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The Role of ICOS in the CXCR5+ Follicular B Helper T Cell Maintenance In Vivo

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ICOS is a new member of the CD28 family of costimulatory molecules that is expressed on activated T cells. Its ligand B7RP-1 is constitutively expressed on B cells. Although the blockade of ICOS/B7RP-1 interaction inhibits T cell-dependent Ab production and germinal center formation, the mechanism remains unclear. We examined the contribution of ICOS/B7RP-1 to the generation of CXCR5+ follicular B helper T (T_{FHI}) cells in vivo, which preferentially migrate to the B cell zone where they provide cognate help to B cells. In the spleen, anti-B7RP-1 mAb-treated or ICOS-deficient mice showed substantially impaired development of CXCR5+ T_{FHI} cells and peanut agglutinin (PNA) germinal center B cells in response to primary or secondary immunization with SRBC.

Expression of CXCR5 on CD4+ T cells was associated with ICOS expression. Adoptive transfer experiments showed that the development of CXCR5+ T_{FHI} cells was enhanced by interaction with B cells, which was abrogated by anti-B7RP-1 mAb treatment. The development of CXCR5+ T_{FHI} cells in the lymph nodes was also inhibited by the anti-B7RP-1 mAb treatment. These results indicated that the ICOS/B7RP-1 interaction plays an essential role in the development of CXCR5+ T_{FHI} cells in vivo. The Journal of Immunology, 2005, 175: 2340–2348.
Materials and Methods

Mice

Female BALB/c, C57BL/6, C57BL/10, B10.D2, CBA/N, C3H/He, DBA/1, AJ, and C.B-17iscid (SCID) mice were purchased from Charles River Japan and Japan SLC. OX40L-deficient mice on C57BL/6 or BALB/c background were obtained from Drs. N. Ishii and K. Sugamura (Tohoku University, Japan) (35). CD28-deficient mice on C57BL/6 background were purchased from The Jackson Laboratory. ICOS-deficient mice on C57BL/6 background have been described previously (36). These mice were bred and maintained in the Oriental Yeast Company. All mice were C57BL/6 background have been described previously (36). These mice were purchased from The Jackson Laboratory. ICOS-deficient mice on C57BL/6 background were obtained from Drs. N. Ishii and K. Sugamura (Tohoku University, Osaka, Japan) (35). CD28-deficient mice on C57BL/6 background were gift from Dr. H. Kikutani (Osaka University, Osaka, Japan and Japan SLC. OX40L-deficient mice on C57BL/6 or BALB/c background were purchased from Nippon Bio-supply Center.

Abs and reagents

Anti-B7RP-1 (HK.53) and anti-mouse OX40L (RM134L) mAbs were generated in our laboratory as previously described (37, 38). Control rat IgG was purchased from Sigma-Aldrich. FITC-conjugated anti-CD4 (RM4-5), biotin- or allophycocyanin-conjugated anti-CD45R/B220 (RA3-6B2), and PE-conjugated anti-ICOS (15F9) mAbs were purchased from eBioscience. Biotin-conjugated anti-OX40 (OX86) mAb, biotin- or PE-conjugated anti-CXCR5 (2G8) mAb, rat IgG isotype controls, hamster IgG control, and PE- or allophycocyanin-streptavidin were purchased from BD PharMingen. Biotin- or FITC-conjugated peanut agglutinin (PNA) was purchased from Vector Laboratories. SRBC were purchased from Nippon Bio-supply Center.

Immunization

Groups of five mice were i.p. immunized with 2 × 108 SRBC in 0.2 ml of PBS to induce GC response in the spleen or immunized with 5 × 10⁶ SRBC/50 μl in the footpads to induce GC response in the popliteal LN. In some groups, mice were i.p. administered with 300 μg of anti-B7RP-1 mAb, anti-OX40L mAb, or control rat IgG at the time of immunization (day 0) and on days 2 and 4. Spleen cells or popliteal LN cells were collected at day 6 or 7, and the induction of PNA+ B220+ GC B cells and CXCR5+ T FH cells were analyzed by flow cytometry. To measure the secondary response, mice were i.p. injected with 2 × 10⁶ SRBC/0.2 ml on day 30 after the first immunization. Some mice were treated with 300 μg of anti-B7RP-1 mAb, anti-OX40L mAb, or control rat IgG on days 30 and 32. Three days after the second immunization, spleen cells were analyzed by flow cytometry.

Flow cytometric analysis

Spleen or LN cells (0.5–1 × 10⁶) were first preincubated with unlabeled anti-CD16/32 mAb (BD Pharmingen) to avoid nonspecific binding of mAbs to FcγR. The GC B cells were determined by staining with FITC-labeled PNA and allophycocyanin-labeled anti-B220 mAb. The T FH cells were determined by staining with FITC-labeled anti-CD4 and biotin-labeled anti-CXCR5 mAb, followed by PE-labeled streptavidin. The expression of ICOS, OX40, and CXCR5 on CD4+ T cells was determined by staining with FITC-labeled anti-CD4 mAb and PE- or biotin-labeled mAbs for respective molecules, followed by allophycocyanin-labeled streptavidin. After washing with PBS, the stained cells (live-gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a FACSCanto or BD LSR II (BD Biosciences), and data were processed using the CellQuest program (BD Biosciences).

Histological examination

The spleens from anti-B7RP-1- or rat IgG-treated BALB/c mice and ICOS-deficient or wild-type C57BL/6 mice on day 7 after SRBC immunization were embedded in Tissue-Tek OCT compound (Sakura FineTechnical), and were frozen in liquid nitrogen bath. PNA staining was conducted as previously described (39). Briefly, 3-μm cryostat sections were fixed with 8% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min on ice. Non-specific binding sites were blocked by incubation for 30 min at room temperature in PBS containing 2% BSA fraction V (Sigma-Aldrich). After removing the solution, the sections were incubated with 5 μg/ml biotin-conjugated PNA for 1 h at 37°C. After washing with PBS, the slides were treated with 0.3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity. The sections were incubated with avidin-biotin-peroxidase complex followed by further amplification with biotinyl tyramide (Catalyzed Signal Amplification System; DAKO) according to the manufacturer’s instructions. Subsequently, the peroxidase color reaction was performed by a 10-min application of freshly prepared 0.05% 3,3′-diaminobenzidine-0.01% hydrogen peroxide solution (WAKO). The sections were counterstained with hematoxylin. For immunohistochemistry, 3-μm cryostat sections were fixed with acetone for 10 min at 4°C. The sections were incubated with 2% BSA in PBS for 30 min at room temperature before incubation with Abs to reduce nonspecific binding of Abs. After removing the solution, the sections were incubated with 20 μg/ml biotin-conjugated anti-CD45R/B220 mAb for 1 h at 37°C. After washing with PBS, the sections were labeled with Alexa-Fluor 594-conjugated streptavidin (Molecular Probes) at 1:100 for 1 h at 37°C, and finally incubated with 20 μg/ml FITC-conjugated anti-CD4 mAb for 1 h at 37°C.

ELISA for SRBC-specific serum Abs

SRBC/c mice were i.p. immunized with SRBC on days 0 and 15 and administered with 300 μg of anti-B7RP-1 mAb, anti-OX40L mAb, or control rat IgG on days −1, 0, and 2. Serum anti-SRBC Abs were measured on day 22 by isotype-specific ELISA. Solute SRBC Ags were prepared as described (40) and protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad). Solute SRBC Ags (5 μg/ml in carbonate buffer, pH 9.6) were coated onto 96-well Immulon 2HB plates (Thermo Labsystems). After blocking with 1% BSA in PBS, SRBC-specific IgM and IgG isotypes were determined by incubating serially diluted serum samples for 2 h at 37°C. After washing with 0.05% Tween 20 in PBS, wells were incubated with biotin-conjugated isotype-specific mAbs, including anti-mouse IgG1 (Serotec) or anti-mouse IgG2a, IgG2b, or IgG3 (BD Pharningen), washed, and then developed with Vectastain ABC kit (Vector Laboratories) and o-phenylenediamine (WAKO). After terminating the reaction with 2N H2SO4, OD at 490/595 nm was measured on a microplate reader (Bio-Rad).

Cytokine production by CD4+ T cell subsets

Spleen cells were collected on day 7 after immunization of BALB/c mice with SRBC. ICOS+, CXCR5+, ICOS−CXCR5−, and ICOS−CXCR5− CD4+ T cells were isolated by FACS sorting and 2 × 10⁶ cells per well were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME on 5 μg/ml immobilized anti-CD3 mAb (2C11). To determine the production of cytokines, cell-free supernatants were collected at 48 h and assayed for IL-2, IL-4, IL-5, and IL-10 by ELISA using OptEIA kits (BD Pharmingen) and IFN-γ by using Mouse IFN-γ ELISA Ready-SET-Go! kit (eBioscience) according to the manufacturer’s instructions.

Adoptive transfer experiments

CD62L−CD4+ naive T cells were purified from the spleen of BALB/c mice by passage through nylon wool columns (WAKO) and by using anti-MACs columns with CD4+ T cell isolation kit and anti-CD62L-coupled microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. Small resting B cells were also purified from the spleen of BALB/c mice as previously described (38). Briefly, spleen cells were treated with a mixture of hybridoma supernatants (anti-Thy-1.2, anti-CD4, and anti-CD8) and low-tox rabbit complement (Cedarlane Laboratories). After Percoll (Amersham Biosciences) gradient centrifugation, small B cells were collected from the 60/70% interface. The purified CD4+ T cells (>95% CD4+ CD62L−; 2 × 10⁵ cells) with or without the purified B cells (>95% B220−; 1 × 10⁵ cells) were i.v. injected into SCID mice (day −1). After 24 h, mice were i.p. immunized with 2 × 10⁶ SRBC and then i.p. administered with 300 μg of anti-B7RP-1 mAb or control rat IgG on days 0, 2, and 4. Seven days after the immunization, spleen cells were analyzed by flow cytometry.

Statistical analysis

The results are expressed as the mean ± SD of five mice in each group. Significant differences between two experimental groups were analyzed by the unpaired Student’s t test. Values of p < 0.01 were considered significant.

Results

Effect of anti-B7RP-1 and anti-OX40L mAbs on primary GC formation and TFH cell development

Because a previous report demonstrated an impaired GC formation in ICOS-deficient mice in response to immunization of SRBC (17), we followed the same protocol and used SRBC as an Ag in this study, which can induce robust polyclonal GC responses in an
adjuvant-independent manner. The splenic GC formation was determined by flow cytometric analysis of PNA\(^+\)B220\(^-\) cells, which have been defined as GC B cells (41). In our preliminary experiments, the PNA\(^+\)B220\(^-\) GC B cells appeared in the spleen at a peak frequency between days 6 and 8 and disappeared on day 30 after i.p. immunization of 2 \(\times\) 10\(^7\) SRBC (data not shown).

To explore the contribution of ICOS/B7RP-1 and OX40/OX40L interactions to the development of GC B cells and CXCR5\(^+\)CD4\(^+\) TFH cells in vivo, we administrated a neutralizing anti-B7RP-1 mAb, a neutralizing anti-OX40L mAb, or control rat IgG on days 0, 2, and 4 after immunization of BALB/c mice with SRBC. Spleen cells were collected on day 7, and the development of PNA\(^+\)B220\(^-\) GC B cells and CXCR5\(^+\) TFH cells was determined by two-color flow cytometry. As represented in Fig. 1A and compiled in Fig. 1B, the control IgG-treated mice developed substantial percentages of PNA\(^+\) GC B cells within total B220\(^+\) B cells (10.4 \(\pm\) 1.3\%) upon SRBC immunization. The anti-OX40L mAb treatment exhibited no significant effect on the development of PNA\(^+\)B220\(^-\) GC B cell population (8.7 \(\pm\) 2.3\%). In contrast, the anti-B7RP-1 mAb treatment significantly reduced the PNA\(^+\)B220\(^-\) GC B cell population (2.9 \(\pm\) 0.3\%). This inhibitory effect of anti-B7RP-1 mAb on GC formation was also confirmed by histological examination, in which the spleen sections from the anti-B7RP-1 mAb-treated mice had smaller GC as compared with those from the control IgG-treated mice (Fig. 2A).

Serum levels of SRBC-specific IgG1, IgG2a, and IgG2b Abs were significantly reduced in the anti-B7RP-1 mAb-treated mice as compared with the control IgG-treated mice, while the levels of IgM Abs were not significantly reduced (Fig. 3). This indicated that the ICOS/B7RP-1 interaction played an important role in inducing the production of Ag-specific IgG Abs, as described by previous reports (14–16, 18, 19).

Concurrently, as represented in Fig. 1C and compiled in Fig. 1D, substantial numbers of CXCR5\(^+\) TFH cells were induced in the control IgG-treated mice (5.6 \(\pm\) 0.9\%) within CD4\(^+\) cells) upon SRBC immunization. Again, the anti-OX40L mAb treatment did not significantly affect the development of CXCR5\(^+\) TFH cells (6.0 \(\pm\) 0.3\%). In contrast, the anti-B7RP-1 mAb treatment significantly inhibited the development of CXCR5\(^+\) TFH cell population (1.7 \(\pm\) 0.1\%). This inhibitory effect of anti-B7RP-1 mAb was also observed when the development of CXCR5\(^+\) TFH cells was monitored 2–10 days after SRBC immunization (Fig. 1E). Immunohistological analysis showed that, whereas many CD4\(^+\) T cells were found in the splenic B cell follicles of the control IgG-treated

![Figure 1](http://www.jimmunol.org)
mice, only a small number of CD4+ T cells infiltrated in the splenic B cell follicles of the anti-B7RP-1-treated mice (Fig. 2B). Similar results were obtained when C57BL/6 mice were i.p. immunized with SRBC and treated with anti-B7RP-1 or anti-OX40L mAb (data not shown). These results indicated that the ICOS/B7RP-1 interaction was essential for the development of CXCR5+ T_{FHI} cells and PNA+ GC B cells in the spleen in response to immunization of SRBC. Unexpectedly, no significant contribution of the OX40/OX40L interaction to either GC formation or CXCR5+ T_{FHI} cell development was observed in this experimental system.

ICOS as well as CD40 and CD28, but not OX40, is essential for the development of GC B cells and T_{FHI} cells

To further confirm the critical contribution of ICOS/B7RP-1, but not OX40/OX40L interaction, we immunized ICOS- or OX40L-deficient mice with SRBC and examined the development of CXCR5+ T_{FHI} cells and PNA+ B220+ GC B cells in the spleen 7 days after. We also included CD28- or CD40-deficient mice, which have been reported to have a defect in GC formation (42, 43), for comparison. As expected, CD40- or CD28-deficient mice did not develop PNA+ B220+ GC B cells in response to SRBC immunization (Fig. 4, A and C). Correspondingly, CXCR5+ T_{FHI} cells were not developed in either CD40- or CD28-deficient mice (Fig. 4, B and D). OX40L-deficient mice on either BALB/c or C57BL/6 background did not exhibit a significant defect in the development of either PNA+ B220+ GC B cells or CXCR5+ T_{FHI} cells (Fig. 4, A–D). In contrast, the development of both GC B cells (Fig. 4C) and CXCR5+ T_{FHI} cells (Fig. 4D) were significantly impaired in ICOS-deficient mice. Immunohistological analysis showed that the development of GC and the infiltration of CD4+ T cells in the splenic B cell follicles were notably reduced in ICOS-deficient mice in response to SRBC immunization (Fig. 2). These results indicated that ICOS as well as CD40 and CD28, but not OX40, were essential for the generation of CXCR5+ T_{FHI} cells and GC B cells in the spleen in response to SRBC immunization.

Effect of anti-B7RP-1 and anti-OX40L mAbs on generation of GC B cells and T_{FHI} cells upon secondary immunization

We further investigated the roles of ICOS/B7RP-1 and OX40/OX40L interactions in the secondary response to SRBC immunization. BALB/c mice were i.p. immunized again with SRBC on day 30 after the first immunization, when almost no primary GC B cells remained in the spleen (data not shown), and treated with anti-B7RP-1 mAb, anti-OX40L mAb, or control IgG on days 30 and 32. Three days after the secondary immunization, the development of PNA+ B220+ GC B cells and CXCR5+ T_{FHI} cells in the spleen was analyzed by flow cytometry. As shown in Fig. 5, the control IgG-treated mice quickly developed both PNA+ GC B cells and CXCR5+ T_{FHI} cells. The anti-B7RP-1 mAb treatment significantly inhibited the induction of both PNA+ GC B cells and CXCR5+ T_{FHI} cells, while anti-OX40L mAb treatment showed no significant effect. These results indicated that the ICOS/B7RP-1,
C57BL/6 mice (or CD28-deficient C57BL/6 mice and wild-type BALB/c mice) results suggested that the CXCR5 SRBC-immunized BALB/c mice. A similar pattern was observed Cytokine production by CD4 T cells expressing ICOS and CXCR5

We also examined cytokine production by isolated ICOS+ CXCR5+, ICOS+CXCR5+, and ICOS+CXCR5+ CD4+ T cells after stimulation with immobilized anti-CD3 mAb. As shown in Fig. 7, the ICOS+CXCR5+ cells produced a high level of IFN-γ and low levels of IL-2 and IL-10 but did not produce detectable levels of IL-4 or IL-5. This agrees with the results obtained with human blood and tonsil CXCR5+ T cells (25, 44). In contrast, ICOS+CXCR5+ cells produced high levels of IL-2, IL-4, IL-5, IL-10, and IFN-γ (Fig. 7).

ICOS/B7RP-1 regulates TFH cell development through T/B cell interaction

Splenic B cells constitutively express B7RP-1 (9). Thus, it is likely that the CXCR5+ TFH cell development is regulated by ICOS/B7RP-1 through T/B cell cognate interaction. To address this possibility, purified naïve CD4+ T cells with or without purified naïve B cells from BALB/c mice were adoptively transferred into SCID mice (day -1), which were then i.p. immunized with SRBC on day 0 and treated with anti-B7RP-1 mAb or control IgG on days 0, 2, and 4. Seven days after the immunization, spleen cells were analyzed for the development of CXCR5+ TFH cells by flow cytometry. As shown in Fig. 8, CXCR5+ TFH cells were only marginally induced by SRBC immunization when SCID mice were reconstituted with CD4+ T cells alone. The cotransfer of B cells markedly enhanced the development of CXCR5+ TFH cells, which was abrogated by the anti-B7RP-1 mAb treatment. These results suggested that the ICOS/B7RP-1 interaction was involved in the development of TFH cells through cognate T/B cell interaction.

Effect of anti-B7RP-1 and anti-OX40L mAbs on development of GC B cells and TFH cells in LN

We next examined the contribution of ICOS/B7RP-1 and OX40/OX40L interactions to the development of PNA+B220+ GC B cells and CXCR5+ TFH cells in LN. BALB/c and C57BL/6 mice were immunized with SRBC in the footpads and treated with control IgG or anti-B7RP-1 and/or anti-OX40L mAbs on days 0, 2, and 4. Six days after the immunization, the induction of PNA+B220+ cells and CXCR5+CD4+ cells in the popliteal LN was analyzed by flow cytometry. In BALB/c mice, the treatment with anti-B7RP-1 mAb, but not anti-OX40L mAb, significantly
inhibited the development of CXCR5+ T<sub>FH</sub> cells in the LN (Fig. 10A) as in the spleen (Fig. 1D). However, the development of PNA<sup>+</sup> GC B cells in LN was not significantly inhibited by anti-B7RP-1 and/or anti-OX40L mAbs (Fig. 9A) unlike in the spleen (Fig. 1B). In contrast, in the LN of C57BL/6 mice, the development of both PNA<sup>+</sup> GC B cells and CXCR5<sup>+</sup> T<sub>FH</sub> cells was substantially inhibited by either anti-B7RP-1 or anti-OX40L mAb alone (Fig. 9, C and D). A further inhibition was observed by mixture of both mAbs. These results suggested that while the ICOS/B7RP-1 interaction played the major role in the development of T<sub>FH</sub> cells in LN, the OX40/OX40L interaction also partially contributed to the development of both GC B cells and T<sub>FH</sub> cells in LN of some strains of mice. It was also noted that the development of GC B cells was not always associated with the development of T<sub>FH</sub> cells, as represented by the LN of anti-B7RP-1 mAb-treated BALB/c mice.

Expression of OX40 on T<sub>FH</sub> cells in LN is variable among mouse strains

To explore the mechanism for the differential contribution of OX40/OX40L interaction to the T<sub>FH</sub> cell development in LN between BALB/c and C57BL/6 mice, we examined the expression of CXCR5, ICOS, and OX40 on CD<sup>+</sup> T cells in the LN of BALB/c and C57BL/6 mice at 6 days after the immunization. As shown in Fig. 10A, CXCR5 was preferentially expressed on CD<sup>+</sup> T cells expressing ICOS but not OX40 in the LN of BALB/c mice, as observed in the spleen (Fig. 6A). In contrast, as shown in Fig. 10B, CXCR5-expressing CD<sup>+</sup> T cells in the LN of C57BL/6 mice expressed both ICOS and OX40, while those in the spleen did not express OX40 (Fig. 6B). We then examined the expression of OX40 on the CXCR5<sup>+</sup> T<sub>FH</sub> cells in the LN and spleen of various strains of mice after SRBC immunization. As shown in Fig. 10C, CXCR5<sup>+</sup> T<sub>FH</sub> cells in the LN of C57BL/6 and C3H/He mice distinctively expressed OX40. In contrast, no significant expression of OX40 was observed on splenic CXCR5<sup>+</sup> CD<sup>+</sup> T cells in all strains tested (Fig. 10D). These results indicated that the expression of OX40 on CXCR5<sup>+</sup> T<sub>FH</sub> cells in LN was variable among mouse strains. This might be responsible for the differential contribution of OX40/OX40L interaction to the development of T<sub>FH</sub> cells and GC B cells in LN of certain strains of mice.

Discussion

ICOS and its ligand B7RP-1 have been implicated in GC formation and Ab production in response to T-dependent Ags (14–19). In this study, we found that blockade of ICOS/B7RP-1 interaction by neutralizing anti-B7RP-1 mAb or ICOS deficiency abolished the development of CXCR5<sup>+</sup> T<sub>FH</sub> cells as well as PNA<sup>+</sup>B220<sup>+</sup> GC B cells in the spleen in response to primary or secondary immunization with SRBC. These results suggest that the critical role played by ICOS/B7RP-1 is to induce the CXCR5<sup>+</sup> T<sub>FH</sub> cells that control GC formation and Ab production.

An optimal GC response requires cognate interactions between Ag-specific T cells and B cells (1). T cells are trapped and activated by APCs in the T cell zone. When B cells migrate into lymphoid organs, they first enter the T cell zone. Most of the B cells move quickly through the T cell zone into the B cell zone.
This clearly indicates that the ICOS/B7RP-1 interaction also abrogated the development of both TFH cells and GC B cells interaction with anti-B7RP-1 mAb at the secondary immunization response mediated by memory T cells and memory B cells. In the ICOS/B7RP-1 interaction was involved in the secondary GC re-
to T-dependent Ags (14–19), it remained unclear whether the possibility.

Further studies are needed to address this possibility.

Although recent studies have shown the impaired GC formation and Ab production in ICOS- or B7RP-1-deficient mice in response to the ICOS signal. Previous studies have suggested that OX40 plays a critical role in the regulation of T cell migration into B cell follicles. In particular, OX40 signaling up-regulated CXCR5 mRNA in CD4+ T cells (30). It has been suggested that the impaired GC formation in CD28-deficient mice may be due to compromised OX40 expression on CD4+ T cells (31). Moreover, OX40L-transgenic mice demonstrated an accumulation of OX40+CD4+ T cells in the B cell follicles of secondary lymphoid organs (32). Furthermore, blockade of OX40/OX40L interaction by OX40-Ig in chronic intestinal inflammation has shown a marked reduction of CXCR5+CD4+ T cells in the lamina propria (46). In contrast, a recent study has indicated that CXCR5 expression on Ag-specific T cells and their migration into the B cell zone were comparable between wild-type and OX40-deficient mice when inoculated with OVA or Heligmosomoides polygyrus (47). In our present study, OX40 expression was not found on splenic CXCR5+CD4+ T cells from eight mouse strains when i.p. inoculated with SRBC (Figs. 6 and 10D). Consistently, the development of CXCR5+CD4+ T cells and GC B cells in the spleen was not significantly affected in anti-OX40L mAb-treated or OX40L-deficient BALB/c and C57BL/6 mice (Figs. 1 and 4). In contrast, CXCR5+CD4+ T cells in the LN of C57BL/6 and C3H/He mice uniquely expressed OX40, while those from the other strains including

((primary follicle), but those B cells that have bound Ag are trapped. Thus, at the border between the T cell zone and the B cell zone, Ag-specific T cells and B cells interact to initiate the GC response (45). It is most likely that the ICOS/B7RP-1 interaction is involved in this process. In the present study, we have dissected the role of ICOS/B7RP-1 interaction in the GC response, especially focusing on the development of CXCR5+ TFH cells, which migrate to the B cell zone where they provide cognate help to B cells (28). Our adoptive transfer experiments showed that the development of CXCR5+CD4+ T cells was enhanced by B cells in an ICOS/B7RP-1-dependent manner (Fig. 8). Therefore, the defect in GC formation and Ab production in ICOS- or B7RP-1-deficient mice might be primarily due to the impaired development of TFH cells. However, it should be noted that it is not yet clear whether ICOS signaling directly induces the CXCR5 expression on CD4+ T cells. So far we tested that an apparent induction of CXCR5+CD4+ T cells was not observed when naive CD4+ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of B7RP-1-transfected P815 cells in vitro (data not shown). We also could not find the CXCR5-expressing CD4+ T cells even when whole spleen cells were stimulated with anti-CD3 mAb or Con A in vitro (data not shown). Therefore, some other signals provided by the splenic microenvironment might be required for the expression of CXCR5 on CD4+ T cells in addition to the ICOS signal. Further studies are needed to address this possibility.

Although recent studies have shown the impaired GC formation and Ab production in ICOS- or B7RP-1-deficient mice in response to T-dependent Ags (14–19), it remained unclear whether the ICOS/B7RP-1 interaction was involved in the secondary GC response mediated by memory T cells and memory B cells. In the present study, we demonstrated that the blockade of ICOS/B7RP-1 interaction with anti-B7RP-1 mAb at the secondary immunization also abrogated the development of both TFH cells and GC B cells (Fig. 5). This clearly indicates that the ICOS/B7RP-1 interaction also plays a critical role in the secondary Ab response mediated memory T cells and memory B cells.

It was notable that the anti-B7RP-1 mAb treatment abrogated the development of TFH cells while sparing the development of GC B cells in the LN of BALB/c mice (Fig. 9, A and B). This suggests that CXCR5+ TFH cells are not always needed for the development of PNA+ GC B cells in LN. Consistent with this notion, an impaired development of GC in the spleen, but not LN, was observed in CXCR5-deficient mice (22). The mechanisms for the B7RP-1- and TFH cell-independent GC formation in the LN of BALB/c mice remain to be determined.

Previous studies have suggested that OX40 plays a critical role in the regulation of T cell migration into B cell follicles. In particular, OX40 signaling up-regulated CXCR5 mRNA in CD4+ T cells (30). It has been suggested that the impaired GC formation in CD28-deficient mice may be due to compromised OX40 expression on CD4+ T cells (31). Moreover, OX40L-transgenic mice demonstrated an accumulation of OX40+CD4+ T cells in the B cell follicles of secondary lymphoid organs (32). Furthermore, blockade of OX40/OX40L interaction by OX40-Ig in chronic intestinal inflammation has shown a marked reduction of CXCR5+CD4+ T cells in the lamina propria (46). In contrast, a recent study has indicated that CXCR5 expression on Ag-specific T cells and their migration into the B cell zone were comparable between wild-type and OX40-deficient mice when inoculated with OVA or Heligmosomoides polygyrus (47). In our present study, OX40 expression was not found on splenic CXCR5+CD4+ T cells from eight mouse strains when i.p. inoculated with SRBC (Figs. 6 and 10D). Consistently, the development of CXCR5+CD4+ T cells and GC B cells in the spleen was not significantly affected in anti-OX40L mAb-treated or OX40L-deficient BALB/c and C57BL/6 mice (Figs. 1 and 4). In contrast, CXCR5+CD4+ T cells in the LN of C57BL/6 and C3H/He mice uniquely expressed OX40, while those from the other strains including

**FIGURE 9.** Effect of anti-B7RP-1 and anti-OX40L mAbs on induction of GC B cells and TFH cells in LN. BALB/c (A and B) or C57BL/6 (C and D) mice were immunized with SRBC in the footpads and administrated with anti-B7RP-1 mAb, anti-OX40L mAb, or control rat IgG on days 0, 2, and 4. Popliteal LN cells were collected on day 6. The induction of GC B cells was determined by staining with PNA and anti-B220 mAb, and expressed as the mean percentages ± SD of PNA+ cells within B220+ cells from five mice in each group (A and C). The induction of TFH cells was determined by staining with anti-CXCR5 and anti-CD4 mAbs, and expressed as the mean percentages ± SD of CXCR5+ cells within CD4+ cells from five mice in each group (B and D). Similar results were obtained in three independent experiments. *, p < 0.01; **, p < 0.001.
distinct subsets producing Th1 or Th2 cytokines in vivo. Further studies are now under way to address this possibility.

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Disclosures

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References


