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CD28 and ICOS play complementary non-overlapping roles in the development of Th2 immunity in vivo

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ABSTRACT

Previous work has shown ICOS can function independently of CD28, but whether either molecule can compensate for the other in vivo is not known. Since ICOS is a potent inducer of Th2 cytokines and linked to allergy and elevated serum IgE in humans, we hypothesized that augmenting ICOS costimulation in murine allergic airway disease may overcome CD28 deficiency. While ICOS was expressed on T cells from CD28^{−/−} mice, Th2-mediated airway inflammation was not induced in CD28^{−/−} mice by increased ICOS costimulation. Further, we determined if augmenting CD28 costimulation could compensate for ICOS deficiency. ICOS^{−/−} mice had a defect in airway eosinophilia that was not overcome by augmenting CD28 costimulation. CD28 costimulation also did not fully compensate for ICOS for antibody responses, germinal center formation or the development of follicular B helper T cells. CD28 and ICOS play complementary non-overlapping roles in the development of Th2 immunity in vivo.

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1. Introduction

CD28 was arguably the most well studied costimulatory molecule until the discovery of ICOS in 1999 expanded the CD28 family [1,2]. Similar to CD28, ICOS has been shown to augment T cell proliferation, Th2 differentiation and production of IL-4 but unlike CD28, ICOS costimulation does not induce high levels of IL-2 production [2,3]. We have previously found an association of ICOS single nucleotide polymorphisms with increased serum IgE levels and allergen sensitivity in a human population, suggesting ICOS plays a role in augmenting allergy in humans [4]. In mouse models of Th2-mediated allergic airway disease, several groups including ours have found that blockade of CD28 completely inhibits Th2-mediated airway inflammation and IgE production [5–8], while ICOS blockade decreases Th2-mediated airway inflammation [9,10]. Fur-

ther, ICOS^{−/−} mice have defects in Th2 differentiation similar to CD28^{−/−} mice [11–15]. These data support a model defined by the requirement of CD28 for Th2 development while ICOS is an important mediator of the magnitude of Th2 responses.

Individuals who have a total defect in ICOS expression due to a deletion within the ICOS gene present clinically with common variable immunodeficiency syndrome (CVID), characterized by hypogammaglobulinemia and recurrent infections of the respiratory and gastrointestinal tract [16–18]. ICOS-null individuals have been found to have profound defects in B cell maturation and immunoglobulin isotype switching. These individuals like ICOS^{−/−} mice have a defect in the production of specialized follicular B helper T cells (T_{FH}). T_{FH} are a novel sub-population of CD4⁺ T cells characterized by expression of the chemokine receptor CXCR5, high ICOS expression and the production of IL-21 [19–21]. Thus similar to CD28, a major role for the ICOS-B7RP-1 pathway in T helper cell-dependent B cell responses has emerged.

Previous work using mice deficient for both ICOS and CD28 has shown that ICOS can play a role independent of CD28 in viral immunity and immunoglobulin isotype switching [22,23].

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However, these reports have not investigated whether augmenting CD28 or ICOS costimulation can compensate for the function of the other molecule. Since costimulatory molecules provide a signal that is additive to the TCR it is possible that increasing activation of ICOS or CD28 may overcome the deficiency of the other for Th2 immunity. We found that while ICOS can be expressed in vivo in CD28^{-/-} mice, augmenting ICOS costimulation did not surmount the requirement for CD28 to induce Th2-mediated airway inflammation or antibody responses. In ICOS^{-/-} mice Th2-mediated airway eosinophilia was significantly less than wild-type mice and augmenting CD28 costimulation had no significant effect. Further, augmenting CD28 costimulation did not restore to wild-type levels antigen-specific antibody responses, germinal center size or the expansion of CD4⁺ CXCR5⁺ T_{FH} in the absence of ICOS. Thus, CD28 and ICOS play complementary non-overlapping roles in the development of Th2 immunity in vivo.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice, ages 4–6 wk, were purchased from National Cancer Institute (Frederick, MD) or Charles River Laboratories (Wilmington, MA). C57BL/6.CD28^{-/-} mice, 4–6 wk old, were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6.ICOS^{-/-} mice were the gift of Dr. Richard Flavell and were bred in our facilities. Wild-type littermate controls from in-house breeding were used in the experiments with ICOS^{-/-} mice. Animals were housed in a specific pathogen-free barrier facility, maintained by the University of Chicago Animal Resources Center (Chicago, IL) and were used under the guidelines of our Institutional Animal Care and Use Committee.

2.2. Animal models

Schistosoma mansoni eggs and antigen were prepared as previously described [24]. The protocol used for animal sensitization and challenge was modified from Padrid et al. [7]. Mice, immunized on day 0 with 5000 inactivated *S. mansoni* eggs delivered by intraperitoneal (i.p.) injection, were challenged on day 7 via intratracheal (i.t.) delivery of 5 µg of soluble egg antigen. Mice were treated i.p. every other day throughout sensitization and challenge with either 50 µg B7RP-1-Fc or 25 µg human Fc (molar equivalent, Amgen); or mice received one dose i.p. of either 100 or 150 µg anti-CD28 (PV-1) [25], hamster isotype control (BioX-Cell), or PBS at the time of sensitization unless noted in the text. In some experiments mice were sensitized i.p. with 10 µg ovalbumin (OVA) in alum. For restimulation ex vivo, lungs or mediastinal lymph nodes were dissociated as previously described [10].

2.3. Bronchoalveolar lavage (BAL)

Mice were sacrificed on day 11. BAL was performed as previously described [10]. Differential cell counts were determined by counting a total of 200 cells. Alternatively, differential cell counts were determined by flow cytometry.

2.4. Serum antibody levels

Total serum IgE was measured by ELISA (BD Biosciences). Antigen-specific IgG1, IgG2a, and IgG2b were measured by coating plates with either SEA (10 µg/ml) or OVA (100 µg/ml) and using secondary isotype specific antibodies (BD Biosciences). OVA specific IgE was measured by coating with an IgE specific antibody (BD Biosciences) and using a biotinylated OVA secondary reagent.

OVA-specific IgE was determined by comparison with a standard curve constructed with the use of purified, mouse, OVA-specific IgE secreted by the hybridoma TOe, a kind gift of Dr. Paul Bryce [26].

2.5. Flow cytometry analysis

BAL cells were stained with anti-CD3, anti-CD4, anti-CD8 and anti-CCR3 (eosinophils). All antibodies were purchased from BD Pharmingen with the exceptions of anti-CD4-allophycocyanin (eBioscience) and anti-CCR3-FITC (R&D Systems). Flow cytometry analysis was performed using either a FACSCalibur or LSR II (BD Pharmingen). Data was analyzed using CellQuest or Flow Jo software.

2.6. Histology

Lungs were fixed by immersion into 4% paraformaldehyde. Spleens were freshly frozen and embedded in Tissue-Tek II OCT embedding compound (Baxter Scientific) and sliced into 6 µm sections. Biotinylated peanut agglutinin (PNA; Vector, B-1075) was used for staining of germinal center B cells. Entire spleen sections were imaged at 4× magnification using an Axioskop microscope and digital camera. Germinal center area was measured using Image J version 1.36b software. An arbitrary standard was set for calibrating distance for all samples at 11.67 pixels per cm. The size of each PNA positive area within the B cell follicles in the entire section was analyzed and 1–2 slides per spleen were analyzed.

2.7. Statistical analysis

Statistics were done using an unpaired Student two-tailed *t*-test, Mann Whitney or two way ANOVA (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). Error bars represent SEM.

3. Results

3.1. ICOS costimulation enhances serum IgE production but does not affect the severity of allergic airway disease

To investigate the effect of augmented ICOS costimulation on Th2-mediated inflammation, we used a model of allergic airway disease that we have previously shown induces a robust Th2 type response in the lung [7,8,10]. A soluble form of the ICOS ligand, B7RP-1-Fc, that has previously been shown to expand the cellular and humoral responses to different antigens was used to augment ICOS costimulation [27,28]. C57BL/6 mice were sensitized i.p. on day 0 with inactivated *S. mansoni* eggs and challenged in the lung on day 7 with *S. mansoni* soluble egg antigen (SEA). Evaluation of the bronchoalveolar lavage (BAL) on day 11 revealed a significant increase in lymphocytes, predominantly CD4⁺ T cells, in the airways of mice treated every other day throughout sensitization and challenge with B7RP-1-Fc by i.p. injection compared to human Fc (HuFc) treated control mice (Fig. 1A and data not shown). No significant difference was found in the total number of eosinophils or in the cellular infiltration in the lungs by histology (Fig. 1A and data not shown). In addition, the levels of IL-5 and IL-4 in the BAL were not significantly different. The level of IFNγ in the BAL in both groups was undetectable (<5 pg/ml). However, the level of TNFα in the BAL was significantly higher in the B7RP-1-Fc treated mice suggesting a pro-inflammatory effect of B7RP-1-Fc (Fig. 1C and data not shown). The most striking difference found was an increase in serum IgE in B7RP-1-Fc treated mice consistent with the role of ICOS in augmenting humoral immunity (Fig. 1B). To determine if an increase in Th2 differentiation had occurred, lung and medias-

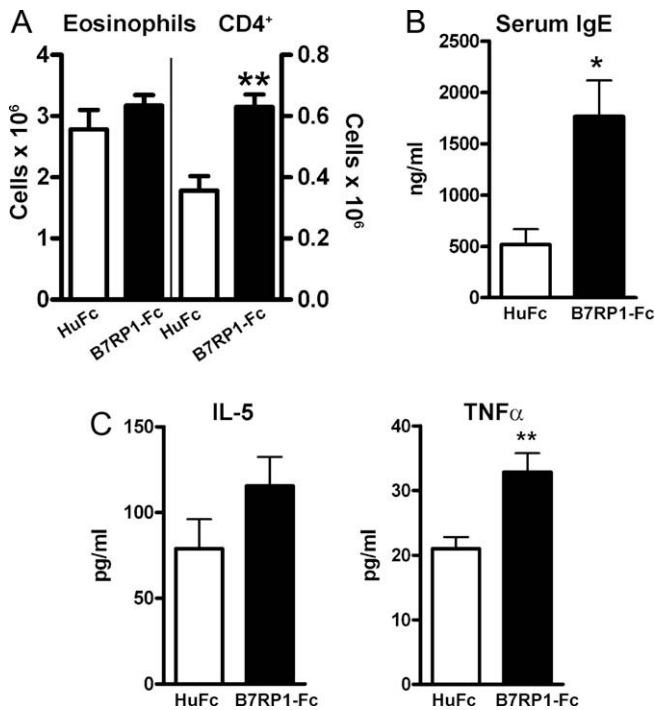


Fig. 1. ICOS costimulation enhances serum IgE production. C57Bl/6 mice sensitized and challenged with *S. mansoni* were injected i.p. every other day with either B7RP1-Fc (black bars) or HuFc (open bars). (A) The total numbers of BAL eosinophils and CD4⁺ T cells, (B) levels of serum IgE and (C) the levels of BAL IL-5 and TNFα were measured. The data are representative of 3 independent experiments with five mice per group.

tinal lymph node (MLN) cells were restimulated ex vivo. Increased IL-5 (lung) and IL-13 (lung and MLN) levels were found in cultures from mice treated with B7RP1-Fc compared to HuFc treated controls (Supp. Fig. S1). The findings suggest that B7RP1-Fc has a modest effect on Th2 inflammation but significantly augments Th2 differentiation and serum IgE.

3.2. ICOS can be expressed in the absence of CD28, but cannot induce Th2-airway inflammation independent of CD28

Previous work has shown that in the absence of CD28, *S. mansoni* cannot induce Th2-mediated airway inflammation or systemic IgE responses [8]. Since ICOS is a potent stimulator of humoral immunity we hypothesized that B7RP1-Fc may compensate for the defect in CD28^{-/-} mice. Given that ICOS expression has been shown to be enhanced by CD28 costimulation in vitro, it was possible that ICOS would not be expressed in vivo in the absence of CD28 [3]. CD28^{-/-} and CD28^{+/+} mice were sensitized i.p. with *S. mansoni* eggs and ICOS expression was examined 7 days later on T cells harvested from the spleen. We found that a population of both CD4⁺ and CD8⁺ T cells from CD28^{-/-} mice did express ICOS although to a lesser extent than CD28^{+/+} T cells (Suppl. Fig. S2).

To determine if ICOS costimulation augmented Th2-mediated inflammation independent of CD28, we administered either B7RP1-Fc or HuFc i.p. to CD28^{-/-} mice every other day throughout sensitization and challenge, as in Fig. 1, compared to sensitization and challenge of CD28^{+/+} mice without treatment. We have previously established that eosinophilia in our model is totally dependent on both sensitization and challenge [7,8]. B7RP1-Fc did not affect total BAL cell counts, eosinophils or lymphocytes in the CD28^{-/-} mice compared to controls (Fig. 2A and data not shown), and did not induce an appreciable increase in peribronchial or perivascular inflammation (Fig. 3E, F). CD28^{-/-} control mice did not

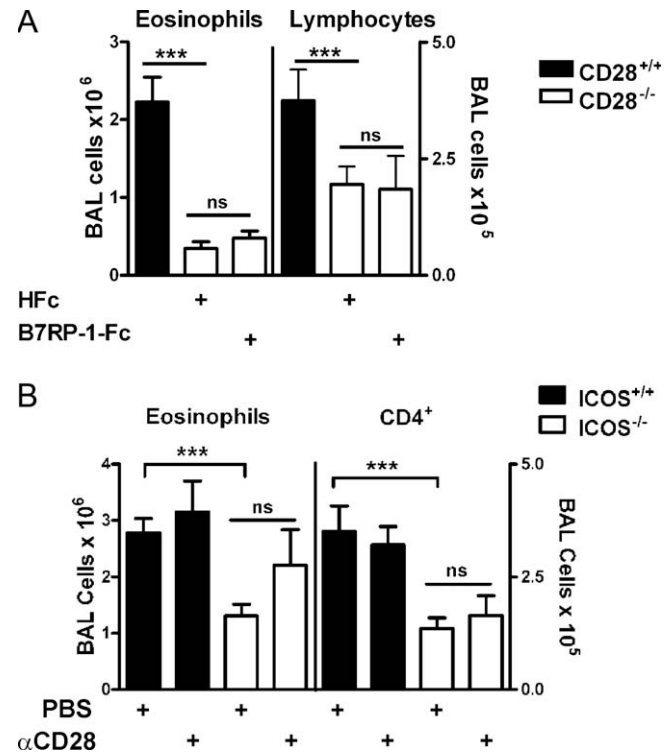


Fig. 2. Responses of CD28^{-/-} and ICOS^{-/-} mice to ICOS or CD28 co-stimulation in *S. mansoni* model of Th2-mediated airway inflammation. Wild-type, CD28^{-/-} or ICOS^{-/-} mice were sensitized and challenged with *S. mansoni*. (A) BAL cell counts from CD28^{+/+} mice (black bars) or CD28^{-/-} mice (open bars) treated every other day i.p. with either control 25 μg HuFc or 50 μg B7RP1-Fc. Data are from two combined experiments that are representative of four independent experiments. (B) BAL cell counts from ICOS^{+/+} (black bars) or ICOS^{-/-} mice (open bars) injected i.p. on day 0 with either 100 μg anti-CD28 or PBS. The data were combined from three independent experiments.

have significant airway inflammation as previously shown (Fig. 2A) [8]. In the absence of CD28, ICOS costimulation can only induce a small, although significant, increase in serum IgE (Fig. 4A). We were unable to detect a significant difference in other isotypes measured, IgG1 or IgG2a (data not shown). Our data show that CD28 is necessary for Th2-mediated airway inflammation and increased ICOS costimulation with B7RP1-Fc cannot overcome the defects in CD28^{-/-} mice.

3.3. CD28 cannot augment Th2 airway eosinophilia in the absence of ICOS

We have previously shown that ICOS blockade decreases airway inflammation in our model [10]. We found that ICOS^{-/-} mice have significantly less airway eosinophilia and CD4⁺ T cells in the BAL after sensitization and challenge (Fig. 2B), confirming that ICOS affects the severity of the response in our model. We hypothesized that augmenting CD28 costimulation may provide an additive signal for T cell activation that would overcome the need for ICOS. We augmented CD28 costimulation by injecting one dose i.p. of anti-CD28 (PV-1) at the time of sensitization in ICOS^{-/-} and ICOS^{+/+} mice compared to controls treated with PBS [29]. Anti-CD28 treatment of ICOS^{-/-} mice did not significantly increase eosinophils or CD4⁺ T cells in the BAL compared to control ICOS^{-/-} mice (Fig. 2B). This was not due to the inability of ICOS^{-/-} T cells to express CD28 (Suppl. Fig. S2). In addition, no differences were detected in peribronchial and perivascular inflammation with anti-CD28 treated ICOS^{-/-} mice compared to control ICOS^{-/-} mice (Fig. 3C, D). Our data suggest that for Th2-mediated airway inflam-

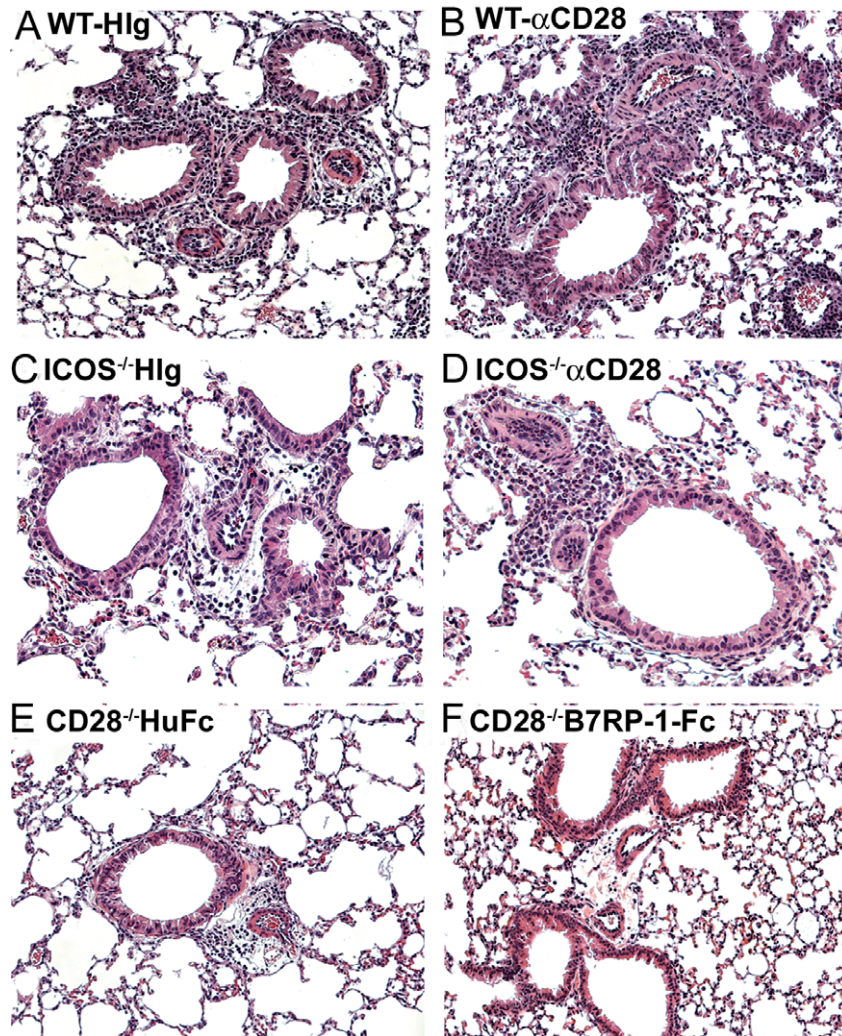


Fig. 3. Augmenting ICOS costimulation does not affect airway inflammation in the absence of CD28. Representative H&E staining from lungs of mice sensitized and challenged with *S. mansoni*. (A, B) Wild-type (WT) mice treated with control Hlg (A) or anti-CD28 (B). (C, D) ICOS^{-/-} mice treated with Hlg (C) or anti-CD28 (D). (E, F) CD28^{-/-} mice treated with HuFc (E) or B7RP-1-Fc (F).

mation ICOS deficiency cannot be overcome by augmenting CD28 costimulation.

3.4. CD28 cannot fully compensate for ICOS for germinal center formation or Ig production

Since ICOS has been found to play an essential role in humoral immunity, we also investigated if increased CD28 costimulation would compensate for ICOS for Th2-mediated isotype switching and germinal center formation. After sensitization and challenge with *S. mansoni*, anti-CD28 significantly increased total serum IgE and antigen-specific IgG1 in ICOS^{-/-} mice (Fig. 4B, C). However the levels of serum IgE in ICOS^{-/-} mice were still significantly decreased compared to ICOS^{+/+} mice. To further investigate the ability of anti-CD28 to overcome the defects in the ICOS^{-/-} mice the immunoglobulin response after sensitization with *S. mansoni* only (without challenge) was tested. We found that anti-CD28 significantly augmented total IgE and SEA-specific IgG1 in ICOS^{-/-} mice compared to isotype control antibody or PBS treated ICOS^{-/-} mice (Suppl. Fig. S3). To more fully examine antigen-specific responses, we sensitized mice with ovalbumin in alum (Fig. 5). Anti-CD28 significantly augmented OVA-specific IgG1, IgG2a, IgG2b and IgE (Fig. 5). The OVA-specific IgG2a and IgG2b responses were relatively small consistent with a Th2 type response. However, we

found that the increased OVA-specific IgE and IgG1 levels in ICOS^{-/-} mice treated with anti-CD28 were significantly less than wild-type mice given anti-CD28 (Fig. 5). These differences could not be overcome by increasing the dose of anti-CD28 (Suppl. Fig. S3).

To determine if augmenting either ICOS or CD28 costimulation in the absence of the other was affecting germinal center formation, spleens were cryosectioned and stained for germinal center B cells using peanut agglutinin (PNA). As previously found, CD28^{-/-} mice had almost no discernible germinal centers by PNA staining and treatment with B7RP-1-Fc had no detectable effect (data not shown) [14]. While ICOS^{-/-} control mice had few germinal centers (Fig. 6), anti-CD28 did induce an increase in the size of germinal centers in ICOS^{-/-} mice (Fig. 6 C, D, and quantified in G). As with serum IgE, the level of response with anti-CD28 in ICOS^{-/-} mice was not as large as in ICOS^{+/+} mice (Fig. 6A, D and G). Increasing the dose of anti-CD28 also did not abrogate the differences in germinal center size found between ICOS^{+/+} or ICOS^{-/-} mice (Fig. 6H).

3.5. CD28 cannot compensate for ICOS in the development of T_{FH}

One possible mechanism by which T cell costimulation may regulate antibody responses is through differentiation of

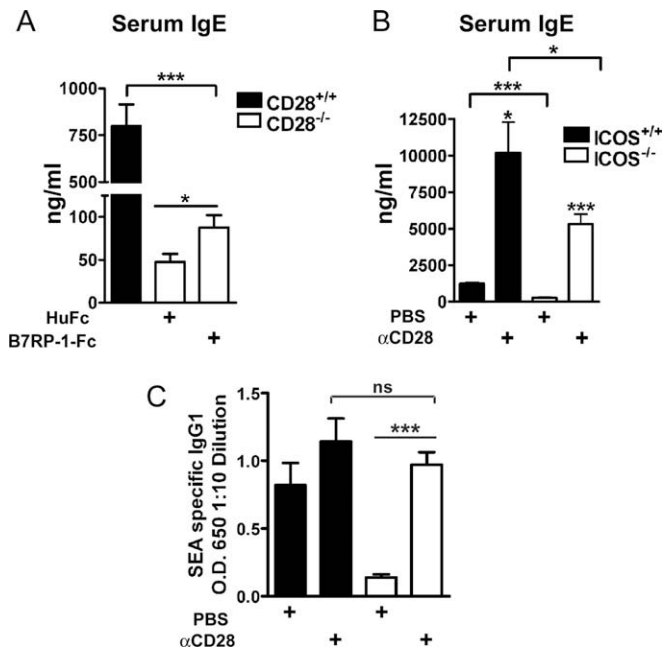


Fig. 4. After sensitization and challenge, anti-CD28 significantly increased serum IgE and antigen-specific IgG1 in $ICOS^{-/-}$ mice. Serum IgE or SEA specific IgG1 was measured on day 11 after sensitization and challenge with *S. mansoni*. (A) Serum IgE from $CD28^{+/+}$ mice ($n = 12$) or $CD28^{-/-}$ mice injected i.p. every other day with HuFc ($n = 15$) or B7RP-1-Fc ($n = 13$). (B, C) Serum IgE (B) and SEA specific IgG1 (C) from $ICOS^{+/+}$ ($n = 3$ each condition) or $ICOS^{-/-}$ mice ($n = 9$ each condition) treated i.p. with 100 μ g anti-CD28 or PBS once at the time of sensitization.

$CD4^{+}CXCR5^{+}$ follicular B helper T cells (T_{FH}). ICOS has previously been associated with the generation and function of these specialized T cells [19–21,30]. Since $ICOS^{-/-}$ mice have been found to be deficient in the number of T_{FH} in vivo, we investigated whether CD28 costimulation could augment the number of T_{FH} in the absence of ICOS [21,30]. Mice were sensitized i.p. with *S. mansoni* and treated one time i.p. with either anti-CD28 or control hamster Ig. Anti-CD28 treatment significantly increased the percentage of $CD4^{+}$ T cells expressing CXCR5 in the spleens of both $ICOS^{-/-}$ and $ICOS^{+/+}$ mice peaking at day 7 (Fig. 7A and Suppl. Fig. S4). However, at day 14 post-sensitization the number of $CD4^{+}CXCR5^{+}$ T cells in $ICOS^{-/-}$ mice treated with anti-CD28 was significantly less than anti-CD28 treated $ICOS^{+/+}$ mice and was not significantly different than control $ICOS^{-/-}$ mice (Fig. 7B). Consistent with previous work $ICOS^{-/-}$ control treated mice had significantly less $CD4^{+}CXCR5^{+}$ T cells than control treated $ICOS^{+/+}$ mice [31]. Anti-CD28 also significantly increased the numbers of $CD4^{+}$ T cells in $ICOS^{+/+}$ mice compared to $ICOS^{-/-}$ mice but had no effect on $CD19^{+}$ B cells (Suppl. Fig. S4 and data not shown). A significant increase in the percentage of $CD4^{+}$ T cells that had up-regulated CD44 was also found in both $ICOS^{-/-}$ and $ICOS^{+/+}$ mice (Suppl. Fig. S4). Thus, although we found a transient increase in the percentage of $CD4^{+}CXCR5^{+}$ T cells in $ICOS^{-/-}$ mice after anti-CD28 treatment, the response was not sustained and by day 14 there is no significant difference between anti-CD28 and control treated $ICOS^{-/-}$ mice. Our findings demonstrate CD28 cannot compensate for ICOS in the development of T_{FH} cells.

4. Discussion

In evaluation of the ability of CD28 and ICOS costimulation to compensate for each other in Th2-mediated immunity, we have found that CD28 is the dominant Th2 costimulatory molecule. Augmenting ICOS costimulation cannot overcome the requirement for CD28 to initiate a Th2 immune response. In contrast, CD28 can partially compensate for lack of ICOS in vivo. While, increased CD28 costimulation did not affect airway eosinophilia in $ICOS^{-/-}$ mice, anti-CD28 significantly increased antigen-specific IgG1 and IgE and germinal center formation in the absence of ICOS. However, the effects of anti-CD28 in the absence of ICOS were not as large as in wild-type mice. Further, anti-CD28 treatment failed to sustain an increase in T_{FH} . Our findings demonstrate that augmenting CD28 costimulation can only partially compensate for ICOS for Th2-mediated immunity and suggest that CD28 and ICOS have complementary non-overlapping roles.

Our prior data with ICOS blockade suggested augmenting ICOS costimulation with B7RP-1-Fc would increase Th2-mediated airway inflammation, but we did not find a significant increase in BAL eosinophilia with B7RP-1-Fc treatment or changes in lung histology in wild-type mice [10]. We did find a small but consistent increase in BAL lymphocytes predominantly $CD4^{+}$ T cells, as well as augmented Th2 cytokine production upon restimulation of lung and draining lymph node cells after B7RP-1-Fc treatment. Further, the most significant effect was on serum IgE. These data suggest that ICOS costimulation expands Th2 development. Our data are consistent with previous studies showing a role for ICOS in expanding Th2 responses in vivo [32,33]. Although we found an effect of B7RP-1-Fc on Th2 development in wild-type mice, B7RP-1-Fc did not affect airway inflammation in the absence of CD28. The lack of response was not due to an absence of ICOS expression in $CD28^{-/-}$ mice. It is perhaps not totally surprising that ICOS cannot compensate for loss of CD28, as CD28 is known to be important for T cell derived IL-2 and IL-4, as well as for survival of T cells [34,35].

While the development of Th2-mediated airway inflammation was dependent on CD28, we also found that $ICOS^{-/-}$ mice had a de-

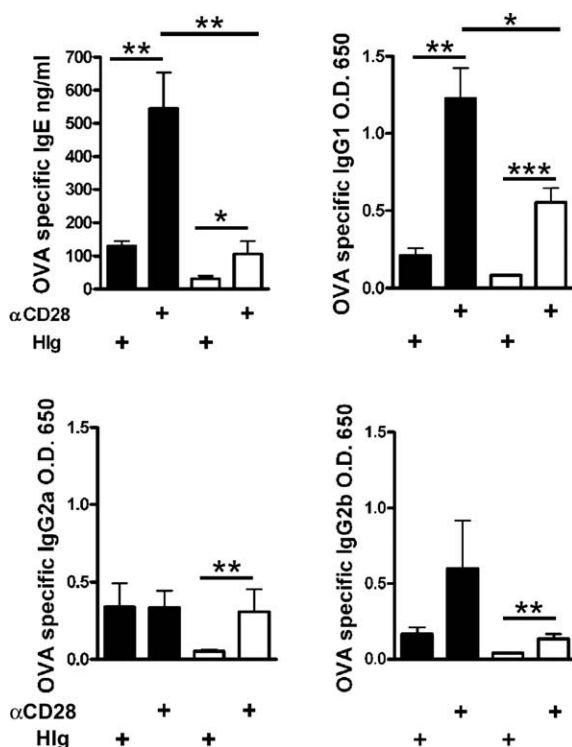


Fig. 5. CD28 cannot fully recover primary antigen-specific IgE and IgG1 responses in $ICOS^{-/-}$ mice. $ICOS^{+/+}$ (black bars) or $ICOS^{-/-}$ (white bars) mice were sensitized i.p. with ovalbumin in alum and treated i.p. on day 0 with either anti-CD28 or Hlg. OVA specific IgE, IgG1, IgG2a and IgG2b were measured on day 14 after sensitization with ova in alum. IgG1 and IgG2b levels were measured at 1:1000 dilution and IgG2a at 1:20.

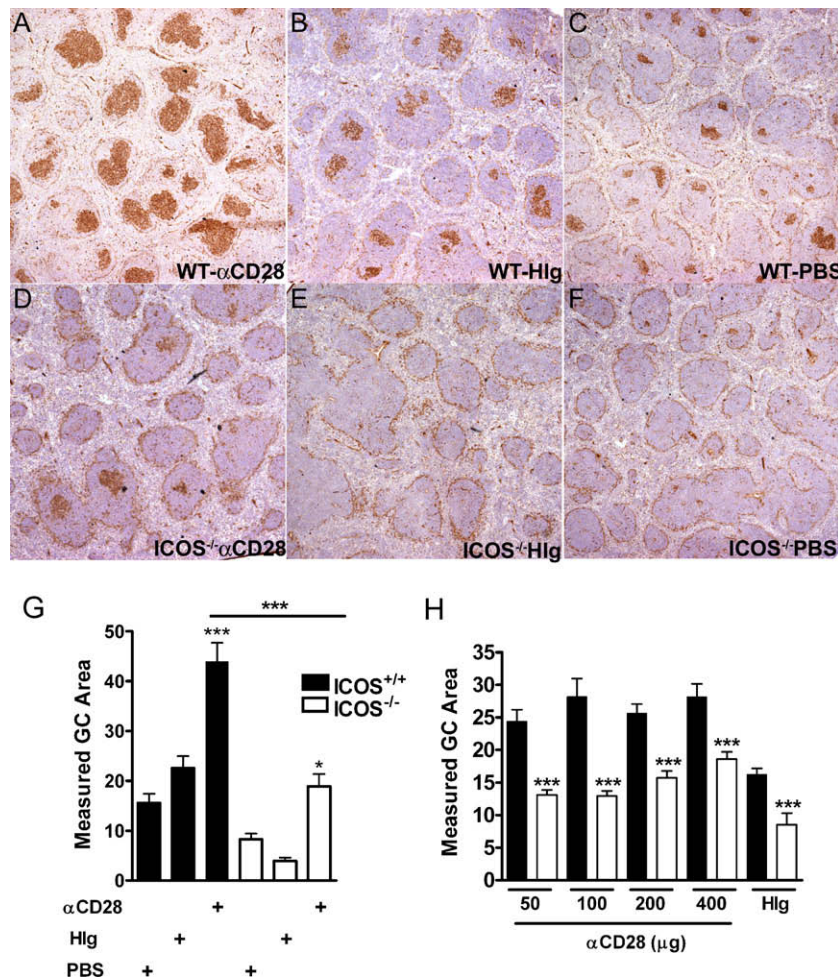


Fig. 6. Augmenting CD28 costimulation cannot fully recover germinal center formation in the absence of ICOS. (A–F) Spleens from ICOS^{+/+} (WT) (A–C) or ICOS^{-/-} mice (D–F) after sensitization with *S. mansoni* were stained with PNA for germinal center B cells. Mice received either anti-CD28 (A, D), Hlg (B, E) or PBS (C, F). All images are at 4× magnification. (G) Germinal center area was measured using Image J software on pictures of 4× magnification (3–4 mice per group were analyzed). Data are representative of two independent experiments. (H) Germinal center area was measured after treatment with 50, 100, 200, 400 μg of anti-CD28 or 400 μg of Hlg.

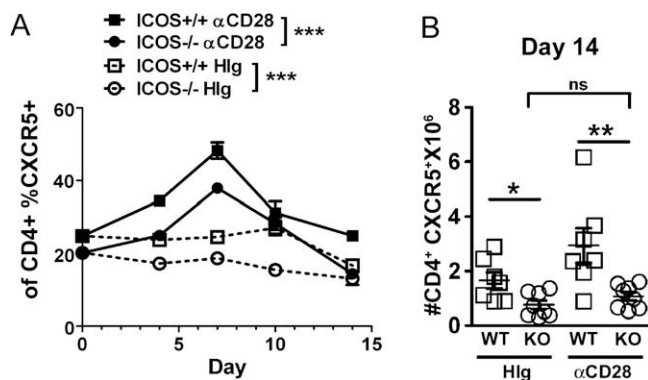


Fig. 7. Anti-CD28 cannot sustain an increase in CD4⁺CXCR5⁺ T cells (T_{FH}) in the absence of ICOS. After sensitization with *S. mansoni*, ICOS^{+/+} and ICOS^{-/-} mice injected i.p. on day 0 with anti-CD28 or Hlg were sacrificed on the days indicated. (A) Percentage of spleen CD4⁺CXCR5⁺ T cells in αCD28 or Hlg treated ICOS^{+/+} mice or ICOS^{-/-} mice. Significance determined by two way ANOVA as indicated in figure. (B) Number of CD4⁺CXCR5⁺ T cells found in the spleen on day 14.

fect in Th2-mediated airway inflammation with a significant reduction in BAL eosinophils and T cells. Previous reports in the literature on allergic airway inflammation in ICOS^{-/-} and B7RP-1^{-/-} mice have been conflicting [11,36,37]. The differences may be due

to the different models used and the variation in backcrossing the mice. In our study, we have used mice backcrossed more than nine generations that are compared to littermate controls. In addition, our model induces a very robust response in the BAL and lung compared to other models using ovalbumin and it is possible that our system is more sensitive to detect differences between ICOS^{-/-} mice and controls. Nevertheless the differences we found in the ICOS^{-/-} mice were only in the BAL and did not translate into significant differences in histology suggesting ICOS modifies the response but is not necessary for a Th2-mediated inflammatory response in the lung.

The most striking defects in Th2-mediated immunity in ICOS^{-/-} mice are in humoral immunity. Interestingly, we found that increased CD28 costimulation *in vivo* did increase immunoglobulin responses and germinal center formation in the absence of ICOS. The effect of anti-CD28 on ICOS^{-/-} mice was not as significant as in wild-type mice suggesting CD28 costimulation did not fully compensate for ICOS costimulation. While anti-CD28 initially increased the frequency of CD4⁺CXCR5⁺ T cells in ICOS^{-/-} mice there was also an effect on T cell activation signified by increased CD44 expression. CXCR5 is a marker for T_{FH} that can be upregulated on all T cells after activation [38]. By day 14 the effect of anti-CD28 on CD4⁺CXCR5⁺ T cells in ICOS^{-/-} mice was abrogated. Our findings suggest that ICOS plays a distinct role from CD28 in modulating the expansion of B cell responses which may be due to its effect on the

persistence of CD4⁺CXCR5⁺ T_{FH} cells. It is interesting that CD28 cannot fully compensate for ICOS *in vivo* since ICOS does not bind any unique signaling molecules from CD28. ICOS like CD28 is known to upregulate phosphoinositide-3 kinase (PI-3K) upon T cell activation but unlike CD28, PI-3K is the only known binding partner for ICOS [9,39]. However, ICOS has been shown to upregulate PI-3K to a greater extent than CD28 [40]. Thus, ICOS costimulation may provide a qualitatively different signal through PI-3K than CD28 or perhaps another binding partner has yet to be identified.

Our study demonstrates that the requirement for ICOS can at least in part be overcome by augmenting CD28 costimulation and suggest that some of the differences in the literature regarding ICOS may be due to the strength of the aggregate costimulation signal induced *in vivo*. These findings may have implications for immunotherapy for humans. Individuals who are deficient for ICOS suffer from humoral immunodeficiency [16,18]. Our work suggests anti-CD28 may benefit these individuals, as well as be useful for boosting responses to vaccines. However, the recent trial of a humanized superagonist anti-CD28 antibody with the deleterious consequence of inducing the systemic inflammatory response syndrome and multi-system organ failure make the current formulation of anti-CD28 for humans unacceptable [41]. Further, the fact that anti-CD28 cannot sustain the T_{FH} response supports the essential role for ICOS in humoral immunity. Future work to determine the downstream signals of ICOS costimulation in T cells may provide novel targets for therapies for autoimmune diseases and allergies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.cellimm.2009.06.013](https://doi.org/10.1016/j.cellimm.2009.06.013).

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