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

Woong-Kyung Suh, Anna Tafuri, Nancy N. Berg-Brown, Arda Shahinian, Suzanne Plyte, Gordon S. Duncan, Hitoshi Okada, Andrew Wakeham, Bernhard Odermatt, Pamela S. Ohashi and Tak W. Mak

_J Immunol_ 2004; 172:5917-5923; doi: 10.4049/jimmunol.172.10.5917 http://www.jimmunol.org/content/172/10/5917

References This article cites 48 articles, 21 of which you can access for free at: http://www.jimmunol.org/content/172/10/5917.full#ref-list-1

Subscription Information about subscribing to _The Journal of Immunology_ is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Inducible Costimulator Plays the Major Costimulatory Role in Humoral Immune Responses in the Absence of CD28

Woong-Kyung Suh, Anna Tafuri, Nancy N. Berg-Brown, Arda Shahinian, Suzanne Plyte, Gordon S. Duncan, Hitoshi Okada, Andrew Wakeham, Bernhard Odermatt, Pamela S. Ohashi, and Tak W. Mak

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.


Received for publication December 12, 2003. Accepted for publication March 1, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Canadian Institute of Health Research, Canadian Network for Vaccines and Immunotherapeutics of Cancer and Chronic Viral Diseases, and the Cancer Research Institute.

2 Address correspondence and reprint requests to Dr. Tak W. Mak, Advanced Medical Discovery Institute, 620 University Avenue, Suite 706, Toronto, Ontario, Canada M5G 2C1. E-mail address: tmak@uhnres.utoronto.ca

3 Current address: L121 Division of Science, Medicine Hat College, Medicine Hat, Alberta, Canada T1A 3Y6.

4 Abbreviations used in this paper: KO, knockout; GC, germinal center; DKO, double knockout; ES, embryonic stem; ICOS, the inducible costimulator; ICOSL, ICOS ligand; KLH, keyhole limpet hemocyanin; PNA, peanut agglutinin; TNP, 2,4,6-trinitrophenol; VSV, vesicular stomatitis virus; WT, wild type.
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<sup>+/+</sup>; 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<sup>+/+</sup>), DHet (Cd28<sup>−/−</sup>), and CD28 KO (Cd28<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 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>2</sub>-Ficoll (25 μg/mouse; Biosearch Technologies, Novato, CA). Anti-TNP IgM was measured on day 7, and anti-TNP IgG3 was measured on day 22 by ELISA using TNP<sub>2</sub>-BSA (Biosearch Technologies) as the Ag.

Anti-vesicular stomatitis virus (anti-VSV) responses

We infected mice with VSV (Indiana strain; 2 × 10<sup>6</sup> PFU i.v.) and bled the animals on days 4, 8, and 12. Neutralizing Ab titers were determined by a plaque formation assay as previously described, using 2-fold serial dilution of the sera (4). Serum concentrations that provide 50% protection of the fibroblast monolayer were considered to be specific titers. On day 12 postinfection, spleens were frozen in liquid nitrogen. The spleen sections 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.
(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 aliphycocyanin-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 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−/−; Icos−/− 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−/+ 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+CD25−/CD62Llow cells (activated/memory T cells) in the total CD4+ T cell population in the peripheral blood decreased with the loss of both genes (mean ± SD for WT, 17.7 ± 3.5%; for CD28 KO, 5.3 ± 0.8%; for DKO, 2.3 ± 1.2%; for ICOS KO, 8.7 ± 3.5% (three mice per genotype at 3 mo of age; p < 0.05 for WT-CD28 KO, CD28 KO-DKO, WT-DKO, and WT-ICOS KO comparisons). These results show that CD28 and ICOS are both positive regulators of peripheral T cell activation.

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 IgG1. 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+CD4+ 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...
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 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 defect 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.

**FIGURE 6.** Severely impaired proliferation and Th2 cytokine production of DKO T cells. WT, CD28 KO, DKO, and ICOS KO mice were immunized with KLH-IFA. On day 10 the draining lymph nodes were collected, and single-cell suspensions were prepared. After depletion of CD8+ and B220+ cells, the cells were restimulated in vitro with 100 μg/ml KLH. A, Impaired proliferation. [3H]thymidine was pulsed during the last 8 h of 2-, 3-, or 4-day culture periods as described in Materials and Methods. Solid lines, With KLH; dotted lines, without KLH. Each data point represents the mean ± SD of triplicate samples. *, p < 0.02; **, p < 0.01. B, Impaired Th2 cytokine production. Activated CD4+ T cells producing IFN-γ, IL-4, or IL-10 were detected by intracellular cytokine staining as described in Materials and Methods. The percentage of cytokine+ cells over the total CD25+CD4+ T cell blasts is indicated in each panel. For A and B, results shown are representative of three independent experiments.
Costimulation by CD28 and ICOS has been implicated to varying extents in 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.

Acknowledgments

We thank M. Saunders for scientific editing.

References