each division of both T cell populations following CD28- and ICOS cross-linking. In both CD4⁺ and CD8⁺ T cell populations that were stimulated with CD28/CD3 beads, the percentage of dead cells declined when cell division proceeded. In contrast, while the response pattern of CD8⁺ T cells to ICOS/CD3 beads was similar to that of CD28/CD3-stimulated T cells, >40% of CD8⁺ T cells were found to be dead in each division (Fig. 2B). These results indicated that while ICOS cross-linking stimulated cell division of both CD4⁺ and CD8⁺ T cells, it only rescued CD8⁺ T cells from cell death.

Expansion of CD8⁺ T cells requires IL-2

To delineate the function of ICOS signals on CD4⁺ and CD8⁺ T cells, we investigated the effect of ICOS cross-linking on the proliferative response of isolated CD4⁺ or CD8⁺ T cells. CD4⁺ and CD8⁺ T cells were enriched from spleen and co-cultured with ICOS/CD3 or CD28/CD3 beads (Fig. 3A). While CD28/CD3 beads were found to stimulate proliferation of both CD4⁺ and CD8⁺ T cells, ICOS/CD3 beads weakly stimulated CD4⁺ T cells, yet unexpectedly did not activate isolated CD8⁺ T cells. These results indicated that the dominant expansion of CD8⁺ T cells in the splenic T cell population by ICOS co-stimulation requires the presence of CD4⁺ T cells, which most likely affect CD8⁺ T cells through the secretion of cytokines.

Since IL-2 is the most potent cytokine produced by CD4⁺ T cells to induce T cell proliferation, and the ICOS-mediated co-stimulatory signal is known to be ineffective for IL-2 production, we thought that the weak expansion of isolated CD4⁺ and CD8⁺ T cells following ICOS ligation may be the result of a shortage of available IL-2 in the cultures. To test this hypothesis, isolated CD4⁺ and CD8⁺ T cells were cultured with antibody-coated beads in the presence or absence of recombinant IL-2. As shown in Fig. 3(B), addition of exogenous IL-2 strongly activated CD8⁺ T cells co-cultured with ICOS/CD3 beads to proliferate to a similar level as those co-cultured with CD28/CD3 beads. Interestingly, recovery of CD4⁺ T cell proliferation by IL-2 is much weaker than that of CD8⁺ T cells. These results indicated that CD8⁺ T cell expansion by ICOS ligation requires the presence of IL-2, and that reactivity of CD4⁺ T cells to IL-2 is rather limited. This conclusion was supported by the fact that addition of anti-IL-2 mAb to the culture of splenic T cells stimulated with ICOS/CD3 beads effectively blocked T cell expansion in a dose-dependent manner (Fig. 3C).

Memory CD4⁺ T cells constitutively express ICOS and produce IL-2 by ICOS stimulation

Since it has been reported that the ICOS signal has little effect on IL-2 production, the requirement of IL-2 for the outgrowth of CD8⁺ T cell population in splenic T cells after ICOS ligation was puzzling. Yoshinaga et al. have reported that there is a small but distinct T cell population that expresses ICOS at a high level on freshly isolated spleen and lymph node cells (6). Indeed, when freshly isolated splenic T cells were stained with
FITC-labeled anti-ICOS mAb, a small population of CD4+ T cells, but not CD8+ T cells, was found to be positive (Fig. 4A). ICOS+ T cells were sorted by flow cytometry and tested for the expression of various activation/memory makers. As shown in Fig. 4(B), almost all ICOS+ CD4+ T cells were CD44high and most were also CD45RBlow and CD62Llow. Furthermore, CD25 and CD69, which are expressed by activated T cells, were not expressed on ICOS+ T cells. This expression pattern is consistent with the notion that ICOS+ T cells are memory phenotype CD4+ cells.

We subsequently tested the response of this ICOS+ T cell population to ICOS ligation. Since all CD44high T cells constitutively express ICOS (Fig. 5A), we sorted CD44high and CD44low cells, and used them as ICOS+ (memory) and ICOS− (naive) CD4+ T cells, respectively, and tested their proliferative response and IL-2 production to ICOS and CD28 cross-linking. As shown in Fig. 5(B), although stimulation with ICOS/CD3 beads had no effect on the proliferative response of CD44low T cell population, CD44high T cells responded vigorously. Furthermore, these CD44high T cells showed substantial IL-2 production by ICOS cross-linking. These results clearly indicated that T cells in spleen and lymph node (data not shown) contain ICOS+ CD44high memory phenotype CD4+ T cells which are fully capable of producing IL-2 by ICOS-mediated co-stimulation. In contrast to CD44high CD4+ T cells, CD44low T cells neither proliferate nor produce IL-2 in response to ICOS/CD3 beads. To further clarify the different nature of ICOS-mediated co-stimulation between CD44low and CD44high CD4+ T cells, we analyzed Bcl-XL expression, which is induced by IL-2 receptor signaling and has anti-apoptotic action (24, 25). As shown in Fig. 5(C), Bcl-XL was strongly induced by ICOS stimulation in CD44high but not CD44low CD4+ T cells, also suggesting the distinct sensitivity to apoptosis between these populations. These results indicated that the co-stimulatory function delivered by ICOS cross-linking for clonal expansion and IL-2 production is limited to CD44high T cells.

**Fig. 1.** The effect of ICOS cross-linking on the expansion of CD4+ or CD8+ cells in whole T cell cultures. Purified BALB/c splenic T cells (5 x 10^5) were co-cultured with indicated antibody-coated beads (5 x 10^5). (A) At the indicated time points, the percentages of CD4+ and CD8+ populations were determined by flow cytometry. Data shown are representative of three independent experiments. (B) Absolute recovered live cell numbers of CD4+ and CD8+ cells were calculated from total live cell numbers and the percentages obtained in (A). Data are representative of three independent experiments.
The ICOS signal induces a distinct expression pattern of IL-2 receptor subunits and IL-2 receptor sensitivity to IL-2 in CD4+ and CD8+ T cell population

The expansion pattern of CD4+ and CD8+ T cells in whole T cell population (Figs 1 and 2) and isolated T cell populations (Fig. 3) induced by ICOS-mediated co-stimulation indicates that the CD8+ T cell population is preferentially responding to IL-2. This suggests that the ICOS signal enhances the sensitivity of CD8+ T cells to IL-2 more markedly than that of CD4+ T cells. To confirm this hypothesis, the response of naive CD4+ T cells to IL-2 was compared with that of naive CD8+ T cells. CD44low CD4+ and CD44low CD8+ T cells were purified by cell sorting, and used as naive cells. These cells were co-cultured with CD3, ICOS/CD3 or CD28/CD3 beads in the presence or absence of rIL-2, and their proliferative response was measured by [3H]Tdr uptake. As shown in Fig. 6, CD28 cross-linking induced strong proliferation of both CD4+ and CD8+ T cells regardless of the presence of rIL-2. In contrast, in the absence of rIL-2, neither CD4+ nor CD8+ T cells showed proliferative responses by ICOS cross-linking. On the other hand, in the presence of IL-2, CD8+ T cells, but not CD4+ T cells, showed vigorous responses. These results clearly indicated that the ICOS signal provides different effects on IL-2 receptor signaling in naive CD4+ and CD8+ T cells.

In order to define the molecular basis of this distinct sensitivity of ICOS-stimulated CD4+ and CD8+ T cells to IL-2, we analyzed IL-2 receptor components in these populations since we postulate that the different sensitivity may be due to a property of IL-2 receptor. To this end, we examined the expression pattern of IL-2 receptor components on CD4+ and CD8+ T cells stimulated with ICOS/CD3 beads.
The IL-2 receptor is composed of three components, a β-chain (CD122) and a γc-chain (CD132) to form a signaling unit, and an α-chain (CD25) to form a high-affinity receptor (26). It is also known that the expression pattern of these IL-2 receptor subunits is different in CD4+ and CD8+ T cell populations (26, 27). As shown in Fig. 7(A), CD25 expression was induced in CD4+ T cells after ICOS cross-linking comparable to CD28 cross-linking. However, CD122 was strongly up-regulated in CD4+ T cells with CD28 cross-linking, but ICOS cross-linking showed no effect on CD122 up-regulation. In CD8+ T cells, weak but significant CD25 expression was up-regulated by both CD28/CD3 and ICOS/CD3 beads. Being strikingly different from CD4+ T cells, CD122 and CD132 were highly expressed on CD8+ T cells in the absence of ICOS cross-linking. These results suggested that the low sensitivity of CD4+ T cells to IL-2 might be caused by the inability of the ICOS signal to up-regulate the IL-2 receptor-signaling subunit, CD122. This hypothesis could be supported by the evidence that the CD44\textsuperscript{high} (ICOS\textsuperscript{high}) CD4+ T cell population, which responded to ICOS stimulation, also expressed CD122 (Fig. 4B).

Next, we tested the biological activity of the IL-2 receptor on CD4+ and CD8+ T cells following ICOS ligation. Since successful IL-2 receptor signaling is known to phosphorylate Stat5 (26), we examined phosphorylation of Stat5 in response to recombinant IL-2 in CD4+ and CD8+ T cells pre-treated with ICOS/CD3 or CD28/CD3 beads. As shown in Fig. 7(B), pre-treatment by CD28/CD3 beads resulted in Stat5 phosphorylation in both CD4+ and CD8+ T cells. In contrast, although strong Stat5 phosphorylation was observed in CD8+ T cells, ICOS cross-linking induced a markedly weaker level of Stat5 phosphorylation in CD4+ T cells.

To clarify reduced IL-2 receptor signaling, we evaluated the expression of Bcl-X\textsubscript{L} in CD4+ T cells pre-treated with ICOS/CD3 beads. In this condition, the result would mostly represent the phenotype of naive CD4+ T cells in the presence of memory CD4+ T cells which could be a source of IL-2. As shown in Fig. 7(C), pre-treatment with ICOS/CD3 beads induced a much weaker Bcl-X\textsubscript{L} induction than that with CD28/CD3 beads. These results are consistent with the notion that ICOS-mediated co-stimulation enhances IL-2 receptor sensitivity of CD8+ T cells, whereas its effect is limited in CD4+ T cells.

**Discussion**

In this study, we investigated the role of ICOS-mediated co-stimulatory signals in cellular responses of T cell subsets. We found that while CD28 ligation induced expansion of both CD4+ and CD8+ populations of splenic T cells, the majority of T cells recovered from culture after ICOS ligation were the CD8+ T cells (Fig. 1). Cell division and expansion analysis of CFSE-stained cells indicated that while CD8+ T cells revealed expansion in each division, only a limited portion of the CD4+ T cells survived each division, resulting in a small accumulation and limited expansion of dividing cells (Fig. 2). These results suggested that the reason why ICOS ligation does not lead to a CD4+ T cell expansion is because, unlike the CD28 co-stimulation signal, the ICOS signal is incapable of inducing an anti-apoptotic effect in activated CD4+ T cells. A similar conclusion has been proposed by Riley et al. for human T cell responses (28).

The fact that administration of anti-IL-2 mAb completely inhibited ICOS-mediated T cell proliferation (Fig. 3C), and that exogenous IL-2 induced naive CD8+ T cell expansion (Fig. 6), indicated that IL-2 is critically required for CD8+ T cell expansion. In contrast, IL-2 had only a limited effect on naive CD4+ T cell expansion induced by ICOS ligation (Fig. 6). This striking difference of naive CD4+ and CD8+ T cells in their reactivity to IL-2 was further studied. Flow cytometric analysis of IL-2 receptors revealed that the expression of β-chain (CD122) on naive CD4+ T cells, which together with γc-chain (CD132) composes the signaling unit of the IL-2 receptor, was increased by CD28 but not ICOS ligation (Fig. 7A). Parallel to the CD122 expression, CD28 ligation, but not ICOS ligation, was capable of inducing proliferation of the CD4+ T cell population, suggesting that CD122 expression may be the factor which determines the sensitivity of T cells to IL-2 under these conditions. In CD8+ T cells, expression of CD122 is at a high level even after sub-optimal anti-CD3 stimulation alone, and CD25 expression was equally enhanced by CD28 or ICOS ligation (Fig. 7A). This is why ICOS ligation is capable of promoting IL-2-dependent proliferation of the CD8+ T cell population. It is noteworthy that memory CD4+ T cells which could respond to ICOS ligation also express CD122 (Fig. 4B).

The signal transduction pathway for CD122 expression is not well understood. We have demonstrated that the different Grb-2 binding capabilities of the phosphatidylinositol-3-kinase (PI3K) binding region in ICOS and CD28 are critical for their
differing abilities for IL-2 promoter activation (29). To define the signaling pathway important for the enhancement of IL-2 receptor signaling by CD28 co-stimulation and CD122 expression on CD4+ T cells, we are currently testing the effect of deletion and point mutations in the CD28 cytoplasmic region. We observed that ICOS ligation of splenic CD4+ T cells, which mostly represent naive CD4+ T cells, failed to induce the expression of the prominent anti-apoptotic molecule Bcl-XL (Fig. 7C). It has been shown that induction of Bcl-XL is the critical event for the manifestation of anti-apoptotic effect of CD28-mediated signaling (30), and that activation of the Akt pathway and IL-2 receptor signaling is involved in Bcl-XL induction (24, 31). Among these two elements, ligation of both CD28 and ICOS results in association and activation of PI3K (32, 33) that initiate the Akt pathway (8, 34), suggesting that failure of the ICOS signal for Bcl-XL induction is not due to a defective Akt pathway. Thus, it is most likely that an insufficient increase in IL-2 receptor sensitivity (i.e. a low reactivity to IL-2) may be the main reason for the induction of CD4+ T cell apoptosis by ICOS ligation.

Since the ICOS-mediated co-stimulatory signal has been generally considered to have little effect on IL-2 production
The observation that CD8+ T cell expansion of splenic T cells stimulated with ICOS/CD3 beads is IL-2 dependent was originally puzzling. However, in this study we detected the presence of a small but distinct population that constitutively expresses ICOS and produced substantial amount of IL-2 after ICOS ligation (Fig. 5B). Yoshinaga et al. have previously reported that a few CD4+ splenic T cells are positively stained by B7RP-1-Fc, a chimeric fusion protein of the ICOS ligand (6). They also showed that these T cells are CD44high and CD45RBlow, a profile of typical memory T cells. Consistent with these reports, the ICOS+ CD4+ T cells that we define have CD44high, CD25− and CD62Llow, i.e. they have a typical memory phenotype. We showed that proliferative response and Bcl-XL up-regulation was potently induced in these cells by exogenous IL-2. The effect of exogenous IL-2 on ICOS-mediated proliferative responses of CD44low CD4+ and CD44low CD8+ T cells. The cells (1 x 10^5) purified from BALB/c splenic T cells by cell sorting were co-cultured with antibody-coated beads (1 x 10^5) in the presence or absence of rIL-2 for 48 h. [3H]TdR incorporation was measured during the last 8 h of the culture period. Data shown are representative of three independent experiments.

Fig. 6. Differing expression pattern of IL-2 receptor subunits and IL-2-mediated signaling in CD4+ and CD8+ T cells after ICOS stimulation. (A) Splenic T cells were stimulated as indicated. At 24 h, the expression patterns of IL-2 receptor subunits in both CD4+ and CD8+ T cells were analyzed by flow cytometry. Data shown are representative of two independent experiments. (B) Splenic T cells were stimulated as indicated for 24 h. CD4+ and CD8+ T cells were isolated from the harvested cultures, starved for 8 h in serum-free medium and then restimulated with rIL-2 for 10 min. Cell lysates were prepared and subjected to western blot analysis for Stat5 phosphorylation. Data shown are representative of two independent experiments. (C) Splenic CD4+ T cells (5 x 10^5) were stimulated as indicated for 24 h. Cell lysates were prepared and equal protein levels of each sample were subjected to western blot analysis for Bcl-XL expression. Data shown are representative of three independent experiments.

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populations by ICOS co-stimulation (Fig. 5B and C) and recently observed that this ICOS+ population not only produces IL-2, but is also a major source of IL-4 and IFN-γ production after ICOS co-stimulation (unpublished data). These results demonstrate a potent co-stimulatory effect of ICOS on the function of memory CD4+ T cells, and a qualitative difference of the ICOS signal between naive and memory CD4+ T cells.

How are these findings of different responses of CD4+ and CD8+ T cell populations to ICOS signaling relevant to physiological settings? It has been reported that the ICOS ligand B7h is widely expressed in non-lymphoid organs, whereas expression of CD28 ligands, B7-1 and B7-2, is rather restricted to lymphoid organs (9). The experimental system adopted in this study, where the ICOS signal is delivered to target T cells by ICOS/CD3 beads, is a particularly adequate system to study the role of ICOS in the immune response under conditions where ICOS-mediated co-stimulation is working in the absence of the B7-CD28 family.

B7h was initially reported as a tumor necrosis factor-α-inducible gene (9), and abundant expression of ICOS was often observed in inflammatory sites (19, 35, 36). Thus, it is likely that ICOS–B7h interaction plays a pivotal role in the development of immune responses at primary inflammatory sites. Indeed, several reports have demonstrated that blockade of the B7h–ICOS interaction during effenter immune response efficiently ameliorates disease progression (19, 20, 36, 37). It is particularly important to emphasize that somatic cells, such as epithelial cells (35, 38) and endothelial cells (39, 40), express B7h but not B7-1 and B7-2. Therefore, in these peripheral tissues, the B7h–ICOS interaction may play a primary role rather than the B7–CD28 interaction. In addition, the dominant expansion of CD8+ T cells by ICOS-mediated co-stimulation may have further important consequences, since MHC class I expression of somatic cells would also be upregulated at inflammatory sites (41), which provides an advantage for the effect of CD8+ T cells.

It should be noted that CD122 is also used as a signaling subunit for the IL-15 receptor (42). Several recent studies suggested that IL-15 is a pivotal survival and growth factor for CD8+ T cells (42). Since it is also known that IL-15 has a proinflammatory role in immune responses (43) and is not secreted by T cells, but is produced by several other cell types (44–47), it is possible that, instead of (or in addition to) IL-2 being produced by migrated memory CD4+ T cells, IL-15 could be a cytokine critically involved in clonal expansion of ICOS-stimulated CD8+ T cells at the inflammatory site.

In summary, our study has demonstrated the different effects of ICOS signaling on each T cell subset, and shown qualitative differences between CD28- and ICOS-mediated signals on naive CD4+ T cell activation. Moreover, the ICOS co-stimulatory effect on CD8+ T cells and memory CD4+ T cells adds new insights for the physiological roles of ICOS other than Tc2 differentiation and Th1/Th2 effector functions.

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Abbreviations
APC antigen-presenting cell
2ME 2-mercaptoethanol
B7h B7 homologous protein
BCA Bicinchoninic acid
CFSE carboxy fluorescein diacetate succinimidyl ester
[^H]TdTR[^H]thymidine
ICOS inducible co-stimulator
NP nitrophilen
PI3K phosphatidylinositol-3-kinase

References
Distinct effect of ICOS ligation on CD4\(^+\) and CD8\(^+\) T cells


