Cutting Edge: The Related Molecules CD28 and Inducible Costimulator Deliver Both Unique and Complementary Signals Required for Optimal T Cell Activation

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Optimal T cell activation requires engagement of CD28 with its counterligands B7-1 and B7-2. Inducible costimulator (ICOS) is the third member of the CD28/CTLA4 family that binds a B7-like protein, B7RP-1. Administration of ICOS-Ig attenuates T cell expansion following superantigen (SAg) administration, but fails to regulate either peripheral deletion or anergy induction. ICOS-Ig, but not CTLA4-Ig, uniquely regulates SAg-induced TNF-α production, whereas IL-2 secretion is modulated by CTLA4-Ig, but not ICOS-Ig. In contrast, both ICOS and CD28 are required for complete attenuation of IL-4 production. Our data suggest that ICOS and CD28 regulate T cell expansion and that ligation of either CD28 or ICOS can either uniquely regulate cytokine production (IL-2/TNF-α) or synergize for optimal cytokine production (IL-4) after SAg administration. The Journal of Immunology, 2001, 166: 1–5.

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Materials and Methods

Mice and in vivo treatment

Six-week-old BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free mouse facility (Millennium Pharmaceuticals, Cambridge, MA). Mice received one dose of 50 μg/mouse of SEB (Sigma, St. Louis, MO) in a single i.v.

Abbreviations used in this paper: ICOS, inducible costimulator; SAg, superantigen; SEB, Staphylococcus aureus enterotoxin B.
Injection on day 0. Control littermates were injected with an identical volume (200 µl) of PBS. For the blocking experiments, mice also received 100 µg/mouse of ICOS-Ig or CTLA4-Ig. Fusion proteins were administered i.p. 90 min before SAg administration on days 0 and 2 for the experimental group of mice sacrificed on day 3; and on days 0, 2, 4, 6, and 8 for the experimental group of mice sacrificed on day 10. Human Ig (100 µg/mouse) was used as an appropriate control.

Immunofluorescence analysis
Spleen and lymph node mononuclear suspensions were subjected to hypotonic erythrocyte lysis, washed, and then stained using mAb directed against CD4 (GK1.5), CD8 (53-6.7), Vß6 (RR4-7), and Vß8 (F23.1) obtained from Pharmingen (San Diego, CA). The Abs anti-CD4 and anti-CD8 were PE labeled, and anti-Vß6 and anti-Vß8 were FITC labeled. Quantitative fluorometric analysis was performed on a FACScaliber instrument (Becton Dickinson, Mountain View, CA).

To obtain the absolute number of spleen or lymph node CD4+Vß8+ and CD8+Vß8+ T lymphocytes, the percentages of these cell types determined by flow cytometry were multiplied by the total number of cells recovered from the spleen or lymph node, respectively.

ICOS-Ig and CTLA4-Ig generation
ICOS-Ig was generated as described previously (11). CTLA4-Ig was obtained from Chimerigen (Allston, MA).

Culture conditions
Triplicates of 1.5 × 10^7 or 3 × 10^7 spleen cells were cultured for 3 days in medium (200 µl/well of RPMI 1640 supplemented with 10% FCS, 50 µM 2-ME, 10 mM HEPES, 200 mM l-glutamine, 10 U/ml penicillin, and 100 µg/ml streptomycin) supplemented with 20 µg/ml SEB or 3 µg/ml Con A. For blocking experiments in vitro, cells were preincubated for 2 h with different concentrations (0, 5, 50, or 100 µg/ml) of either ICOS-Ig, CTLA4-Ig, or human Ig and then stimulated with SEB (20 µg/ml) for 72 h. For assessment of proliferation, cells were harvested after an 8-h pulse labeled with 1 µCi of [3H]thymidine (Amersham, Little Chalfont, U.K.).

Cytokine measurement
Blood was collected at 1, 1.5, or 2 h after enterotoxin challenge. Serial dilutions of serum samples were assayed using commercial ELISA kits for TNF-α, IFN-γ, IL-2, IL-4, and IL-10 (Endogen, Boston, MA).

Results and Discussion
The response of peripheral T lymphocytes in BALB/c mice to the i.v. administration of SEB consists of 1) a rapid cytokine production in the serum, which peaks 90–120 min after SEB injection; 2) a transient proliferative expansion of CD4+Vß8+ and CD8+Vß8+ T cells, which reaches a maximum level at 3–4 days following SEB treatment; and 3) a deletion phase of mature Vß8+ lymphocytes via apoptosis, which is clearly present 10 days after SEB injection and lasts over 30 days (16, 19, 20, 22–24, 30–32). The surviving SEB-specific Vß8+ T cells in vivo are anergic as assessed by their low proliferative response to a second enterotoxin stimulation in vitro (16, 19, 20, 22, 24).

Effect of ICOS on SEB-induced Vß8+ T cell proliferation
On day 3, SEB treatment resulted in 2-fold and 5-fold increases in the total number of peripheral CD4+Vß8+ and CD8+Vß8+ T cells, respectively, when compared with that observed in PBS-treated controls (Fig. 1, A and B). The size of the peripheral Vß8+ T cell population from PBS-treated mice is not different from that of PBS+hlg-; PBS+ICOS-Ig-, or PBS+CTLA4-Ig-treated littermates, indicating that the administration of the fusion proteins alone does not have any detectable effect on cell numbers (data not shown). However, administration of ICOS-Ig (100 µg/mouse) reduces the expansion of both CD4+ and CD8+Vß8+ T cells in response to SEB by 70% in both secondary lymphoid tissues (Fig. 1, A and B). The suppression of T cell expansion was comparable in magnitude to that observed using CTLA4-Ig. These data support recent work in CD28-deficient mice or using CD28 mAbs that T cell expansion requires CD28 engagement (29, 33). Administration of both CTLA4-Ig together with ICOS-Ig failed to further suppress T cell expansion (data not shown). No significant responses of Vß8+ T cells to SEB in control mice or experimental littermates were detected (data not shown) indicating the specificity of SEB for Vß8+ T lymphocytes.

![FIGURE 1. Effect of SEB on the clonal expansion of peripheral Vß8+ T cells following ICOS-Ig and CTLA4-Ig administration. Mice received a single i.v. injection of SEB, and on day 3 the absolute number of spleen (A) or lymph node (B) CD4+ Vß8+ (black circles) or CD8+ Vß8+ T cells (gray circles) was determined by flow cytometry as described in Materials and Methods. ICOS-Ig or CTLA4-Ig was administered i.p. on days 0 and 2. Each dot represents a single mouse, and each bar represents the mean of each experimental or control group. Open symbols indicate PBS-treated control littermates. *, A significant difference between control and test group of mice was determined using Student’s t test (p < 0.001). C, SEB-induced Vß8+ T cell proliferation in vitro following ICOS-Ig or CTLA4-Ig administration. Spleen cells from untreated mice (0.5 × 10^6/well) were incubated for 2 h with either ICOS-Ig (△), CTLA4-Ig (□), or hlg (●) at the indicated concentrations. SEB (20 µg/ml) was added to all of the wells but those control wells containing medium (○). Proliferation assessment was determined after an 8-h pulse label with 1 µCi of [3H]thymidine on 48-h microtiter well cultures.](http://www.jimmunol.org/)

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To further confirm that ICOS is involved in SAg-induced T cell expansion, proliferation assays in vitro were performed. Splenic cells from unprimed mice were preincubated with different concentrations of ICOS-Ig, CTLA4-Ig, or human Ig and then stimulated with SEB. Fig. 1C shows that a decrease in T cell proliferation was detected when ICOS-Ig or CTLA4-Ig was present in the culture. At 50 μg/ml, ICOS-Ig and CTLA4-Ig induced a 3.5-fold and a 2.5-fold decrease, respectively, in SEB-driven Vβ8⁺ T cell proliferation (Fig. 1C). Taken together, these data suggest that clonal expansion of SEB-reactive Vβ8⁺ T cells can be suppressed by either CD28 or ICOS blockade. The precise mechanisms whereby these two molecules regulate T cell expansion are unclear. However, we have observed that mice treated with CTLA4-Ig or mice genetically deficient in CD28 show a reduced ICOS expression after SEB administration (J.-A. G., unpublished observations). This suggests that CD28/B7 interactions are in part required for ICOS induction, which then can interact with its ligand B7RP-1, which is constitutively expressed on B cells and macrophages, to regulate T cell clonal expansion. These data differ from our previous observation with soluble Ags, whereby CTLA4-Ig, but not ICOS-Ig, inhibits proliferation of resting CD4⁺ T cells (11). The precise reasons for this difference remains to be further elucidated. However, after SAg treatment, both B7RP-1-positive B cells and macrophages, are involved in Ag presentation to Vβ8⁺ specific T cell, whereas murine dendritic cells, which do not express the ICOS ligand (10), are likely the principle cell population that most effectively presents processed peptides from soluble proteins to Ag-specific CD4⁺ T cells.

Effect of ICOS on SEB-induced Vβ8⁺ T cell clonal deletion

Clonal size can be regulated either by depletion of Ag-stimulated cells or by anergy induction. CD28 has been demonstrated to provide an anti-apoptotic signal via the up-regulation of BCL-xL (34, 35) and at least in part, through the stabilization of IL-2 mRNA, to support T cell expansion (36). However, despite the suboptimal proliferation of peripheral CD4⁺Vβ8⁺ and CD8⁺Vβ8⁺ T cells detected in SEB-treated mice following either ICOS-Ig or CTLA4-Ig administration, these lymphocytic populations were reduced during the enterotoxin-driven deletion phase as much as the same populations in the SEB-treated control littersmates (data not shown). This indicates that neither ICOS- nor CD28-mediated signals regulate clonal deletion in response to SEB and supports the observation that responsive cells undergo depletion in CD28-deficient mice (29).

Effect of ICOS on SEB-induced Vβ8⁺ T cell anergy

The Vβ8⁺ T lymphocytes that have not been eliminated by apoptosis in vivo 10 days after the single i.v. administration of SEB are anergic. Subsequently, these cells show a very low proliferative response in vitro to a second restimulation with the enterotoxin (18–27). To determine whether costimulatory signals mediated by ICOS are required for the induction of Vβ8⁺ T cell anergy in response to SEB, the proliferative response to SEB in vitro of splenocytes from ICOS-Ig-treated mice after SEB priming in vivo was evaluated. As expected, spleen Vβ8⁺ T cells from SEB-treated mice failed to proliferate to the enterotoxin in vitro (Fig. 2A). Whereas CTLA4-Ig administration in vivo was able to significantly reduce SEB-induced Vβ8⁺ T cell anergy, ICOS-Ig did not show any effect on the impairment of these cells to mount a proliferative response upon SEB restimulation (Fig. 2A). An increase in the dose of SEB (25 or 50 μg/ml) did not modify the suboptimal T cell activation observed with lower doses (data not shown).
Effect of ICOS on SEB-induced cytokine production

Cytokine production after SAg administration is a consequence of T cell-derived stimulation (25, 38). We next investigated whether CD28 and ICOS would also deliver comparable signals required for cytokine production. CD28-mediated costimulation is critical for SEB-induced early secretion of IL-2 (Fig. 3); however, by 2 h post SEB administration, IL-2 production is comparable in both control Ig- and CTLA4-Ig-treated mice (Fig. 3). Administration of ICOS-Ig had no significant effect on IL-2 production and did not synergize with CTLA4-Ig for additional suppression of IL-2 secretion (Fig. 3). In contrast, ICOS, but not CD28, delivered an important signal for TNF-α secretion (Fig. 3). Similar to IL-2, no synergistic effects between ICOS and CD28 were observable (Fig. 3). These results differ from data using CD28-deficient mice, which exhibit a marked protection from repeated peritoneal administrations of Toxin Shock Syndrome toxin-1 (TSST-1), which is a consequence of abrogated TNF-α production (39). Likewise, SEB-induced TNF-α production was partially protected by anti-B7-2, but not B7-1 blockade (40). The precise reasons for these differences are at present unknown, although different toxins and routes of administration might use different signals. IFN-γ production was completely inhibited by CTLA4-Ig, although ICOS-Ig alone reduced IFN-γ levels by 50% 2 h post SEB administration (Fig. 3). Finally, CD28 signaling was important for IL-4 production, whereas ICOS-Ig administration alone was ineffective (Fig. 3). However, unlike IL-2, IFN-γ, and TNF-α, the combination of CD28 and ICOS blockade completely inhibited IL-4 production (Fig. 3). Thus CD28 delivers signals preferential for IL-2 and IFN-γ, ICOS signals for TNF-α, whereas CD28 and ICOS synergize for complete inhibition of IL-4 production in response to SEB.

In conclusion, ICOS/B7RP-1 plays an important role in mediating T cell responses to SAg and is at least as important as the signals that results from CD28/B7-1 and B7-2 interaction. CD28 and ICOS also have distinct roles in regulating cytokine production; IL-2 (strictly CD28 dependent), TNF-α production (strictly ICOS dependent), IFN-γ (CD28 dependent and in part ICOS dependent). For other cytokines such as IL-4, signaling through both ICOS and CD28 are required for IL-4 production. We suggest that ICOS is an important costimulatory molecule involved in optimal T cell expansion, but not in either peripheral deletion or anergy and can deliver both unique and complementary signals to CD28 for cytokine secretion.

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References


