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ICOS Costimulation Expands Th2 Immunity by Augmenting Migration of Lymphocytes to Draining Lymph Nodes\textsuperscript{1}

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The T cell costimulatory molecule ICOS regulates Th2 effector function in allergic airway disease. Recently, several studies with ICOS\textsuperscript{−/−} mice have also demonstrated a role for ICOS in Th2 differentiation. To determine the effects of ICOS on the early immune response, we investigated augmenting ICOS costimulation in a Th2-mediated immune response to Schistosoma mansoni Ags. We found that augmenting ICOS costimulation with B7RP-1-Fc increased the accumulation of T and B cells in the draining lymph nodes postimmunization. Interestingly, the increased numbers were due in part to increased migration of undivided Ag-specific TCR transgenic T cells and surprisingly B cells, as well as non-TCR transgenic T cells. B7RP-1-Fc also increased the levels of the chemokines CCL21 and CXCL13 in the draining lymph node, suggesting ICOS costimulation contributes to migration by direct or indirect effects on dendritic cells, stromal cells and high endothelial venules. Further, the effects of B7RP-1-Fc were not dependent on immunization. Our data support a model in which ICOS costimulation augments the pool of lymphocytes in the draining lymph nodes, leading to an increase in the frequency of potentially reactive T and B cells. The Journal of Immunology, 2008, 181: 1019–1024.

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Materials and Methods

\textbf{Animals}

Female C57BL/6 mice (4- to 6-wk-old) were purchased from The Division of Cancer Treatment at the National Cancer Institute or Charles

The data on humoral immunity in the ICOS\textsuperscript{−/−} mice correlated with findings in humans who are ICOS deficient and suffer from common variable immunodeficiency (14). ICOS-deficient individuals were found to have profound defects in B cell maturation and Ig isotype switching. Initially, no defects in T cell function were found. However, investigators have now demonstrated that ICOS-deficient individuals have a T cell defect in IL-10 production, as well as in IL-17 (15). These findings suggest that ICOS costimulation is necessary for the maintenance of a mature B cell compartment. A major role for the ICOS-B7RP-1 pathway in the Th cell-dependent B cell responses has emerged and has been confirmed in humans.

A focus of our laboratory has been the role of ICOS in Th2-mediated diseases in both mice and humans (16, 17). We and others have published data that in vivo blockade of ICOS affects Th2 effector function (16, 18). However, ICOS\textsuperscript{−/−} mice have a defect in Th2 differentiation and ICOS has been shown to play a role in the initial expansion of primary and activated T cells in both Th1 and Th2 immune responses (8–10, 19, 20). We hypothesized that ICOS-B7RP-1 interactions are most influential on Th2 cells after initial activation when ICOS is known to be highly expressed (1). We have found that increased ICOS costimulation with B7RP-1-Fc regulates Th2-dependent immune responses by augmenting the number of Ag-specific T cells in the secondary lymphoid tissue partially through increased migration. Moreover, we found that ICOS costimulation has a dramatic effect on the migration of B cells and non-Ag-specific T cells into the lymph nodes. Surprisingly, the effect of B7RP-1-Fc on T and B cells does not require immunization, suggesting that ICOS costimulation affects homeostasis of the pool of T and B cells in the lymph nodes.
River Laboratories. DO11.10 BALB/c TCR transgenic mice, which express a TCR specific for OVA peptide 323–329 presented in the context of I-A^d were bred in house (21). BALB/c and B6.Tcrb^-/-Terd^-/- mice were purchased from The Jackson Laboratory. ICOS^-/- mice were a gift from Richard Flavell (Yale University, School of Medicine, New Haven, CT), and were backcrossed to C57BL/6 mice. All animals were housed in a specific pathogen-free facility maintained by the University of Chicago Animal Resources Center. The studies detailed herein conform to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Flow cytometry and reagents

Before staining, cells were incubated with anti-FcR (2.4G2) hybridoma supernatant. Dead cells were excluded by the addition of propidium iodide. All Abs were purchased from BD Pharmingen with the exception of anti-CD4 (eBiosciences) and anti-KJ1–26, which was directly conjugated by the Flow Cytometry Facility, University of Chicago (Chicago, IL). Flow cytometry analysis was performed using a FACSscan (BD Biosciences), a FACSCalibur (BD Biosciences), or an LSRII (BD Biosciences). Data was analyzed using CellQuest software. B7RP-1-Fc and human-Fc (HFc),^4 were supplied by Amgen. OVA peptide 323–339 was produced by the University of Chicago Peptide Synthesis Facility.

Schistosoma mansoni

C57BL/6 mice were immunized i.p. on day 0 with 5 × 10^5 inactivated S. mansoni eggs. Mice received an i.p. injection of either 25 μg HFc or 50 μg B7RP-1-Fc on days 7, 9, 11, and 13 after immunization.

Adoptive transfer

Pooled spleen and lymph node cells from naive TCR transgenic DO11.10 donor mice were used. In some experiments, donor cells were labeled with CFSE (Molecular Probes) before transfer as previously described (22). Before transfer, the percentage of CD4^+ KJ1–26^+ TCR transgenic T cells was determined by flow cytometry. Five × 10^6 TCR transgenic T cells were injected into BALB/c mice 2 days before immunization. Recipient mice were sensitized s.c. on day 0 with 50 μg OVA peptide in alum. On days 7, 9, 11, and 13 mice received an i.p. injection of either 25 μg HFc or 50 μg B7RP-1-Fc. Animals were sacrificed at indicated time-points during the experiment and TCR transgenic T cells were detected in draining lymph nodes by labeling with anti-CD4 and KJ1–26 Abs.

Lymph node, lung, and spleen restimulation ex vivo

Lungs were disassociated by agitating the tissue in digestion buffer containing hyaluronidase, (Sigma-Aldrich), DNase1 (Boehringer Mannheim), and collagenase P (Boehringer Mannheim) as previously described (16). Cells were plated at 2 × 10^6 cells per well (lungs) or 4 × 10^6 cells per well (lymph node (LN) and spleen) in a 96-well U-bottom plate. T cell-depleted, irradiated splenocytes were mixed with lung cells at a 1:1 ratio. Cells were cultured with media alone or soluble egg Ag (SEA, 5 μg/ml). After 48 h, supernatants were analyzed by ELISA.

Cytokine and chemokine analysis

Cytokine production (IL-4, IL-5, IL-10, IL-13, and IFN-γ) by lymph node, lung and spleen cultures was measured by ELISA according to the manufacturer’s protocol (BD Pharmingen or for IL-13, R&D Systems) or by Cytokine Bead Array (BD Pharmingen). For chemokine measurements, lymph node, lung, or spleen were placed into preweighed sterile tubes containing a solution of PMSF, leupeptin, and aprotinin. Tubes were reweighed to calculate the weight of each tissue obtained. Tissues were then placed into preweighed sterile tubes containing a solution of PMSF, leupeptin, and aprotinin. Tubes were reweighed to calculate the weight of each tissue obtained. Tissues were then homogenized using a hand-held homogenizer for ~20 s. Homogenized tissue was spun down for 10 min at 3000 rpm and the supernatant was collected for chemokine analysis by ELISA (BD Pharmingen). Chemokine concentration measured in ng/ml was divided by the weight of the tissue in grams.

Statistical analysis

All statistics were done using an unpaired Student’s two-tailed t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Error bars represent SEM.

^4 Abbreviations used in this paper: HFc, Human-Fc; DLN, draining lymph node; SEA, soluble egg antigen; LN, lymph node; Tg^+, DO11.10 TCR transgenic T cell.

Results

Augmenting ICOS costimulation increases effector cell migration to the lungs in the absence of challenge

Our previous work provided evidence that ICOS enhanced Th2 cell effector function in the lung (16). However, an important role for ICOS in the primary immune response has been established by several recent studies (9, 10, 19, 23). We hypothesized that ICOS-B7RP-1 interactions affect Th2 cells after initial activation. To induce a robust Th2 immune response, C57BL/6 mice were sensitized i.p. with inactivated S. mansoni eggs, and 1 wk later, the mice were treated with either B7RP-1-Fc or isotype control (HFc) every other day for 1 wk and sacrificed on day 14 post sensitization (Fig. 1A). ICOS costimulation augmented the total numbers of CD4^+ , CD8^+ , and CD19^+ lymphocytes within the DLN (inguinal and aortic) (B), spleen (D), or lungs (F) were determined. Cytokine levels were measured from the DLN, spleen, and lung cells restimulated for 48 h with SEA (C, E, and G). The data shown are representative of three independent experiments.

To evaluate the Ag-specific Th2 responses, ex vivo DLN, spleen, and lung cells were restimulated with S. mansoni SEA in vitro and IL-5 production was measured. In the DLN cultures, spontaneous IL-5 production was significantly greater in the cultures from the B7RP-1-Fc treated animals compared with controls, but otherwise no differences were detected in the DLN or spleen (Fig. 1, C and E). No significant differences in either IFN-γ or IL-10 production were found (data not shown). Interestingly, cells from the lungs of B7RP-1-Fc-treated mice produced significantly
greater amounts of IL-5 when restimulated with SEA compared with controls (Fig. 1G). This was surprising since no differences in the total number of CD4+ or CD8+ T cells recovered from the lung tissue were found between the control group and the B7RP-1-Fc treated group (Fig. 1F). Further, the mice were not challenged with Ag in the lung. Similar to the DLN cultures, spontaneous IL-5 production from cultures of lung lymphocytes from B7RP-1-Fc treated mice was also elevated when compared with the response from control mice (Fig. 1G). Thus, ICOS costimulation augments the Ag-specific response in the lung even in the absence of local Ag challenge.

**ICOS-mediated costimulation increases both Ag-specific and nonspecific populations within the DLN as a result of increased migration**

While the data above suggested that ICOS costimulation increased the population of Ag-responsive cells in vivo, it was not possible to track Ag-specific cells in the *S. mansoni* model. To evaluate the specific T cell response, we used an adoptive transfer model of DO11.10 cells that were immunized s.c. with OVA in alum. Mice were treated with B7RP-1-Fc or HFc as indicated in the figure. The total number of cells within the DLN (axillary) was determined. Tg CD4+ T cells (A), Tg CD4+ T cells day 14 only (B), non-transgenic CD4+ T cells (C), and CD19+ cells (D) are shown. Each time point is the mean ± SEM of three to four mice per group except day 0 (n = 2). The data are representative of three independent experiments.

![FIGURE 2. B7RP-1-Fc treatment expands the number of cells within the lymph nodes in an adoptive transfer model. BALB/c mice adoptively transferred with DO11.10 cells were immunized s.c. with OVA in alum. Mice were treated with B7RP-1-Fc or HFc as indicated in the figure. The total number of cells within the DLN (axillary) was determined. Tg CD4+ T cells (A), Tg CD4+ T cells day 14 only (B), non-transgenic CD4+ T cells (C), and CD19+ cells (D) are shown. Each time point is the mean ± SEM of three to four mice per group except day 0 (n = 2). The data are representative of three independent experiments.](http://www.jimmunol.org/images/article/1243202f.jpg)

**ICOS costimulation augments chemokine levels in the DLN**

The major chemokine receptor on naive T and B cells is CCR7, while the receptor CXCR5 is important for B cell homing within the lymph node (24, 25). CCL21 (SLC), one of the ligands for CCR7, is expressed by high endothelial venules, dendritic cells, and stromal cells in the T cell areas of lymphoid tissue (26). CXCL13 (BLC), produced by stromal cells directs B cells to B cell follicles and is the ligand for CXCR5 (25). Since ICOS costimulation affected migration of both T and B cells into the draining lymph node, we postulated that the effects may be mediated through increased production of some chemokines. The levels of CCL21 and CXCL13 were measured in the supernatant of DLN, lung, and spleen tissue homogenates prepared on day 14 of the adoptive transfer model described above. Both CCL21 and CXCL13 were significantly elevated in B7RP-1-Fc treated mice compared with controls in the DLN but not the lungs or spleen (Fig. 4). ICOS costimulation may directly or indirectly influence
the production of chemokines known to mediate T and B cell homing within the lymph node.

**FIGURE 3.** B7RP-1-Fc treatment post-sensitization increases migration of T and B cells into the DLN. BALB/c mice adoptively transferred with CFSE labeled DO.11.10 cells were immunized and treated with B7RP-1-Fc or HFc as in Fig. 2. A, Representative dot plot showing the division of Tg CD4+ T cells into CFSE<sup>high</sup> or CFSE<sup>low</sup> on day 14. B, The total number of CFSE<sup>high</sup>Tg+ CD4+ T cells (upper panel) and CFSE<sup>low</sup>Tg+ CD4+ T cells in treated mice (■) compared with controls (□). Percentage (C, E) and absolute numbers (D, F) of non-Tg+ CD4+ T cells and CD19+ B cells that were CFSE<sup>high</sup> on day 14. Hatched bar, before treatment; □ HFc; ■ B7RP-1-Fc. Each bar is the mean ± SEM of four mice. The data are representative of three independent experiments.

**FIGURE 4.** Increased ICOS costimulation with B7RP-1-Fc post-sensitization augments chemokine levels within the DLN but not spleen or lung. CCL21 and CXCL13 levels in the cell lysates from B7RP-1-Fc-treated mice (■) compared with control HFc treated mice (□) on day 14. The mice were immunized and treated as described in Figs. 2 and 3. For CXCL13 the LN and lung are on the left axis and the spleen is on the right axis. The data are from three mice per group and are representative of two independent experiments.

**B7RP-1-Fc increases T and B cells in the lymph nodes in the absence of immunization**

In the adoptive transfer studies, we found that both Ag-specific and non-specific T cells were increased in the DLN of mice treated with B7RP-1-Fc. The data suggested that augmenting ICOS costimulation may affect lymphocyte migration in the absence of Ag stimulation. We investigated the effects of B7RP-1-Fc treatment in B6 mice in the absence of immunization. Interestingly, after only two doses of B7RP-1-Fc i.p., increased numbers of T and B cells were found in the inguinal lymph nodes (Fig. 5A). The numbers of both CD4 and CD8 T cells were increased. The effect was specific to the lymph nodes, as no increases in cell numbers were found in the spleen (Fig. 5B). As a control, we evaluated the effect in ICOS<sup>−/−</sup> mice and found no differences between B7RP-1-Fc treated mice and controls (Fig. 5C). Further, in B6. Tcrb<sup>−/−</sup>Tcρ<sup>−/−</sup> mice the...
increased the levels of the chemokines CCL21 and CXCL13, surprisingly, B cells and nonspecific T cells. B7RP-1-Fc also part to increased migration of undivided Ag-specific T cells and the lymph nodes. The increased numbers were due at least in B7RP-1-Fc led to increased accumulation of T and B cells in the immune response are not entirely clear. In the current study, B cell responses. Yet, the mechanisms by which ICOS regulates Th2 responses in vivo, as well as a major role in T cell dependent migration of lymphocytes and enhancing the likelihood of an effector response. While our data suggested there was no effect of B7RP-1-Fc on T cell proliferation, ICOS may also increase the T cell pool in the lymph node by promoting T cell survival as recently reported (28). Further, the need for a “boost” from ICOS may be more apparent in instances of weak stimuli and may account for the differential dependence on ICOS in different Th2-mediated infectious parasite models (20, 29).

Interestingly, the effects of B7RP-1-Fc were not dependent on Ag sensitization. The T cells expressing the highest level of ICOS in the absence of activation are memory T cells and NK T cells (1, 30). Augmenting ICOS costimulation may stimulate memory T cells or NKT cells to directly or indirectly promote increased migration of T and B cells. It is also possible that B7RP-1 expressed on lymph node stromal cells costimulates T cells and plays a role in recruiting lymphocytes. While the effect may be due to a TCR independent signal, we cannot rule out T cell activation with environmental Ags. Interestingly, mice transgenic for B7RP-1-Fc developed lymphoid hyperplasia and hypergammaglobulinemia as they aged, suggesting a response to environmental Ags is sufficient (5). However, the timing of our effect suggests a more acute role for ligation of ICOS on memory T cells in increasing the pool of lymphocytes in the lymph node.

After B7RP-1-Fc treatment, we found an increase in the chemokines CXCL13, the ligand for CXCR5, and CCL21, the ligand for CCR7. Our data suggest the mechanism by which B7RP-1-Fc augments the pool of lymphocytes in the lymph node and effector tissues are due to effects on chemokine production. Both germinal center B cells and follicular B helper T cells express CXCR5. ICOS has previously been shown to be highly expressed on follicular B helper T cells and to contribute to their development and function in vivo (31–37). While ICOS is not known to directly stimulate chemokine production, it is possible that T cell cytokines regulate the secretion of chemokines by the high endothelial venules, dendritic cells, and stromal cells. TNF-α has been shown to affect lymphoid structure in tissues and germinal center formation (38, 39), suggesting TNF-α may regulate chemokine production. Interestingly, we have found in our mouse model of allergic airway disease that B7RP-1-Fc treatment augments the level of TNF-α in the airways (our unpublished data). A possible mechanism for the increased migration of lymphocytes in our D011.10 model is increased chemokine secretion, as a result of increased TNF-α in response to ICOS costimulation of T cells. A cell:cell contact mechanism between T cells and the chemokine producing cells may also be an underlying mechanism. Nevertheless, our data suggest that ICOS can regulate T:B interactions by augmenting migration of lymphocytes and enhancing the likelihood of an efficient B cell response. A greater understanding of how ICOS regulates the accumulation of T and B cells in the DLN, as well as at sites of inflammation, such as the lung, will provide insight into the mechanisms by which T cells perpetuate local immune responses in diseases like asthma and allergy.

FIGURE 5. B7RP-1-Fc response does not require immunization. C57BL/6 mice were treated on days 0 and 2 with either B7RP-1-Fc (n = 8) or HFc (n = 8) i.p. On day 9 the number of CD4⁺, CD8⁺, and CD19⁺ lymphocytes was determined in the inguinal lymph nodes (A) and spleen (B). Data are from two independent experiments combined. The same protocol was repeated in ICOS−/− mice (C), LN is shown (B7RP-1-Fc n = 5; HFc n = 4) and TCRγ⁻/− mice (D), LN is shown (B7RP-1-Fc n = 4; HFc n = 3).

effect of B7RP-1-Fc was completely abrogated (Fig. 5D). In conclusion, we find that B7RP-1-Fc costimulation of T cells augments the number of lymphocytes in the lymph nodes independent of immunization.

Discussion
The discovery of ICOS as a costimulatory molecule primarily expressed on activated Th2 cells led to the hypothesis that ICOS was important for Th2 effector function. However, subsequent studies have determined the role of ICOS in the expansion of both Th1 and Th2 responses in vivo, as well as a major role in T cell dependent B cell responses. Yet, the mechanisms by which ICOS regulates the immune response are not entirely clear. In the current study, we have found that augmenting ICOS costimulation with B7RP-1-Fc led to increased accumulation of T and B cells in the lymph nodes. The increased numbers were due at least in part to increased migration of undivided Ag-specific T cells and surprisingly, B cells and nonspecific T cells. B7RP-1-Fc also increased the levels of the chemokines CCL21 and CXCL13, suggesting ICOS costimulation contributes to migration by direct or indirect effects on dendritic cells, stromal cells and high endothelial venules. Interestingly, the effects of B7RP-1-Fc were not dependent on immunization.

Our data support a model in which ICOS costimulation augments the pool of lymphocytes in the lymph node, leading to an increase in the number of potentially reactive T and B cells. The frequency of distinct Ag-specific CD4⁺ T cell clones in the mouse has recently been shown to be very low and has been suggested to be advantageous to maintaining a diverse repertoire (27). ICOS may increase the repertoire of Ag-specific lymphocytes migrating to the lymph node and tissue and increase the chances of a robust response. While our data suggested there was no effect of B7RP-1-Fc on T cell proliferation, ICOS may also increase the T cell pool in the lymph node by promoting T cell survival as recently reported (28). Further, the need for a “boost” from ICOS may be more apparent in instances of weak stimuli and may account for the differential dependence on ICOS in different Th2-mediated infectious parasite models (20, 29).


