Inducible Costimulatory Molecule-B7-Related Protein 1 Interactions Are Important for the Clonal Expansion and B Cell Helper Functions of Naive, Th1, and Th2 T Cells[1]

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Inducing T cell responses requires at least two distinct signals: 1) TCR engagement of MHC-peptide and 2) binding of CD28 to B7.1/2. However, the recent avalanche of newly described costimulatory molecules may represent additional signals which can modify events after the initial two-signal activation. Inducible costimulatory molecule (ICOS) is a CD28 family member expressed on T cells rapidly following activation that augments both Th1 and Th2 T cell responses and has been implicated in sustaining rather than initiating T cell responses. Although it is known that blockade of ICOS-B7-related protein 1 (B7RP-1) in vivo dramatically reduces germinal center formation and Ab production, the mechanism(s) remains unclear. An optimal T cell-dependent Ab response requires T and B cell activation, expansion, differentiation, survival, and migration, and the ICOS-B7RP-1 interaction could be involved in any or all of these processes. Understanding this will have important implications for targeting ICOS-B7RP-1 therapeutically. We have therefore used a double-adoptive transfer system, in which all of the above events can be analyzed, to assess the role of ICOS-B7RP-1 in T cell help for B cell responses. We have shown that ICOS signaling is involved in the initial clonal expansion of primary and primed Th1 and Th2 cells in response to immunization. Furthermore, while ICOS-B7RP-1 interactions have no effect on the migration of T cells into B cell follicles, it is essential for their ability to support B cell responses. *The Journal of Immunology,* **2003, 170: 2310–2315.**

It is well accepted that activation of naive T cells requires two distinct signals. Signal one is derived from recognition of MHC-peptide complexes by the TCR and signal two is provided by the binding of the T cell-expressed costimulatory molecule CD28 to its ligands B7.1/2 on APC (1). However, the recent identification of newly defined costimulatory molecules has suggested that multiple interactions may be required to elicit optimal costimulation of T cells (2). Inducible costimulatory molecule (ICOS)[3] is a recently described member of the CD28 family of costimulatory molecules (3, 4) that is expressed on T cells in the spleen, lymph nodes, and Peyer's patch (4–7). Since ICOS is not expressed on resting T cells but is induced on all activated T cells within 24–48 h (4), ICOS signaling has been proposed to be involved in sustaining primary and/or memory T cell responses rather than initiating primary responses. Furthermore, ICOS has been shown to augment both Th1 and Th2 responses (3, 8–12). Importantly, the ICOS ligand, B7-related protein 1 (B7RP-1) (6, 7, 13–16), is expressed constitutively on resting B cells and dendritic cells and is up-regulated on monocytes, keratinocytes, and a population of $CD3^+$ cells after activation (3). Furthermore, ICOS-B7RP-1 interactions are thought to be important in B-T cell cooperation. This hypothesis is supported by the observation that ICOS-deficient mice do not generate germinal centers or Abs after immunization with soluble Ag (8, 17, 18). Although it is known that blockade of ICOS-B7RP-1 interactions in vivo dramatically reduces germinal center formation and Ab production, the mechanism(s) underlying this remains unclear. An optimal T cell-dependent Ab response requires T and B cell activation, expansion, differentiation, survival, and migration and the ICOS-B7RP-1 interaction may be involved in any or all of these processes. Understanding these pathways may have important implications for targeting ICOS-B7RP-1 therapeutically. We have therefore used a B and T cell double-adoptive transfer system, in which all of the events listed above can be analyzed, to assess the role of ICOS-B7RP-1 interactions in T cell help for B cells. Using this system, we have previously shown that naive unpolarized (primary) Th1 and Th2 T cells are capable of producing help for B cell clonal expansion and Ab production in vivo (19). By modifying this system, we have been able to assess the requirement for ICOS signaling in B cell responses supported by primary, Th1, or Th2 T cells. In this study, we have dissected the role of ICOS in T cell clonal expansion and in T cell help for B cells in vivo. We have shown that ICOS is important for the initial accumulation of primary T cells and primed Th1 and Th2 T cells in response to immunization. Furthermore, although it has no effect on the migration of these cells into B cell follicles, it is essential for their ability to support maximal B cell clonal expansion and Ab production.

Materials and Methods

Animals

BALB/c ($H-2^{d/d}$, IgM^a) mice were purchased from Harlan-Olac (Oxon, U.K.). IgH^b BALB/c ($H-2^{d/d}$, IgM^b) mice were bred in-house (Central Research Facilities, University of Glasgow, Glasgow, U.K.). Mice homozygous for the

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[3] Abbreviations used in this paper: ICOS, inducible costimulatory molecule; B7RP-1, B7-related protein 1; HEL, hen egg lysozyme; PLN, peripheral lymph node; tg, transgenic; CD40L, CD40 ligand.

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cOVA peptide$_{323-339}$/I-A^d-specific DO11.10 TCR transgenes (detected using the clonotypic mAb KJ1.26) on the BALB/c background (20) were used as donors. Similarly, mice heterozygous for the anti-hen egg lysozyme (HEL) *IgM^a* and *IgD^a* transgenes on the BALB/c background (MD4) (21) were screened for the expression of the MD4 transgenes by flow cytometry; positive animals were used as donors. Six-week-old male IgH^b mice were used as recipients. All animals were specified pathogen free and were maintained under standard animal house conditions with free access to both water and standard rodent pellets at the University of Glasgow Central Research Facilities in accordance with local and home office regulations.

Preparation of cell suspensions for adoptive transfer

Peripheral lymph nodes (axillary, brachial, inguinal, cervical; PLN), mesenteric lymph nodes, and spleens from MD4 BALB/c and DO11.10 BALB/c mice were pooled and forced through Nitex (Cadisch Precision Meshes, London, U.K.) using a syringe plunger. The suspensions were washed in sterile RPMI 1640 (Life Technologies, Paisley, U.K.). Cells were washed by adding 1 ml of washing agent before the suspensions were centrifuged at 450 × *g* for 5 min and the supernatant was discarded. Th1 and Th2 cells were prepared as described below. The percentage of IgM^{a+}B220$^+$ MD4 B cells or KJ1.26$^+$CD4$^+$ DO11.10 T cells in these preparations was determined by flow cytometric analysis as described below. Cell suspensions containing 1–6 × 10^6 transgenic (tg) T and 1–6 × 10^6 tg B cells in 100 μl were mixed and 200 μl was injected i.v. into unirradiated, age- and sex- matched IgH^b BALB/c recipients as described previously (19, 22, 23).

T cell purification and T cell cultures

T cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml Fungizone (all from Life Technologies), and 0.05 μM 2-ME (complete medium; Sigma-Aldrich, Dorset, U.K.). CD4$^+$ T cells were purified from pooled lymph node and spleen cells from DO11.10 BALB/c or DO11.10 (BALB/c × C57BL/6)F$_1$ mice by negative selection as described previously (19). Briefly, B cells, monocytes, and CD8$^+$ T cells were removed using anti-CD19, anti-CD11c, and anti-CD8 mAbs (all from Serotec, Oxford, U.K.) respectively, then anti-IgG MACS beads (Miltenyi Biotec, Auburn, CA). APC were obtained by irradiating BALB/c spleen cells (2000 rad for 10–20 min). T cell differentiation was induced by culturing 2 × 10^5/ml CD4$^+$ T cells with 2 × 10^6/ml APC and 0.3 μM OVA$_{323-339}$ (Genosys, Cambridgeshire, U.K.). In addition, Th1 cell cultures included 5 ng/ml IL-12 and 10 μg/ml anti-IL-4 mAb (11B11), whereas Th2-polarizing cultures incorporated 5 ng/ml IL-4, 1/500 anti-IL-12, and 1/500 anti-IFN-γ serum. After 3 days of culture, the cells were washed and harvested for adoptive transfer.

Ags and Ag administration

Chicken OVA (fraction V) was obtained from Sigma-Aldrich and HEL was obtained from Biozyme (Gwent, U.K.). Conjugated OVA-HEL (0.5 mM) was prepared as described previously (19). Animals were injected s.c. with 130 μg of cOVA-HEL in 100 μl of saline/50% IFA (Sigma-Aldrich).

Ab treatments

Mice were injected with 100 μg of mAb 12A8, control Ab (matched isotype of irrelevant specificity), or PBS i.p. on the day of immunization and 2 days later. mAb 12A8 is a nondepleting rat anti-mouse ICOS that blocks the binding of ICOS ligand (9, 12, 24).

Flow cytometry

PLN were harvested between days 1 and 10 after Ag exposure. Cell suspensions were prepared as described above. Aliquots of cells were incubated with FcR blocking buffer (anti-CD16/32 hybridoma supernatant, 10% mouse serum; Diagnostic Scotland, Edinburgh, U.K.) and 0.1% azide (Sigma-Aldrich) for 5 min at 4°C to prevent binding of Ab to cells via Fc regions. For detection of CD4$^+$ DO11.10 tg T cells, the cell suspensions were incubated with PE-conjugated anti-CD4 (BD PharMingen, Oxford, U.K.) and biotinylated clonotypic anti-TCR Ab, KJ1.26 (produced from the original hybridoma (25)) for 40 min at 4°C. The cells were washed in FACS buffer (PBS, 2% FCS, and 0.1% azide) and then incubated with FITC-conjugated streptavidin (Vector Laboratories, Peterborough, U.K.) for 40 min at 4°C. For detection of B220$^+$ MD4 B cells, cell suspensions were stained as above substituting PE-conjugated anti-B220 (BD PharMingen) for PE-conjugated anti-CD4 and biotinylated HEL or anti-IgM^a (BD PharMingen) for biotinylated KJ1.26. Two-color analysis was performed on 20,000 events.

Ab ELISAs

To detect tg B cell-derived anti-HEL IgM^a in serum, Immulon 2 plates (Costar; Corning Glass, Corning, NY) were coated with HEL (20 μg/ml) in PBS at 4°C overnight. Plates were then washed at least three times with PBS-Tween 20 (0.05%; Sigma-Aldrich) before being blocked with PBS-FCS (10% v/w) for 1 h at 37°C. Plates were washed and incubated with serially diluted serum samples for 3 h at 37°C before further washing. IgM^a levels in serum were determined by incubation with biotinylated anti-IgM^a (2 μg/ml; BD PharMingen) for 1 h at 37°C. Plates were then washed and incubated with Extravidin (1/1000; Sigma-Aldrich) for 1 h at 37°C. Plates were washed again and tetramethylbenzidine Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. All ELISAs were read on a plate reader at 630 nm.

Immunohistochemistry

PLN were frozen in liquid nitrogen in OCT-embedding medium (Miles Diagnostic Division, Elkart, IN) in cryomoulds (Miles Diagnostic Division) and stored at −70°C. Tissue sections (6–10 μm) were cut on a cryostat (ThermoShandon, Cheshire, U.K.) and stored at −20°C. Sections were brought to room temperature in acetone for 10 min, air dried, and rehydrated with PBS before being incubated in 0.1% azide/3% H_2O_2 for 45 min, changing the solution three times, to block endogenous peroxidase. Avidin solution (Vector Laboratories) was added for 15 min to block unmasked endogenous biotin, then biotin solution (Vector Laboratories) was added to block excess avidin. Finally, tissues were incubated with Fc blocking buffer for 30 min. Sections were washed in PBS after each treatment. The tg T or B cells were detected on sections by incubation with biotinylated KJ1.26 (1/500 in PBS/2% goat serum; Vector Laboratories) or biotinylated anti-IgM^a (1/500 in PBS/2% goat serum), respectively, for 30 min. Sections were then washed three times in PBS before being incubated with an avidin-biotin complex-alkaline phosphatase for 30 min. Again PBS was used to wash the sections three times before incubation in the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium/Tris-HCl (pH 9.5) for 45 min in the dark. B and T cell areas were detected on KJ1.26$^+$ or IgM^{a+} stained sections by incubation with biotinylated B220 or Thy1.2 (1/500 in PBS/2% goat serum; BD PharMingen) for 30 min. Sections were then washed three times in PBS before being incubated with avidin-biotin complex-HRP (Vector Laboratories) for 30 min. After an additional three washes in PBS, the HRP substrate 3,3′-diaminobenzidine (Vector Laboratories) was added for 10 min before washing in H_2O, incubating with 3,3′-diaminobenzidine-enhancing solution, and a final wash in H_2O. Subsequent exposure to acetone/2% acetic acid, 70% ethanol, 95% ethanol, 95% ethanol, and then 100% ethanol dehydrated the tissues, before clearing in Histoclear (Bs & S, Edinburgh, U.K.) and immediate mounting in Immunomount (Vector Laboratories).

Statistics

Results are expressed as mean ± SEM or mean + range. To test significance, Student's unpaired *t* tests were performed. A value of $p \leq 0.05$ was regarded as significant.

Results

ICOS-B7RP-1 interactions are required for fulminant T and B cell responses in vivo

Primary OVA-specific TCR tg T cells and HEL-specific tg B cells were transferred into naive syngeneic IgH^b BALB/c mice. Recipients were subsequently immunized with cOVA-HEL/IFA s.c. on day 0 in the presence or absence of anti-ICOS treatment and control mice received no Ag preparation (Fig. 1*A*). In accordance with previous studies (19, 22, 23, 26), accumulation of tg T cells peaked 3 days after immunization (data not shown). Treatment with anti-ICOS mAb resulted in decreased accumulation of tg T cells (Fig. 1*C*). Fig. 1*B* shows FACS plots from one animal from each experimental group; plots are representative of at least three animals per group. When analyzed as a group, the anti-ICOS-treated group showed significantly reduced T cell clonal expansion ($p \leq 0.05$), expanding to only 48% of the untreated group 3 days after immunization (3.46 ± 0.08% KJ1.26$^+$ T cells vs 1.55 ± 0.56% in the anti-ICOS-treated group). This decrease in tg T cell numbers may be the result of decreased migration of tg T cells to lymph nodes, increased death of tg T cells, or decreased division of tg T cells

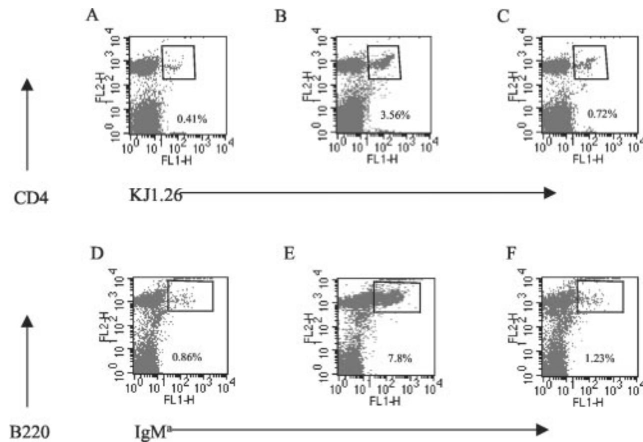

FIGURE 1. Effect of anti-ICOS mAb treatment on CD4$^+$KJ1.26$^+$ T cell and B220$^+$IgM^{a+} B cell expansion in vivo. Nontransgenic IgH^b BALB/c mice were coinjected with CD4$^+$KJ1.26$^+$ T cells and B220$^+$IgM^{a+} B cells. Recipients were then immunized (*B*, *C*, *E*, and *F*) with 130 μg of cOVA-HEL in IFA and i.p. anti-ICOS injections (*C* and *F*) were performed on the day of immunization and 2 days later. CD4$^+$KJ1.26$^+$ T cell expansion was assessed by flow cytometry on day 3 (*A–C*), and B220$^+$IgM^{a+} B cell expansion was assessed on day 5 (*D–F*). Plots are representative of groups that contained at least three animals. Similar results were obtained in an additional three experiments.

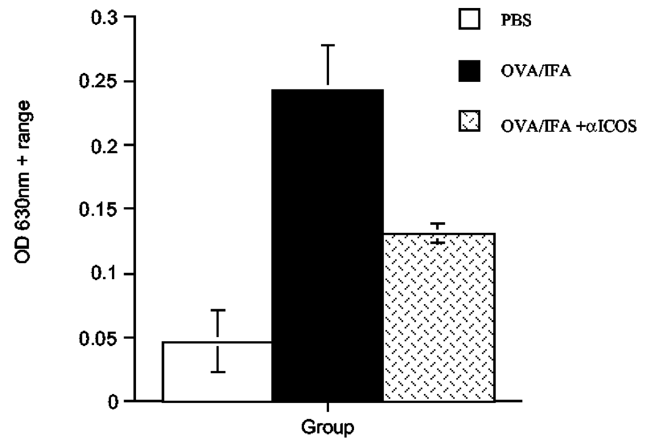

FIGURE 2. Effect of anti-ICOS mAb treatment on Ag-specific B cell Ab production in vivo. Serum samples were collected 10 days after immunization from animals described in Fig. 1 and analyzed for anti-HEL-specific IgM^a by ELISA. Data represent the mean OD + range for two mice per group.

following anti-ICOS treatment. To assess this, we have stained tg T cells with CFSE before transfer and examined the effect of treatment with anti-ICOS on cell division. Treatment with anti-ICOS did not prevent tg T cells from dividing (data not shown).

We next assessed the requirement of ICOS-B7RP-1 interactions between Ag-specific T and B cells in vivo. Fig. 1, *D–F*, shows Ag-specific B cell clonal expansion, which peaked on day 5 (data not shown) as previously reported (19, 22, 23, 26). Earlier studies have shown that this expansion is dependent on help provided by tg T cells (19, 22). As can be seen in Fig. 1*F*, recipients treated with anti-ICOS mAb also showed less Ag-specific B cell clonal expansion compared with untreated mice. Data from experimental groups showed that B cells from anti-ICOS mAb-treated animals expanded significantly less than untreated groups, reaching only 29% of untreated levels 5 days after immunization ($p \leq 0.05$; 5.46 $\pm$ 1.29% IgM^{a+} B cells vs 1.59 $\pm$ 0.25% IgM^{a+} B cells in the anti-ICOS-treated group). In addition, analysis of HEL-specific serum IgM^a revealed that anti-ICOS mAb treatment resulted in decreased Ab production by tg B cells 5 days after immunization (Fig. 2).

Th1 and Th2 cells require ICOS signaling for maximal clonal expansion in vivo

Because the above results indicated an unexpected role for ICOS in the clonal expansion of primary T cells and their ability to help B cells, we next investigated its role in the expansion of Th1 and Th2 cells. We hypothesized that ICOS might be particularly important in the ability of primed Th2 cells to help B cells. We used the adoptive transfer system to track Ag-specific Th1 and Th2 T cell responses in the presence or absence of anti-ICOS mAb. Representative FACS plots in Fig. 3, *A–H*, show that both Th1 and Th2 cells require ICOS signaling to achieve maximal clonal expansion in response to immunization in vivo. When data from all animals in each experimental group were collated, treatment with anti-ICOS mAb resulted in Th1 and Th2 cells expanding to only 56 and 52%, respectively, relative to control IgG-treated mice (1.3 $\pm$ 0.24% KJ1.26$^+$ Th1 T cells vs 0.73 $\pm$ 0.11% in the anti-ICOS-treated group and 2.07 $\pm$ 0.21% KJ1.26$^+$ Th2 T cells vs 1.07 $\pm$ 0.31% in the anti-ICOS-treated group). In contrast to previous studies (19), Th1 and Th2 cell accumulation peaked on day 5 (data not shown) as opposed to day 3; this may reflect the use of a different adjuvant (IFA vs CFA) in the two studies.

ICOS-B7RP-1 interactions are required for Th1 and Th2 cells to support fulminant B cell responses in vivo

We have previously shown that Th1 and Th2 cells are able to help B cells in a similar manner in vivo (19). We therefore wished to determine whether ICOS was involved in both of these scenarios. To further investigate the role of ICOS signaling in Th1 and Th2 responses, we used the double-adoptive transfer system to determine whether treatment with anti-ICOS mAb altered the ability of Th1 and Th2 cells to support Ag-specific B cell responses in vivo. In previous studies, we have shown that both Th1 and Th2 cells support B cell clonal expansion and Ab production and that this was dependent on CD40-CD154 interactions (19). In this study, we demonstrate that Th1 and Th2 cells also depend on ICOS-B7RP-1 interactions to support B cell clonal expansion because treatment with anti-ICOS mAb (Fig. 3, *K*, *L*, *O*, and *P*) resulted in reduced B cell clonal expansion. In the anti-ICOS mAb group, B cells expanded significantly less ($p \leq 0.05$), 14 and 11%, compared with the control IgG-treated Th1 cell- and Th2 cell-transferred groups respectively (3.39 $\pm$ 0.66% KJ1.26$^+$ Th1 T cells vs 0.46 $\pm$ 0.08% in the anti-ICOS-treated group and 8.29 $\pm$ 0.31% KJ1.26$^+$ Th2 T cells vs 0.92 $\pm$ 0.38% in the anti-ICOS-treated group). Fig. 4 shows that treatment with anti-ICOS mAb also significantly reduced B cell Ab production by both Th1 and Th2 T cell-transferred groups, further confirming that ICOS signaling is involved in Th1 and Th2 T cell support of B cell responses.

Localization of Ag-specific T and B cells following treatment with anti-ICOS mAb

It is widely accepted that T cells migrate into B cell follicles to provide help for B cells and we have previously shown that tg Ag-specific primary Th1 or Th2 T cells migrate to B cell follicles following immunization (19). This migration appears to be an essential component of T cell help for B cells and our studies above demonstrated a blockade of T cell responses by anti-ICOS. We therefore investigated whether anti-ICOS mAb blocked migration of T cells, i.e., was the migration of these cells dependent on

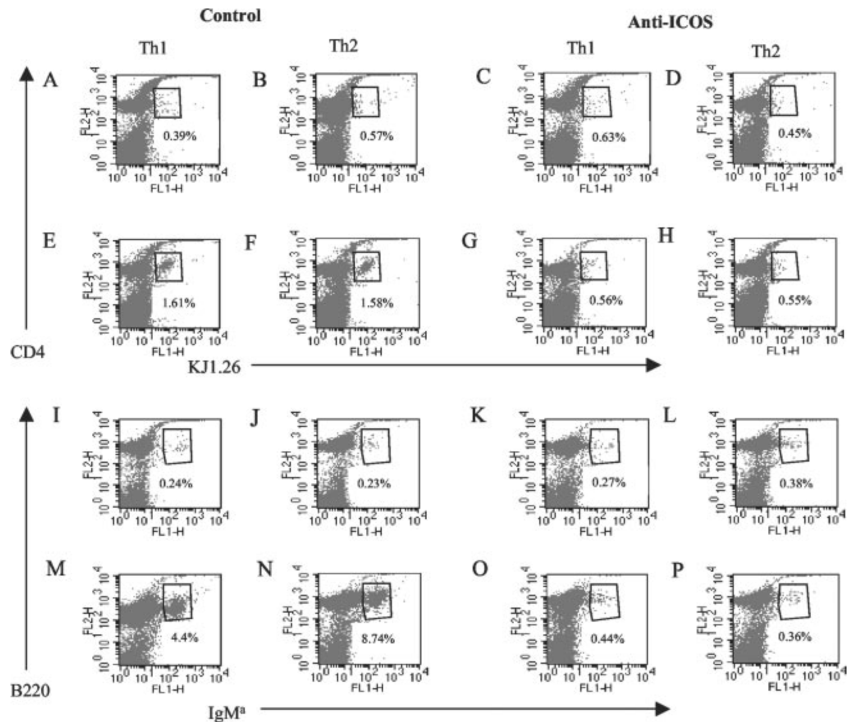

FIGURE 3. Th1 and Th2 T cells require ICOS-ICOS ligand interactions to fully expand and support B cell responses in vivo. Nontransgenic IgH^b BALB/c mice were injected with $CD4^+KJ1.26^+$ Th1 (*A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O*) or Th2 (*B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P*) T cells along with $B220^+IgM^{a+}$ B cells. Recipients were then immunized (*E–H* and *M–P*) with 130 μg of cOVA-HEL in IFA. Intraperitoneal anti-ICOS injections (*C*, *D*, *G*, *H*, *K*, *L*, *O*, and *P*) were performed on the day of immunization and 2 days later. $CD4^+KJ1.26^+$ T cell expansion and $B220^+IgM^{a+}$ B cell expansion was assessed by flow cytometry on day 5. Plots are representative of groups that contained at least three animals. Similar results were obtained in another two experiments.

ICOS-B7RP-1 interactions? We took lymph nodes from mice transferred with primary Th1 or Th2 cells and immunized in the presence or absence of anti-ICOS and determined the anatomical location of Ag-specific T cells. In confirmation of previous studies, tg T cells did not move into B cell follicles in unimmunized animals that received primary (data not shown), Th1 (Fig. 5*A*), or Th2 (data not shown) T cells (19, 22, 23, 26). Furthermore, following immunization, Ag-specific T cells in all of these groups migrated into follicles. However, Fig. 5 demonstrates that treatment with anti-ICOS mAb does not prevent primary (Fig. 5*C*), Th1 (Fig. 5*E*), and Th2 (Fig. 5*G*) T cells from migrating into B cell follicles in response to immunization.

Since there was no defect in T cell migration, we next determined any effects of ICOS blockade on B cell clonal expansion and germinal center development. To observe the tg B cell response in situ, the lymph node sections described above were stained with an Ab against the tg B cell marker IgM^a. As with previous studies (19, 22, 23, 26), tg B cells did not clonally expand in unimmunized animals (Fig. 6*E*). However, exposure to Ag in adjuvant resulted in tg B cell clonal expansion in animals that were transferred with primary (Fig. 6*A*), Th1 (Fig. 6*C*), and Th2 (data not shown) T cells. Treatment with anti-ICOS mAb resulted in diminished clonal expansion in all groups (Fig. 6, *B*, *D*, and *F*), confirming the FACS results and showing that ICOS-B7RP-1 interactions are required for primary, Th1, and Th2 T cells to provide B cell help in vivo.

Discussion

In this study, we have dissected the role of ICOS in T cell clonal expansion and T cell help for B cells in vivo. We have shown that ICOS is important for the initial clonal expansion of primary and primed Th1 and Th2 T cells in response to immunization. Furthermore, although it has no effect on the migration of these cells into B cell follicles, it is essential for their ability to support B cell responses.

It has previously been suggested that ICOS interactions are not involved in the initial response of naive T cells to Ag (as ICOS is not expressed on resting T cells (4)). This conclusion is supported by in vitro studies where naive tg T cell responses to peptide remained intact despite ICOS-B7RP-1 interactions being blocked by an ICOS-Ig fusion protein (27). To investigate the role of ICOS signaling in Ag-specific naive immune responses directly in vivo, we transferred primary $CD4^+KJ1.26^+$ DO11.10 OVA-specific T cells into naive mice which then received anti-ICOS mAb before and after immunization. Our results show that treatment with anti-ICOS mAb resulted in a significantly reduced primary T cell response. Therefore, although ICOS may not be involved in the initial interactions between T cell and APC, we suggest that it is important in the generation of primary T cell responses. We therefore envisage a scenario where the initial two signal interactions (where signal one is MHC II/peptide binding the TCR, and signal two is provided by CD28 binding B7.1/2) are of sufficient duration to allow modification/amplification by a third signal such as ICOS. Indeed, evidence for the longevity of APC-T cell interactions in vivo has been recently provided (28). Since the DO11.10 cells

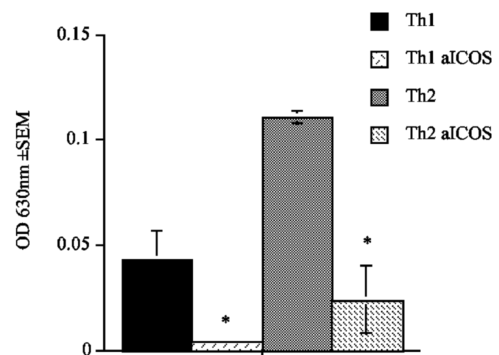

FIGURE 4. Effect of anti-ICOS mAb treatment on Ag-specific B cell Ab production in vivo. Serum samples were collected 5 days after immunization from animals described in Fig. 3 and analyzed for anti-HEL-specific IgM^a by ELISA. Data represent the mean OD ± SEM for three mice per group. *, $p \leq 0.05$.

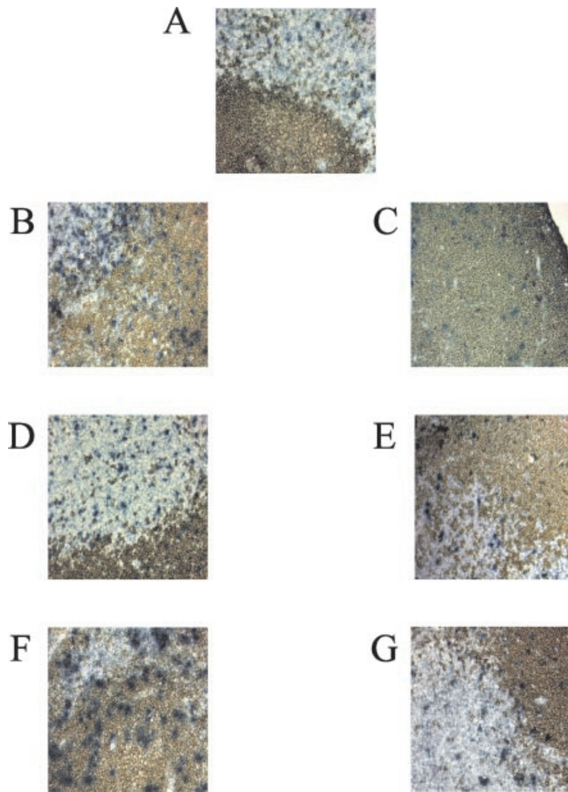

FIGURE 5. Visualization of Ag-specific T cell in vivo responses after treatment with anti-ICOS mAb. Lymph nodes taken from mice described in Figs. 1 and 3 were removed 7 days after immunization and stained with KJ1.26 in blue and the B cell marker B220 in brown. Unimmunized mice that received unpolarized (data not shown), Th1 (*A*), or Th2 cells (data not shown) showed paracortical localization of KJ1.26$^+$ T cells that did not migrate into B cell follicles. Seven days after immunization, KJ1.26$^+$ T cells could be observed in the B cell follicles of unpolarized (*B*), Th1 (*D*), and Th2 (*F*) recipient mice. Treatment with anti-ICOS mAb at days 0 and 2 did not prevent this migration of unpolarized (*C*), Th1 (*E*), or Th2 (*G*) cells. Where data are not shown, the pattern of migration is the same as that illustrated by the representative micrograph shown.

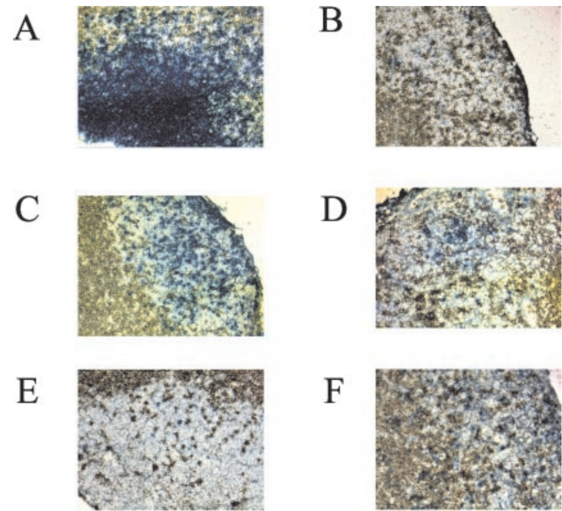

FIGURE 6. Visualization of Ag-specific B cell in vivo responses after treatment with anti-ICOS mAb. Lymph nodes taken from mice described in Figs. 1 and 3 were removed 7 days after immunization and stained for the tg B cell marker IgM^a in blue and the T cell marker CD4 in brown. Unimmunized mice that received unpolarized (data not shown), Th1 (*E*), and Th2 cells (data not shown) had few tg IgM^{a+} B cells resident in the B cell follicles. Following immunization, IgM^{a+} B cell clonal expansion was observed in the unpolarized (*A*), Th1 (*C*)-, and Th2 (data not shown)-transferred groups. Treatment with anti-ICOS mAb resulted in decreased clonal expansion in animals transferred with unpolarized (*B*), Th1 (*D*), and Th2 (*F*) tg T cells.

were unaffected by ICOS blockade in vitro, our results highlight the importance of studying responses in the more complex environment of in vivo models. Our results are supported by experiments with ICOS$^{-/-}$ T cells which exhibit reduced proliferation in response to anti-CD3 in vitro (17, 18).

We also assessed whether ICOS interactions were required for the generation of a primary B cell response by adoptively transferring OVA-specific T cells and HEL-specific B cells into naive recipients before immunizing with chemically coupled OVA-HEL/IFA (as described previously in Refs. 19, 22, and 23). Treatment with anti-ICOS mAb resulted in reduced B cell clonal expansion and Ab production, indicating that ICOS-B7RP-1 interactions are important in T cell help for B cells. ICOS has previously been reported to be involved in B-T cell cooperation as ICOS is expressed by activated T cells which have migrated into germinal centers and B7RP-1 is expressed by resting B cells (7). Furthermore, ICOS-deficient mice have impaired humoral immunity and isotype switching (8, 17, 18). This defect appears to result from a lack of T cell help as isotype switching in response to T cell-independent Ags remains intact (18). Our data support a role for ICOS in B-T cell cooperation because it appears that ICOS blockade results in T cells being rendered unable to help B cells. However, it is possible that the diminished B cell response observed results from the decreased number of T cells or a change in their differentiation. Additional experiments using ICOS or B7RP-1 KOs localized on T cells or APC are being designed to test this hypothesis.

ICOS has mainly been regarded as a molecule involved in costimulation of previously activated T cells because it is not expressed on resting T cells but is rapidly up-regulated on activated T cells (3–7, 27). Furthermore, since ICOS is expressed on Th1 and Th2 cells but expression is only sustained on Th2 cells after chronic stimulation (3), it has been suggested that ICOS signaling may be involved in the induction of both Th1 and Th2 responses but is of heightened importance in Th2 responses (3, 9, 29). However, more recent evidence supports a role for ICOS in both Th1 and Th2 responses (11, 12, 24). To investigate the role of ICOS signaling in the activation of primed Th1 and Th2 cells, we adoptively transferred OVA-specific in vitro-primed T cells into naive recipients. These experiments are especially informative because the Th1 and Th2 cells have the same specificity, whereas others have extrapolated between different studies using different Ags and adjuvants (11, 30). Recipients were also transferred with HEL-specific B cells before treatment with anti-ICOS mAb and immunization with OVA-HEL/IFA. These experiments demonstrate that ICOS signaling is important in the clonal expansion of previously primed T cells and that blocking ICOS signaling prevents Th1 and Th2 cells from providing help for B cell clonal expansion and Ab production. We suggest that the reduced B cell response is due to a block in B-T cell interactions. In support of this, ICOS-B7RP-1 interactions can enhance up-regulation of CD154 (CD40 ligand (CD40L)) in T cells, a molecule known to be involved in T-B cell cooperation (7, 19). However, it remains formally possible that the diminished T cell level is responsible. Again, work is underway in our laboratory to discriminate between these two possibilities.

Previous studies have shown that reduced B cell responses may result from effects on T cell migration (23, 31). Therefore, to assess whether the reduced B cell response after anti-ICOS treatment

was due to a defect in T cell migration, we removed lymph nodes from recipients and examined the location of Ag-specific T cells. We have been able to show that after transfer of all types (primary, Th1, and Th2), Ag-specific T cells were observed in B cell follicles. Thus, the reduced B cell responses observed after anti-ICOS mAb treatment were not the result of a defect in T cell migration. ICOS-B7RP-1 interactions can enhance up-regulation of other factors involved in B cell help, such as CD154 (CD40L), in T cells (7). Indeed, this may be the role that ICOS interactions play in the induction of humoral immunity. However, we have previously shown that migration of T cells into B cell follicles is also not CD154 dependent (19), suggesting that after ICOS blockade, the T cell reaches the follicle in a CD154-independent migration but remains unable to mediate B cell help due to a failure to up-regulate CD154. In this respect, the role of ICOS is distinct from CD28 because mice rendered deficient in CD28 signaling by over-expression of CTLA-4 Ig fail to generate germinal centers due to a failure of T cells to up-regulate CXCR-5 and migrate into B cell follicles (32).

In this study, we present evidence that ICOS-B7RP-1 interactions are involved in the response of primary T cells as well as Th1 and Th2 T cells to Ag. Furthermore, these interactions are critical for primary, Th1, and Th2 T cells to provide help to Ag-specific B cells following immunization. The defect in B cell clonal expansion and Ab production observed after anti-ICOS mAb treatment does not result from a failure of Ag-specific T cells to migrate to B cell follicles since primary, Th1, and Th2 tg T cells were observed in B cell follicles after exposure to Ag in adjuvant. The defect in T cell help is not clear but may be a result of tg T cells failing to up-regulate other costimulatory molecules, such as CD154 (CD40L), involved in T-B cell interactions.

References

1. Lafferty, K. J., and J. Woolnough. 1977. The origin and mechanism of the allograft reaction. *Immunol. Rev. 35:231.*
2. Coyle, A. J., and J. C. Gutierrez-Ramos. 2001. The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. *Nat. Immunol. 2:203.*
3. Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Gonzalo, et al. 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity 13:95.*
4. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature 397:263.*
5. Beier, K. C., A. Hutloff, A. M. Dittrich, C. Heuck, A. Rauch, K. Buchner, B. Ludewig, H. D. Ochs, H. W. Mages, and R. A. Kroczek. 2000. Induction, binding specificity and function of human ICOS. *Eur. J. Immunol. 30:3707.*
6. Mages, H. W., A. Hutloff, C. Heuck, K. Buchner, H. Himmelbauer, F. Oliveri, and R. A. Kroczek. 2000. Molecular cloning and characterization of murine ICOS and identification of B7h as ICOS ligand. *Eur. J. Immunol. 30:1040.*
7. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, et al. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature 402:827.*
8. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature 409:102.*
9. Gonzalo, J. A., J. Tian, T. Delaney, J. Corcoran, J. B. Rottman, J. Lora, A. Al-garawi, R. Kroczek, J. C. Gutierrez-Ramos, and A. J. Coyle. 2001. ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses. *Nat. Immunol. 2:597.*
10. Riley, J. L., P. J. Blair, J. T. Musser, R. Abe, K. Tezuka, T. Tsuji, and C. H. June. 2001. ICOS costimulation requires IL-2 and can be prevented by CTLA-4 engagement. *J. Immunol. 166:4943.*
11. Kopf, M., A. J. Coyle, N. Schmitz, M. Barner, A. Oxenius, A. Gallimore, J. C. Gutierrez-Ramos, and M. F. Bachmann. 2000. Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection. *J. Exp. Med. 192:53.*
12. Rottman, J. B., T. Smith, J. R. Tonra, K. Ganley, T. Bloom, R. Silva, B. Pierce, J. C. Gutierrez-Ramos, E. Ozkaynak, and A. J. Coyle. 2001. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat. Immunol. 2:605.*
13. Ling, V., P. W. Wu, H. F. Finnerty, K. M. Bean, V. Spaulding, L. A. Fouser, J. P. Leonard, S. E. Hunter, R. Zollner, J. L. Thomas, et al. 2000. Cutting edge: identification of GL50, a novel B7-like protein that functionally binds to ICOS receptor. *J. Immunol. 164:1653.*
14. Wallin, J. J., L. Liang, A. Bakardjiev, and W. C. Sha. 2001. Enhancement of $CD8^+$ T cell responses by ICOS/B7h costimulation. *J. Immunol. 167:132.*
15. Brodie, D., A. V. Collins, A. Iaboni, J. A. Fennelly, L. M. Sparks, X. N. Xu, P. A. van der Merwe, and S. J. Davis. 2000. LICOS, a primordial costimulatory ligand? *Curr. Biol. 10:333.*
16. Swallow, M. M., J. J. Wallin, and W. C. Sha. 1999. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFα. *Immunity 11:423.*
17. Dong, C., A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature 409:97.*
18. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. Chan, G. Duncan, et al. 2001. ICOS is essential for effective T-helper-cell responses. *Nature 409:105.*
19. Smith, K. M., L. Pottage, E. R. Thomas, A. J. Leishman, T. N. Doig, D. Xu, F. Y. Liew, and P. Garside. 2000. Th1 and Th2 $CD4^+$ T cells provide help for B cell clonal expansion and antibody synthesis in a similar manner in vivo. *J. Immunol. 165:3136.*
20. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{low}$ thymocytes in vivo. *Science 250:1720.*
21. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature 334:676.*
22. Garside, P., E. Ingulli, R. R. Merica, J. G. Johnson, R. J. Noelle, and M. K. Jenkins. 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science 281:96.*
23. Smith, K. M., F. McAskill, and P. Garside. 2002. Orally tolerized T cells are only able to enter B cell follicles following challenge with antigen in adjuvant, but they remain unable to provide B cell help. *J. Immunol. 168:4318.*
24. Ozkaynak, E., W. Gao, N. Shemmeri, C. Wang, J. C. Gutierrez-Ramos, J. Amaral, S. Qin, J. B. Rottman, A. J. Coyle, and W. W. Hancock. 2001. Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat. Immunol. 2:591.*
25. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med. 157:1149.*
26. Smith, K. M., J. M. Davidson, and P. Garside. 2002. T-cell activation occurs simultaneously in local and peripheral lymphoid tissue following oral administration of a range of doses of immunogenic or tolerogenic antigen although tolerized T cells display a defect in cell division. *Immunology 106:144.*
27. McAdam, A. J., T. T. Chang, A. E. Lumelsky, E. A. Greenfield, V. A. Boussiotis, J. S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V. K. Kuchroo, et al. 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of $CD4^+$ T cells. *J. Immunol. 165:5035.*
28. Stoll, S., J. Delon, T. M. Brotz, and R. N. Germain. 2002. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science 296:1873.*
29. Tesciuba, A. G., S. Subudhi, R. P. Rother, S. J. Faas, A. M. Frantz, D. Elliot, J. Weinstock, L. A. Matis, J. A. Bluestone, and A. I. Sperling. 2001. Inducible costimulator regulates Th2-mediated inflammation, but not Th2 differentiation, in a model of allergic airway disease. *J. Immunol. 167:1996.*
30. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat. Rev. Immunol. 2:116.*
31. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity 1:327.*
32. Breitfeld, D., L. Ohl, E. Kremmer, J. Ellwart, F. Sallusto, M. Lipp, and R. Forster. 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med. 192:1545.*