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The Inducible Costimulator Plays the Major Costimulatory Role in Humoral Immune Responses in the Absence of CD28

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CD28 plays crucial costimulatory roles in T cell proliferation, cytokine production, and germinal center response. Mice that are deficient in the inducible costimulator (ICOS) also have defects in cytokine production and germinal center response. Because the full induction of ICOS in activated T cells depends on CD28 signal, the T cell costimulatory capacity of ICOS in the absence of CD28 has remained unclear. We have clarified this issue by comparing humoral immune responses in wild-type, CD28 knockout (CD28 KO), and CD28-ICOS double-knockout (DKO) mice. DKO mice had profound defects in Ab responses against environmental Ags, T-dependent protein Ags, and vesicular stomatitis virus that extended far beyond those observed in CD28 KO mice. However, DKO mice mounted normal Ab responses against a T-independent Ag, indicating that B cell function itself was normal. Restimulated CD4+ DKO T cells that had been primed in vivo showed decreased proliferation and reduced IL-4 and IL-10 production compared with restimulated CD4+ T cells from CD28 KO mice. Thus, in the absence of CD28, ICOS assumes the major T cell costimulatory role for humoral immune responses. Importantly, CD28-mediated ICOS up-regulation is not essential for ICOS function in vivo.


A n optimal T cell response requires two types of signals (1). One is delivered by the TCR upon recognition of specific peptide-MHC complexes displayed on the surface of APCs. The second signal is provided by stimulatory or inhibitory coreceptors that bind to their cognate ligands on APCs. CD28 is a key stimulatory coreceptor expressed on naïve T cells. Ligation of CD28 by its ligand, CD80 (B7-1) and CD86 (B7-2), augments and sustains TCR signaling and leads to increased production of IL-2, cell cycle progression, and enhanced cell survival (1). The crucial role played by CD28 in the proliferation and differentiation of T cells has been highlighted by studies of CD28 knockout (KO) mice. CD28-deficient T cells hypoproliferate upon antigenic stimulation, and CD28 KO mice have reduced Ab responses with impaired germinal center (GC) formation (2–4). However, some immune responses remain intact in the absence of CD28, perhaps because a prolonged TCR signal overcomes the need for costimulation (5, 6), and/or other costimulatory molecules can substitute for CD28 (7). CD28-mediated costimulation is regulated by the CD28-related molecules CD152 (CTLA-4), which is expressed on the T cell surface after T cell activation, CD152 binds B7-1 and B7-2 with greater affinity than CD28 and down-regulates T cell activation by inhibiting TCR/CD28-mediated signaling pathways (1).

Another CD28-related molecule, inducible costimulator (ICOS), is induced in activated T cells (8). ICOS binds to a member of the B7 family called ICOS ligand (ICOSL; also known as B7RP1 (9), B7-H2 (10), B7h (11), GL50 (12), and LICOS (13)). Unlike CD80 and CD86, whose expression is largely restricted to APCs, ICOSL is expressed in multiple nonlymphoid organs (11, 12). ICOS ligation enhances T cell proliferation and the production of various cytokines, including IFN-γ, IL-4, and IL-10 (8, 10, 14, 15). However, in contrast to CD28, ICOS ligation augments only a minimal amount of IL-2 that is readily consumed by proliferating T cells (14, 15). Although ICOS-ICOSL interaction appears to provide costimulatory signals to both CD4+ and CD8+ T cells (16), analyses of mice and humans deficient in ICOS or ICOSL highlight the critical role of ICOS costimulation for Ab isotype switching, affinity maturation, and GC response (17–23).

Interestingly, CD28, ICOS, and CD152 are not only structurally related, but also cross-regulate each other’s expression and/or functions. CD152 ligation inhibits the induction of ICOS and ICOS-mediated signaling, although this effect can be readily over-ridden by IL-2 (15). Furthermore, CD28 ligation up-regulates ICOS expression ~8- to ~20-fold above the level achieved by TCR stimulation alone (15, 24, 25). The CD28-mediated ICOS up-regulation is more prominent in CD4+ T cells than in CD8+ T cells (25). Thus, the impact of CD28 deficiency in CD28 KO mice should be mediated by two components: the lack of CD28 itself, and the compromised ICOS induction. One prediction would be that inactivation of the Icos gene in CD28 KO mice might have little further impact on CD28 KO phenotypes.
CD28 and ICOS appear to play overlapping costimulatory roles in humoral immune responses. However, the degree of synergy between CD28 and ICOS in humoral immunity remains unclear. CD28 influences the ICOS expression level in vitro, but whether this has critical in vivo consequences is unknown. We addressed these issues by comparing the Ab responses in wild-type (WT), CD28 KO, and double-knockout (DKO) mice. We found that DKO mice exhibit enormous defects in Ab responses against environmental Ags, T-dependent protein Ags, and viral infection. These defects are far beyond the scope observed in CD28 KO mice. We show that these defects are due to the impaired proliferation and cytokine production of T cells activated in the absence of both CD28 and ICOS. Thus, CD28 and ICOS act independently, but cooperatively, in T cell costimulation during humoral immune responses, and ICOS can assume crucial costimulatory functions in the absence of CD28.

Materials and Methods

Mice

The Icos<sup>−/−</sup> embryonic stem (ES) cell clone was generated as previously described (17). We disrupted the second exon of the Cd28 locus in Icos<sup>−/−</sup> ES cells using the same strategy that had been used for generation of the original CD28 KO mice (2), except that a hygromycin resistance cassette (hygro) was used in place of the neomycin resistance cassette (neo; Fig. 1A). We bred two strains of mice derived from two independent ES clones that had Icos (neo) and Cd28 (hygro) on the same chromosome (as judged by the cosegregation pattern of the two alleles) to obtain WT (Cd28⁺/+; Icos<sup>−/−</sup>), DHet (Cd28<sup>−/+</sup>; Icos<sup>−/−</sup>), and DKO (Cd28<sup>−/−</sup>; Icos<sup>−/−</sup>) mice. DKO mice derived from the two strains of mice showed the same phenotypes. We bred one strain of mice derived from one ES clone that had Icos (neo) and Cd28 (hygro) on the opposite chromosomes to generate WT (Cd28⁺/+; Icos<sup>−/−</sup>), CD28 Het (Cd28<sup>−/+</sup>; Icos<sup>−/−</sup>), and CD28 KO (Cd28<sup>−/−</sup>; Icos<sup>−/−</sup>) animals. CD28 KO mice from this strain showed the same phenotypes as the original CD28-deficient mice (2). For some experiments we used ICOS KO mice (17) as controls. All the animals described above were of a mixed 129/Ola×C57BL/6 genetic background and within 2 wk of age difference in all experiments. All live animal experiments were conducted with the approval of the University Health Network animal care committee (Toronto, Canada).

Antibodies

All Abs were purchased from BD PharMingen (San Diego, CA), except for anti-ICOS-PE (eBioscience, San Diego, CA).

Ab analysis

Basal Ig levels were determined using an ELISA kit (Southern Biotechnology Associates, Birmingham, AL) according to the manufacturer’s instructions. To measure anti-keyhole limpet hemocyanin (anti-KLH) responses, we injected mice s.c. with KLH emulsified in IFA (50 µg/mouse). Anti-KLH IgM was measured on day 7, and anti-KLH IgG1 and IgG2a were measured on day 14 by ELISA. For anti-2,4,6-trinitrophenol (TNP)-Ficoll responses, we injected mice i.p. with TNP<sub>24</sub>-Ficoll (25 mg/mouse) for 5 weeks. TNP-specific spleen cells were stained with peanut agglutinin (PNA), anti-CD4 Ab, and anti-B220 Ab to evaluate GC reaction as previously described (4).

T cell analysis

For CD154 staining, we cultured total lymph node cells in RPMI 1640 medium containing 10% FCS plus antibiotics (complete medium) in the presence of 1 µg/ml anti-CD3 Ab (2C11; BD PharMingen) and anti-CD154-PE Ab or isotype control-PE Ab for 16 h. After stimulation, cells were washed and then stained with anti-CD4-allophycocyanin Ab. CD154 expression was assessed on CD4<sup>+</sup> T cells by flow cytometry. For intracellular cytokine analysis, we injected mice s.c. with KLH-IFA (50 µg/mouse) and isolated the draining lymph nodes 10 days later. We prepared single-cell suspensions in complete medium and then depleted CD8<sup>+</sup> and B220<sup>+</sup> cells using Ab-conjugated magnetic beads (Dynal Laboratories, Chantilly, VA). Cells were then cultured at 5 × 10<sup>6</sup> cells/ml/well in 12-well plates in the presence of 100 µg/ml KLH. After 90-h incubation, the cells were washed and restimulated with PMA (10 ng/ml) plus ionomycin plus brefeldin A (10 µg/ml).

FIGURE 1. Disruption of the Cd28 locus in an Icos<sup>−/−</sup> ES cell clone. A, Targeting strategy. The targeting vector was designed to replace the second exon of the Cd28 gene (■) with the hygromycin resistance gene (hygro). S, SalI; X, XbaI; RI, EcoRI. B, Southern blot analysis. Genomic DNAs from WT (Cd28⁺/+; Icos<sup>−/−</sup>), double-heterozygote (DHet; Cd28<sup>−/+</sup>; Icos<sup>−/−</sup>), and double-homozygote (DKO; Cd28<sup>−/−</sup>; Icos<sup>−/−</sup>) mice were digested with XbaI and subjected to Southern blotting using the probe shown in A. C, Flow cytometric analysis. Total splenocytes from WT, DHet, and DKO mice were stimulated with Con A (1 µg/ml) for 48 h and stained with anti-TCRβ-FITC plus anti-CD28-PE, or anti-TCRβ-FITC plus anti-ICOS-PE. Activated T cells (TCRβ<sup>+</sup>) were gated based on high forward scattering.
(100 ng/ml) for 4 h in the presence of GolgiStop (BD PharMingen). Cells were then stained with anti-CD4-FITC and anti-CD25-PE, followed by allophycocyanin-conjugated Abs to IFN-γ, IL-4, and IL-10 using an intracellular cytokine staining kit (BD PharMingen). For proliferation assays, cells were prepared as described above at 2 × 10^5/ml/well in 96-well, U-bottom plates with or without KLH (100 μg/ml) and pulsed with [3H]thymidine (1 μCi/well) for the last 8 h of a 2-, 3-, or 4-day culture period.

Statistical analysis

Student’s t test was used to determine the statistical significance of differences between genotypes.

Results

Generation of Cd28<sup>−/−</sup>; Icos<sup>−/−</sup> mice

The genes encoding CD28, CD152, and ICOS are clustered on the same chromosome: chromosome 1 in mice (26) and chromosome 2 in humans (27). The mouse Cd28 and Icos genes are separated by only ~1.5 cm (26), precluding the generation of DKO animals by conventional breeding steps. We therefore used a hygromycin resistance cassette (hygro) to target the Cd28 gene in an Icos<sup>+/+</sup> ES clone in which one Icos allele had already been disrupted by insertion of a neomycin resistance cassette (neo; Fig. 1A). As described in Materials and Methods, we obtained three ES clones that were used to derive WT, CD28 KO, and DKO mice.

Disruption of the Cd28 allele in DKO mice was demonstrated by Southern blot analysis (Fig. 1B). The Cd28 and Icos mutations were confirmed to be null, as assessed by flow cytometric analysis of the expression of CD28 and ICOS proteins on the surface of activated T cells (Fig. 1C). Examination of T, B, NK, and NKT cell populations in primary and secondary lymphoid organs revealed normal differentiation and distribution of these cells in the absence of CD28 and ICOS (data not shown). However, the percentage of CD4<sup>+</sup>CD62L<sup>low</sup> cells (activated/memory T cells) in the absence of CD28 and ICOS proteins on the surface of activated T cells (Fig. 1C). Examination of T, B, NK, and NKT cell populations in primary and secondary lymphoid organs revealed normal differentiation and distribution of these cells in the absence of CD28 and ICOS (data not shown). However, the percentage of CD4<sup>+</sup>CD62L<sup>low</sup> cells (activated/memory T cells) was normal differentiation and distribution of these cells in the absence of CD28 and ICOS proteins on the surface of activated T cells (Fig. 1C).

Reduced serum IgG1 concentration in CD28 KO and DKO mice: Serum samples were prepared from the tail blood of 3-mo-old WT ( ), CD28 KO ( ), and DKO ( ) mice, and the concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were determined by ELISA (n = 8–16 mice/genotype). There were significant differences in IgG1 concentration among the genotypes compared with WT:CD28 KO, CD28 KO:DKO, and WT:DKO (horizontal lines). * p < 0.05, by Student’s t test.

Drastically reduced basal IgG1 in DKO mice

Basal serum Ig levels provide an unbiased indication of the efficiency of immune responses evoked by environmental Ags. Deficiency of either CD28 or ICOS results in a reduction of basal serum IgM. Previous reports have shown that CD28 KO mice retain ~20% of the mean WT IgG1 concentration (2), whereas ICOS KO mice show ~25–30% WT IgG1 (17, 18). In this study we compared serum concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in 3-mo-old WT, CD28 KO, and DKO mice (Fig. 2). Among these isotypes, a statistically significant difference from the WT mean was apparent only for IgG1. CD28 KO mice retained ~10% of the mean WT IgG1 level, whereas DKO mice showed only ~1.2% of WT IgG1. Other Ab isotypes tended to be decreased in CD28 KO and DKO mice, but these differences lacked statistical significance. These data indicate that in the absence of CD28, ICOS plays a critical role in promoting Ab isotype switching to IgG1. Consistent with this idea, the bulk of ICOS<sup>−/−</sup>CD4<sup>+</sup> T cells in nonmanipulated mice express IL-4, the key cytokine for isotype switching to IgG1 (28).

Profound defect in Ab isotype switching induced by a T-dependent Ag

Although CD28 is the major costimulatory molecule for responses to many immunological challenges, the magnitude of the impact of CD28 deficiency varies depending on the immunization protocol (2, 29, 30). For this study we chose an immunization protocol that revealed a partial defect in CD28 KO mice and allowed us to measure the impact of CD28-ICOS double deficiency. When CD28 KO mice were immunized with KLH emulsified in IFA, we found that a small, but significant, reduction in anti-KLH IgG1 production occurred relative to the WT mean (Fig. 3A). Importantly, this partial anti-KLH IgG1 response was completely abolished in DKO mice. Under the same conditions, CD28 KO mice produced normal levels of IgG2a, whereas this isotype was nearly undetectable in the sera of DKO mice (Fig. 3A). In addition, whereas the IgG2a defect in DKO mice became much less significant after secondary immunization, the IgG1 defect remained profound (Fig. 3B).

The level of IgM in DKO mice was not reduced compared with that in WT or CD28 KO mice when the animals were nonmanipulated or actively immunized (Fig. 2, IgM; Fig. 3A, IgM). These data thus suggested that B cell function itself was not affected by the combined loss of CD28 and ICOS. To confirm this, we immunized WT and DKO mice with the T-independent Ag, TNP-Ficoll. This Ag directly stimulates B cell responses without the need for thymus-derived T cell help (31). As predicted, DKO mice produced normal levels of anti-TNP IgM and IgG3 (Fig. 3C). Taken together, our results show that Ab isotype switching is heavily dependent on ICOS-mediated T cell help when CD28 is not present.
Severe impairment of antiviral humoral immunity in DKO mice

The overall effectiveness of humoral immunity against pathogenic challenges depends not only on the amount of Ab synthesized, but also on its affinity/avidity. Higher affinity Abs are generated through somatic hypermutation of Ig genes in GC B cells. Although individual inactivation of the Cd28 and Icos genes leads to a greatly compromised GC response (3, 4, 17, 18, 23), both CD28 KO and ICOS KO mice display only a partial defect in the production of neutralizing Ab during VSV infection (2, 32). In this study we infected WT, CD28 KO, ICOS KO, and DKO mice with VSV and measured the production of neutralizing IgM and IgG Ab. As expected, anti-VSV IgM production on day 4 was equivalent in all four genotypes (Fig. 4A). By days 8 and 12, CD28 KO mice produced anti-VSV IgG, but displayed a partial defect in this response. Neutralizing IgG was ~8-fold lower than the WT mean titer on both days 8 and 12 postinfection (Fig. 4A), confirming

FIGURE 3. Impaired Ab isotype switching in CD28KO and DKO mice in response to a T-dependent Ag. A. Primary response. WT (●), CD28 KO (○), and DKO (□) mice were s.c. injected with KLH-IFA, and serum anti-KLH Ab titers were determined on day 7 (IgM) or day 14 (IgG1 and IgG2a) postinfection (n = 8 mice/genotype). B. Secondary responses. The mice described in A were boosted using the same protocol 4 wk after the primary injection. Anti-KLH IgG1 and IgG2a titers were determined 7 days later. C. Intact Ab responses against a T-independent Ag. Four WT (●) and three DKO (□) mice were i.p. injected with TNP-Ficoll, and serum titers of anti-TNP IgM and IgG3 Ab were determined by ELISA. *, p < 0.05; **, p < 0.01.

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FIGURE 4. Severe defects in anti-VSV humoral immunity in DKO mice. A. WT (n = 3; □), CD28 KO (n = 2; □), DKO (n = 8; □), and ICOS KO (n = 2; □) mice were i.v. injected with VSV, and the neutralizing Ab titers were determined on the indicated days by a plaque formation assay as described in Materials and Methods. #, Below detection limit; arrow, GCs are shown in B. Results shown are representative of two independent experiments. B. Impaired GC response. Spleens were taken on day 12 postinfection, and GCs were stained as described in Materials and Methods. Results shown are representative sections obtained from two mice per genotype.
earlier reports (2, 4). In contrast, ICOS KO mice showed only minimal defects, as previously reported (32). However, the impact of the combined loss of CD28 and ICOS was far greater. Five of 8 DKO mice did not produce any detectable neutralizing IgG by day 8 postinfection, and two of these five mice did not show any neutralizing IgG, even by day 12 (Fig. 4A). Moreover, the titers of neutralizing anti-VSV IgG synthesized by DKO mice were, on the average, 64-fold lower than those of WT mice (Fig. 4A). These defects in mounting a neutralizing Ab response were consistent with the reduced number and size of GCs in CD28 and ICOS KO spleens and a complete absence of GC in DKO spleen (Fig. 4B). Thus, CD28 and ICOS have largely overlapping roles in antiviral Ab response. When CD28 is not present, ICOS can support a reduced, but substantial, level of neutralizing Ab response against virus. However, the complete absence of GC reaction and the severe defect in neutralizing Ab response in DKO mice suggest that the functions of CD28 and ICOS during the process of GC reaction and T-B collaboration may not occur in a linear pathway.

Impaired proliferation and Th2 cytokine production by DKO CD4+ T cells

To determine the molecular basis of the Ab response defects, we first examined the ability of WT, CD28 KO, DKO, and ICOS KO T cells to express CD154 (CD40L), a molecule that plays a key role in T-B collaboration. No major defects in CD154 induction were detected in CD4+ T cells of CD28 KO, DKO, or ICOS KO mice after 16-h anti-CD3 treatment (Fig. 5). This is consistent with previous reports that CD154 induction is largely independent of costimulatory signals (7, 23, 33).

We next examined proliferation and cytokine production by CD4+ T cells primed in vivo with KLH-IFA. Upon restimulation in vitro with KLH, CD28 KO T cells showed a 2-fold decrease in proliferation compared with WT T cells, whereas ICOS KO T cells had little defect (Fig. 6A). However, inactivation of the Icos gene in the CD28 KO mice resulted in an additional ~2-fold decrease in T cell proliferation, indicating that the ICOS-mediated costimulation becomes significant when CD28 costimulation is not provided.

Consistent with previous reports (17, 21, 34, 35), the frequencies of IL-4- or IL-10-producing CD4+ T cells were lower in CD28 KO and ICOS KO T cell culture compared with WT controls (Fig. 6B). Importantly, this defect is far more pronounced in DKO T cells. However, the frequency of IFN-γ-producing CD4+ T cells was increased in CD28 KO, DKO, and ICOS KO T cell culture compared with that in WT controls (Fig. 6B). This is in line with the previous findings that Th1 differentiation is favored over Th2 in the absence of CD28 or ICOS costimulation (18, 36–38). Collectively, these results indicate that costimulation via both CD28 and ICOS is required for the full expansion and differentiation of Th2 cells during humoral immune responses.

**Discussion**

By comparing humoral immune responses in WT, CD28 KO, and DKO mice, we have shown that the combined inactivation of the Cd28 and Icos genes leads to profound defects in Ab isotype switching and the generation of neutralizing Abs. These defects correlate with a severe defect in the GC response and the expansion and differentiation of Th2 cytokine-producing cells. The partially impaired humoral responses displayed by CD28 KO mice are almost completely abrogated when ICOS is also eliminated. We therefore conclude that ICOS is a critical costimulatory molecule for humoral immune responses in its own right, and that CD28-mediated up-regulation of ICOS expression is not essential for the in vivo function of ICOS.

The synergistic effects of CD28 and ICOS on Ab responses are consistent with our current knowledge of the roles of CD28, ICOS, and CD152 in the GC reaction or T-B collaboration in general. Although both CD28 KO and ICOS KO mice show greatly reduced GC responses, the roles of CD28 and ICOS during the process of GC response appear to be different. Signaling via CD28 is required for the development of a GC response, but the established GCs can be maintained without CD28 signaling (30, 39). In contrast, disruption of ICOS-ICOSL interaction does not affect the migration of T cells into B cell follicles (40). Moreover, primed T cells and B cells reconstituted in naive irradiated recipient animals cannot generate Ab upon antigenic rechallenge in the absence of ICOS-ICOSL interaction, indicating that the ICOS pathway is important in T-B collaboration as well as in T cell priming (21).

The fact that the combined loss of CD28 and ICOS completely abrogates the GC response is consistent with the distinctive (but overlapping) roles of CD28 and ICOS during GC reaction and T-B collaboration. CD152 plays a critical role to down-regulate the size and duration of GCs (30). Therefore, the costimulatory mechanism that plays a dominant role at the later stage of the GC response must be under the control of CD152, as proposed by Walker et al. (30). Consistent with this model, ICOS plays a dominant T cell costimulatory role in the absence of CD28 and can be controlled by CD152 (15).

In a normal humoral response, CD28-mediated signals during T cell activation presumably induce optimal expression of ICOS.
The combined signals emanating from CD28 and ICOS can then lead to the differentiation of Th cells expressing high levels of cytokines, including IL-4 and IL-10, cytokines known to stimulate B cell growth, differentiation, and isotype switching (41–43). Activation of the CD40-CD154 pathway during T-B contact should facilitate CD28- and ICOS-mediated T cell help as CD40 signaling up-regulates CD86 (44) and sustains ICOSL expression on activated B cells (45). The CD40-CD154 pathway should also guide the Ab response toward the Ags that were recognized and presented by B cells. The severe defect in Ab responses in CD40- or CD154-deficient mice (46, 47) is consistent with the potential roles of the CD40 pathway in boosting and/or coordinating CD28 and ICOS pathways during T-B collaboration.

The role of costimulation in Th1/Th2 differentiation has been controversial, as engagement of CD28 and ICOS can enhance the expression of both Th1 and Th2 cytokines by T cells (8, 10, 14, 15, 34). In contrast, many studies have shown that these costimulators preferentially facilitate Th2 responses, especially after repeated and/or chronic T cell stimulation (17–19, 25, 28, 34, 35, 38, 48). Our results support the view that CD28 and ICOS play a cooperative role in Th2 differentiation. First, DKO mice show the greatest deficit in the IL-4-dependent IgG1 response in both nonmanipulated and immunized mice. Secondly, CD4+ T cells that are differentiated in the absence of CD28 and ICOS display a greatly impaired production of IL-4 and IL-10 with a modest enhancement of IFN-γ expression.
Costimulation by CD28 and ICOS has been implicated to be a key feature in various autoimmune diseases, allergy, allograft rejection, and infectious diseases (1, 16). It has yet to be determined what functional interplay occurs between CD28 and ICOS during disease progression. Our DKO mice will be useful tools for addressing these questions.

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References


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