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Cutting Edge: Critical Role of Inducible Costimulator in Germinal Center Reactions¹

Chen Dong,^{2,3} Ulla-Angela Temann,³ and Richard A. Flavell⁴

Inducible costimulator (ICOS) is a new member of the CD28/CTLA-4 family that is expressed on activated and germinal center (GC) T cells. Recently, we reported that ICOS-deficient mice exhibited profound defects in T cell activation and effector function. Ab responses in a T-dependent primary reaction and in a murine asthma model were also diminished. In the current study, we investigate the mechanism by which ICOS regulates humoral immunity and examine B cell GC reactions in the absence of ICOS. We found that ICOS^{-/-} mice, when immunized with SRBC, had smaller GCs. Furthermore, IgG1 class switching in the GCs was impaired. Remarkably, GC formation in response to a secondary recall challenge was completely absent in ICOS knockout mice. These data establish a critical role of ICOS in regulation of humoral immunity. *The Journal of Immunology*, 2001, 166: 3659–3662.

Germinal centers (GC)⁵ are critical for T-dependent humoral immune responses. In GC, activated B cells undergo Ag-driven changes in their Ig loci, i.e., Ab class-switching recombination and somatic hypermutation of Ig variable regions, which result in production of high-affinity neutralizing Abs. B cell development into memory or plasma cells may also require GC.

T cells provide critical help for B cell maturation in GC. CD40-CD154 interactions are essential for B cell activation and GC formation (1). Cytokines, particularly IL-4, -5, -6, -10, and -13 Th2 cytokines are also important for B cell proliferation and class switching. IL-4 is critical for GC formation in Peyer's patches (2), but not for secondary GC in lymph nodes (3).

T cell activation and function is mediated by members of the CD28/CTLA-4 costimulator family. CD28 is critical for T cell activation and differentiation and hence also for GC in response to protein Ag (4, 5). ICOS is a novel CD28-related costimulator that is expressed by activated T cells (6, 7). Its ligand, B7H/B7-RP1, was also recently identified as a B7 family member, which is constitutively expressed on B cells and induced in nonlymphoid tissues by TNF- α (7, 8). When analyzing mice deficient for the inducible costimulator (ICOS) gene, we found that ICOS regulates production of Th2 effector cytokines, especially IL-4 and IL-13 (9). IgG1 Ab production in response to a primary protein immunization and IgG1 and IgE production in a recalled reaction were both compromised (9). These data indicate that ICOS is an essential mediator of Th2 function in humoral immune responses. Since ICOS is expressed by human GC T cells and its ligand by B cells (6–8), we investigate in the current study whether it regulates B cell response through a GC-dependent mechanism. We found that ICOS^{-/-} mice were deficient in B cell expansion and IgG1 class switching in primary GC and were unable to form secondary GC. These data indicate ICOS as a critical regulator of humoral immunity.

Materials and Methods

Mice

Generation and maintenance of ICOS-deficient mice has been described previously (9). Eight- to 10-wk-old wild-type and knockout mice were used for the current study.

Immunization

SRBC were purchased from Colorado Serum (Denver, CO) and diluted 10-fold in HBSS medium immediately before use. Wild-type and knockout mice ($n = 2$) were immunized with 0.2 ml of diluted SRBC. Primary GC response in challenged mice was assayed 6 days after immunization. To measure secondary response, 24 days after first immunization, mice were boosted with 0.2 ml of SRBC. Three days later, immunized mice were sacrificed and their spleens were analyzed by immunohistochemical staining.

Immunohistochemical analysis

Spleens from sacrificed mice were embedded in OCT and frozen sections were prepared. B and T cells in unimmunized spleens were identified by a biotinylated B220 (Caltag, South San Francisco, CA) and an anti-Thy1.2 Ab (PharMingen, San Diego, CA) followed by alkaline

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⁵ Abbreviations used in this paper: GC, germinal center; ICOS, inducible costimulator; PNA, peanut agglutinin; AP, alkaline phosphatase; CD40L, CD40 ligand; CR, complement reception.

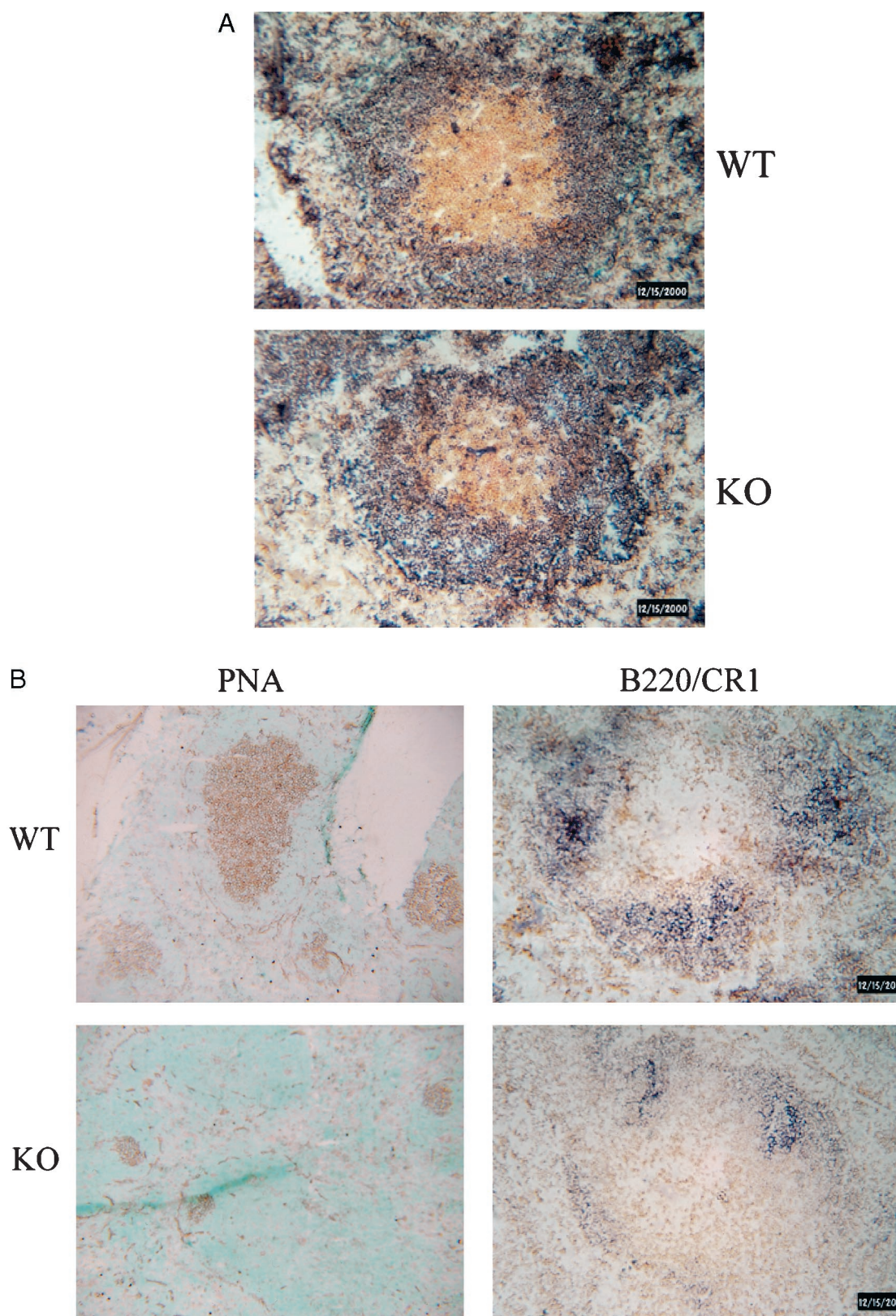


FIGURE 1. Defective primary GC reaction in $ICOS^{-/-}$ mice. **A**, Normal B and T cell localization in spleen of $ICOS^{-/-}$ mice. T and B cells were identified by staining with anti-Thy1.2 (brown) and anti-B220 (purple). **B**, GC in the spleens of SRBC-immunized $ICOS^{+/+}$ and $^{-/-}$ mice were identified by PNA staining or by anti-CR1 (purple) and B220 (brown) Abs. **C**, B cells switched to IgG1 isotype in wild-type (WT, *top*) and knockout (KO, *bottom*) spleens were stained by anti-mouse IgG1 (purple) and B220 Abs (brown). Original magnification: **A** and **B**, $\times 100$; **C**, $\times 200$.

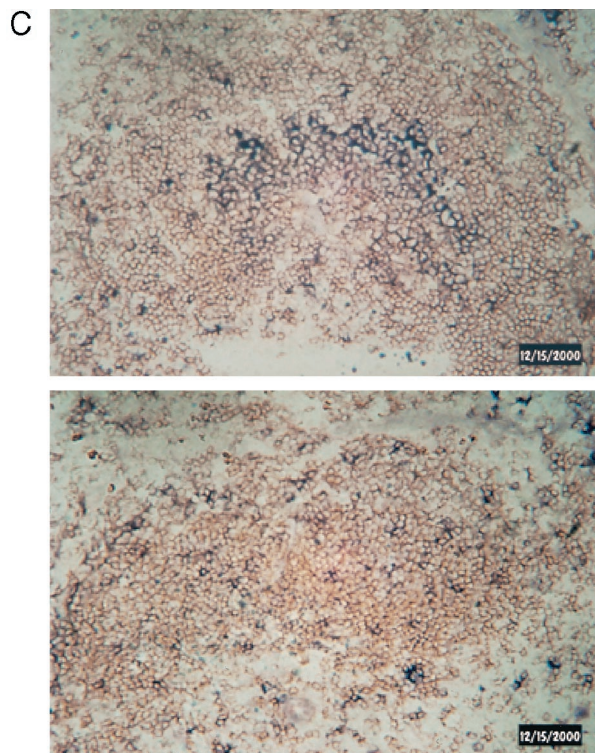


FIGURE 1. Continued.

phosphatase-conjugated streptavidin (Zymed, San Francisco, CA) and HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA). B cells in GC were stained with HRP-conjugated peanut agglutinin (PNA; EY Laboratories, San Mateo, CA) or fluorescein-labeled anti-complement receptor (CR)1 Ab (provided by Mark Shlomchik, Yale University) followed by an anti-fluorescein Ab conjugated with alkaline

phosphatase (AP; Vector Laboratories). IgG1⁺ B cells were identified by an anti-mouse IgG1 Ab from Southern Biotechnology Associates (Birmingham, AL) that is conjugated with AP. Substrates for HRP and AP were purchased from Zymed.

Analysis of CD40 ligand (CD40L) expression by in vitro activated T cells

Lymph node cells from wild-type or knockout mice were treated with 2.5 μ g/ml Con A. Four hours later, expression of ICOS and CD40L by activated CD4 T cells was analyzed by flow cytometry.

Results and Discussion

ICOS^{-/-} mice and mice treated with an ICOS blocker exhibited deficiencies in Ab responses that are mediated by Th2 cytokines (9, 10). Since ICOS is expressed on GC T cells and its ligand is expressed on B cells, we investigated in this study whether ICOS is important for GC reactions. We used SRBC as an Ag to induce strong GC formation in mouse spleen, which can also help avoid the biased T cell responses elicited by different adjuvants.

A previous study showed that the splenic GC reaction peaked at 6 days after SRBC challenge (11). We followed the same protocol and used immunochemical methods to compare GC responses in wild-type and knockout mice. There is no apparent defect in B and T cell localization in the spleens of knockout mice (Fig. 1A) and no significant GC formation could be identified in unimmunized ICOS^{+/+} or ^{-/-} mice by PNA staining (data not shown). Six days after SRBC immunization, a dramatic GC reaction occurred in wild-type spleens with a large cluster of PNA⁺ B follicles (Fig. 1B). In contrast, although knockout spleens contained roughly similar numbers of PNA⁺ clusters, the size of each GC was greatly reduced (Fig. 1B). Staining with anti-CR1, another GC marker confirmed this observation (Fig. 1B). These results indicated that although ICOS is not essential for GC formation, it is critical for B cell expansion in GC. We also analyzed Ab class switching by

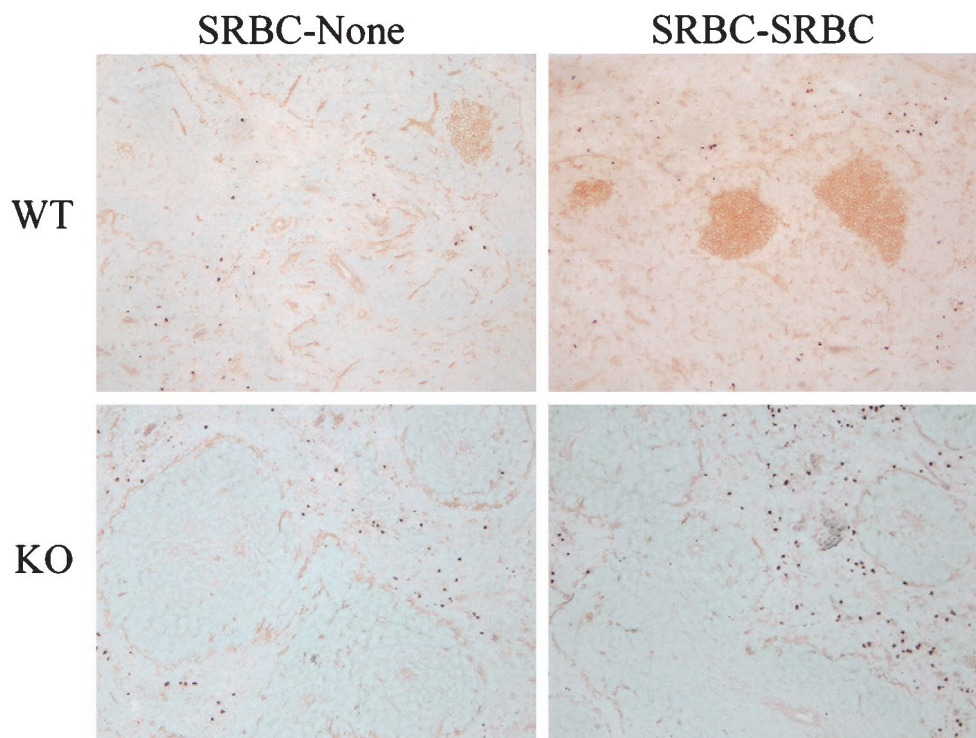


FIGURE 2. ICOS is essential for secondary GC reaction. Twenty-four days after primary immunization, ICOS^{+/+} and ^{-/-} mice were boosted with SRBC as described in the legend to Fig. 1, and secondary GC formations were characterized 3 days later with PNA staining of spleen sections. Mice with primary but without secondary immunization were used as controls. WT, Wild type; KO, knockout.

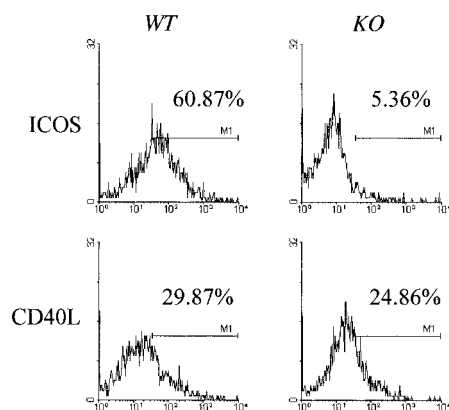


FIGURE 3. CD40L expression by activated ICOS^{-/-} CD4 T cells. ICOS^{+/+} and ^{-/-} lymph node cells were treated with Con A for 4 h. Expression of ICOS and CD40L on CD4⁺ cells was analyzed by flow cytometry. WT, Wild type; KO, knockout.

GC B cells. Most PNA⁺ GC (12 of 14) in wild-type spleen contained large numbers of IgG1⁺ cells, whereas only 5 of 26 GC in the knockout were found to be IgG1⁺ (Fig. 1C). This result correlated with our earlier finding that ICOS is required for IL-4 production by Th2 effector cells, since IgG1 class switch is dependent on IL-4.

We then investigated whether there is any defect of ICOS^{-/-} mice in a secondary response to SRBC. Twenty-four days after the first immunization, few GC could be found in either wild-type or knockout spleens. Three days after a secondary boost of SRBC, wild-type mice quickly developed large PNA⁺ GC in their spleens (Fig. 2). However, not a single GC could be identified in the knockout mice (Fig. 2). This result indicates that ICOS is essential for secondary GC formation. This contrasted sharply with the IL-4-deficient mice which mount a normal if not stronger GC reaction (3).

The above data support a critical role for ICOS in mediating humoral immune responses. However, how ICOS works in GC reactions is not clear. It is not likely to occur through a CD40L-dependent mechanism, as in vitro activated ICOS^{-/-} CD4 T cells could express normal levels of CD40L (Fig. 3). We previously reported that ICOS^{-/-} effector T cells exhibited a deficiency in

IL-2, IL-4, and IL-13 production. Whether these cytokines alone or together account for GC defects in ICOS^{-/-} animals is unclear and requires further investigation.

From this study, we showed that ICOS^{-/-} mice were defective in B cell expansion and class switch in primary GC, which is likely CD40L independent but does correlate well with our earlier report on IL-4 deficiency in ICOS^{-/-} T cells (9). On the other hand, ICOS is essential for secondary GC formation, which is probably through an IL-4-independent mechanism. These results indicate an essential role of ICOS in GC reactions and support its function in humoral immunity.

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