Immunity
Article

Roquin Differentiates the Specialized Functions of Duplicated T Cell Costimulatory Receptor Genes *Cd28* and *Icos*

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DOI 10.1016/j.immuni.2008.12.015

SUMMARY

During evolutionary adaptation in the immune system, host defense is traded off against autoreactivity. Signals through the costimulatory receptor CD28 enable T cells to respond specifically to pathogens, whereas those through the related costimulatory receptor, ICOS, which arose by gene duplication, are critical for affinity maturation and memory antibody responses. ICOS ligand, unlike the pathogen-inducible CD28 ligands, is widely and constitutively expressed in the immune system. Here, we show that crosstalk between these two pathways provides a mechanism for obviating the normal T cell dependence on CD28. Several CD28-mediated responses—generation of follicular helper T cells, germinal center formation, T helper 1 cell-dependent extrafollicular antibody responses to *Salmonella* and bacterial clearance, and regulatory T cell homeostasis—became independent of CD28 and dependent on ICOS when the E3 ubiquitin ligase Roquin was mutated. Mechanisms to functionally compartmentalize ICOS and CD28 signals are thus critical for two-signal control of normal immune reactions.

INTRODUCTION

The appearance of high-affinity memory antibody responses through B cell selection in germinal centers in phylogeny—in birds and mammals—coincides with the appearance of the inducible T cell costimulator (*Icos*) gene (Bernard et al., 2007). ICOS is critical in man and mouse for this specialization of the antibody response (Bossaller et al., 2006; Dong et al., 2001; Grimbacher et al., 2003; McAdam et al., 2000; Tafuri et al., 2001), and it is most highly expressed on a specialized subset of effector $CD4^+$ T cells within the germinal center, T follicular helper (Tfh) cells (Hutloff et al., 1999). Phylogeny, sequence homology, and genomic localization indicate that *Icos* emerged by duplication of the adjacent and evolutionarily more ancient gene, *Cd28*, encoding a general T cell costimulatory receptor (Bernard et al., 2007). Genomic analysis has indicated that gene duplication provides an important substrate for evolution (Ohno et al., 1968), but little is known about how duplicated paralogous genes resolve adaptive conflicts while acquiring specialized functions that confer a selective advantage. This general question of conflict between paralogs is particularly acute in the case of *Cd28* and *Icos* because substitution by ICOS for the role normally played by CD28 has the potential to violate the two-signal mechanism of discrimination between pathogens and self.

Little is known about the extent to which ICOS can substitute for CD28 to promote in vivo immune responses. This question becomes particularly important in a context in which the CD28 system is not active: a response against self. Biochemically, both *Cd28* and *Icos* encode T cell costimulatory receptors sharing 39% amino acid identity and, in transformed cell lines, largely overlapping capacity to activate the PI3K intracellular signaling pathway and enhance T cell receptor (TCR)-induced gene expression, cytokine synthesis, and cytokine proliferation (Rudd and Schneider, 2003). Some biochemical specialization exists: CD28 is the main inducer of IL-2, whereas ICOS is a poor IL-2 inducer because of its inability to recruit the signaling adaptor Grb2 (Harada et al., 2003; Watanabe et al., 2006). Also, ICOS lacks a unique motif present within the polyproline cytoplasmic domain of CD28; such a motif is specific for binding the protein tyrosine kinase Lck and important for CD28-dependent Treg cell differentiation in the thymus (Tai et al., 2005). In addition to this biochemical specialization, CD28 and ICOS exhibit highly specialized patterns of expression. CD28 is constitutively expressed on most T cells, whereas ICOS is expressed at very low amounts on naive T cells, is upregulated in all effector and memory T cell subsets, including Treg cells, and is most highly expressed on the specialized Tfh cells located within germinal centers (Beier et al., 2000; Gross et al., 1992; Hutloff et al., 1999).

CD28 has a well-understood role in T cell discrimination of self and microbial antigens because microbial antigens are accompanied by ligands for pathogen-recognition receptors such as those of the Toll-like receptor family, which induce expression

of the CD28 ligands, CD80 and CD86 (Greenwald et al., 2005; Sharpe and Freeman, 2002). Self-antigen peptides presented in the absence of CD80 and CD86 signaling engage the TCR, but not CD28, inducing tolerance responses such as anergy or apoptosis (Harding et al., 1992; Matzinger, 1994). Simultaneous delivery of two signals—engagement of the TCR by antigenic peptide-major histocompatibility complex (MHC) complexes and engagement of CD28 by CD80 and CD86—delivers a qualitatively and quantitatively enhanced intracellular signal to induce T cell immune responses selectively in cells that recognize microbial antigens (Janeway and Bottomly, 1994; Lafferty et al., 1980; Matzinger, 1994). By contrast with CD80 and CD86, ICOS ligand (ICOSL) is widely and constitutively expressed in the absence of microbial stimuli on antigen-presenting cells and several nonhemopoietic tissues, including endothelium (Brodie et al., 2000; Ling et al., 2000; Swallow et al., 1999). Should ICOS retain overlapping costimulatory function sufficient to substitute for CD28, the constitutive presence of ICOSL could bypass the need for microbial induction of costimulatory ligands. Although this might be a benefit in the case of processed microbial antigens that are retained in an inert state for months or years within germinal centers, it would potentially undo a key self-pathogen discrimination mechanism and lead to autoimmunity.

Analysis of mice and humans lacking ICOS, CD28, or both has emphasized the specialization of these two costimulatory receptors. T cell-dependent immunity is dramatically depressed in *Cd28*-deficient mice (Shahinian et al., 1993), which show a complete absence of T cell-dependent germinal centers and Tfh cells, impaired extrafollicular antibody responses to *Salmonella* coupled with reduced pathogen clearance (McSorley and Jenkins, 2000; Mittrucker et al., 1999), and a dramatic (>80%) reduction in Treg cells (Ferguson et al., 1996; Salomon et al., 2000; Tai et al., 2005; Tang et al., 2003; Walker et al., 1999). By contrast, *Icos*$^{-/-}$ mice remain competent to form germinal center B cells, Tfh cells, and Treg cells, albeit in reduced numbers (Akiba et al., 2005; Bossaller et al., 2006; Burmeister et al., 2008; Dong et al., 2001; McAdam et al., 2000; Tafuri et al., 2001). Once germinal centers are established, CD28 is dispensable for Tfh cell and germinal center survival and selection of somatically mutated B cells into the memory pool (Walker et al., 2003), but these processes are profoundly impaired in mice and humans that lack ICOS or ICOSL, who have a near-total absence of memory B cells (Bossaller et al., 2006; Dong et al., 2001; Grimbacher et al., 2003; McAdam et al., 2000; Tafuri et al., 2001). Although combined deficiency of CD28 and ICOS leads to a more severe defect in T cell-dependent antibody response than deficiency of either alone (Suh et al., 2004), this is consistent with sequential specialized roles and does not resolve the question of whether ICOS might substitute for CD28 in either a beneficial or harmful way.

The mechanisms that control expression and activity of CD28, ICOS, and their ligands are only beginning to be revealed. Here, we address the question of specialization versus overlap between ICOS and CD28 and the consequences of changes in their control mechanisms by analyzing an autoimmune mouse mutant, *sanroque*, with a defect in the *Rc3h1* gene encoding the E3 ubiquitin ligase Roquin, that does not affect the sequence of *Cd28* or *Icos* but alters the stability of *Icos* messenger RNA (mRNA) (Li et al., 2007; Vinuesa et al., 2005a; Yu et al., 2007). We show that ICOS can indeed substitute for CD28 in the induction of in vivo immune responses, including primary antibody responses, germinal center B cell formation, and homeostasis of Foxp3^{+} Treg cells. These overlapping functions of the ICOS costimulatory receptor are normally compartmentalized by Roquin's control on *Icos* mRNA, so that the important role of CD28 in self-pathogen discrimination is not normally overturned by ICOS. Pathological breakdown of this compartmentalization may be important in autoimmune disease, whereas controlled breakdown in Tfh cells may be a physiological adaptation to assist selection of high-affinity antibodies in germinal centers.

RESULTS

Rc3h1$^{san/san}$ T Cells Overexpress ICOS in the Absence of CD28

Homozygosity for the M199R "*Rc3h1*$^{san/san}$" mutation in Roquin causes aberrant overexpression of ICOS on the surface of both naive T cells and memory and effector T cells (Vinuesa et al., 2005a). Signaling through CD28 during T cell priming has previously been shown to be essential for optimal upregulation of ICOS (McAdam et al., 2000). To examine whether signaling through CD28 is necessary for the overexpression of ICOS by *Rc3h1*$^{san/san}$ T cells, we generated *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice and used flow cytometry to assess ICOS expression on peripheral blood CD44^{lo} (naive) and CD44^{hi} (effector and memory) CD4^{+} T cells. CD28 deficiency only exerted a 30% correction of ICOS expression on *Rc3h1*$^{san/san}$ T cells. The expression of ICOS on naive CD4^{+} T cells and effector and memory CD4^{+} T cells was still more than 2-fold higher in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice compared with *Rc3h1*$^{+/+}$ mice (Figures 1A and 1B), indicating that the M199R substitution in Roquin uncouples ICOS expression from CD28-driven T cell activation.

As shown previously, ICOS is hyperinduced in activated CD44^{hi} cells in *Rc3h1*$^{san/san}$ mice (Figure 1B; ICOS mean fluorescence intensity [MFI] in naive CD44^{lo} cells = 35.90 ± 9.50; ICOS MFI in activated cells = 131.00 ± 13.00). This hyperinduction is also seen after activation of *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice (Figure 1B): Indeed, a comparable ~4-fold increase in ICOS expression is seen in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ activated cells (ICOS MFI = 91.15 ± 12.45) compared with naive cells from the same mice (ICOS MFI = 21.80 ± 1.80). These data indicate that ICOS is still overexpressed on *Rc3h1*$^{san/san}$ CD4^{+} T cells lacking CD28.

Mutant Roquin Rescues Germinal Center Formation in CD28-Deficient Mice

Given the ability of *Rc3h1*$^{san/san}$ CD4^{+} T cells to express ICOS independently of CD28 and the potential for ICOS to transmit comparable costimulatory signals to CD28, we asked whether the *Rc3h1*$^{san/san}$ mutation would restore CD28-dependent antibody responses in the absence of CD28. Signaling through CD28 has been shown to be essential for the initiation of T cell-dependent antibody responses and formation of germinal centers (Ferguson et al., 1996; Walker et al., 2003). Previously, it has been shown that *Rc3h1*$^{san/san}$ mice form spontaneous germinal centers but mount normal antibody responses to foreign antigens (Vinuesa et al., 2005a) (Figure 2A). We therefore examined the CD28 dependence of the antibody response to a model

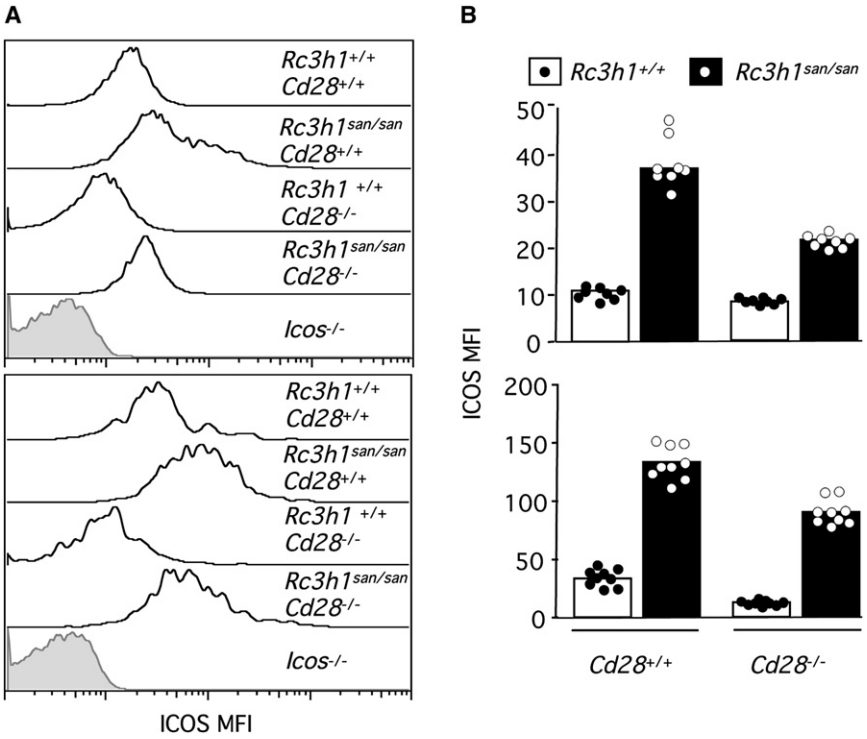

Figure 1. Increased ICOS Expression on Naive and Effector-Memory *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ CD4$^+$ T Cells

(A) Representative flow-cytometric histograms showing ICOS MFI of CD4$^+$CD44^{lo} (top panel) or CD4$^+$CD44^{hi} (bottom panel) on peripheral blood T cells from mice with the indicated genotypes. Specificity of ICOS staining is determined by the use of *Icos*$^{-/-}$ mice as a control.

(B) Bar graphs show ICOS MFI for the mice shown in (A). Each circle represents one mouse, and bars represent the median values for each group. ICOS MFI is different between all groups in the CD44^{hi} analysis, with a statistical significance of $p < 0.0001$. Within the CD44^{lo} comparison, all groups also have a statistically significant difference of $p < 0.0001$, with the exception of *Rc3h1*$^{+/+}$ *Cd28*$^{+/+}$ versus *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$, in which significance reached $p = 0.0011$. Data are representative of four experiments.

protein antigen—chicken gammaglobulin (CGG). Whereas anti-CGG IgG1 was undetectable in *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ mice 14 days after immunization, *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice mounted a robust antibody response, albeit lower than that seen in mice expressing CD28 (Figure 2A). Thus, the *Rc3h1*$^{san/san}$ mutation relieves the dependence on CD28 for this antibody response.

To analyze the cellular basis for the *Rc3h1*$^{san/san}$ effect on CD28 dependence, we next examined the formation of germinal centers 8 days after intraperitoneal immunization with sheep red blood cells (SRBCs). Consistent with previous reports, CD28-deficient mice failed to form GL-7$^+$ CD95$^+$ B220$^+$ cells after immunization (Figure 2C), and we were unable to observe PNA staining in the IgD$^+$ B cell follicles (Figure 2D). The absence of CD28 eliminated the formation of spontaneous germinal centers in unimmunized *Rc3h1*$^{san/san}$ mice (Figure 2B), but remarkably, *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice mounted robust germinal center responses upon SRBC immunization that were greater than those of immunized wild-type (*Rc3h1*$^{+/+}$ *Cd28*$^{+/+}$) mice (Figure 2E).

The *Rc3h1*$^{san/san}$ mutation could relieve the CD28 dependence of germinal center responses either by acting within the B cells or in helper T cells or other cells needed to support the B cell expansion. To distinguish between these alternatives, we made mixed bone marrow chimeras in which sublethally irradiated C57BL/6 Ly5a recipients were reconstituted with a 1:1 mix of *Rc3h1*$^{san/san}$.Ly5b and *Rc3h1*$^{+/+}$.Ly5a bone marrow or a control *Rc3h1*$^{+/+}$.Ly5b and *Rc3h1*$^{+/+}$.Ly5a mix. Analysis of these chimeras after SRBC immunization revealed that the increase in germinal center B cells observed in recipients of *Rc3h1*$^{san/san}$.Ly5b: *Rc3h1*$^{+/+}$.Ly5a mixed bone marrow consisted of nearly equal cell numbers of Roquin wild-type (Ly5a) and Roquin mutant (Ly5b) origin. This indicates that most of the increase in germinal center response caused by the *Rc3h1*$^{san/san}$ mutation is B cell extrinsic (Figure 2F).

In order to conclusively confirm that the *Rc3h1*$^{san/san}$ mutation rescued selection of antigen-specific germinal center B cells in *Cd28*$^{-/-}$ mice, and that this is not due to B cell intrinsic factors, we used the "SW$_{HEL}$" B cell transfer system (Paus et al., 2006; Phan et al., 2003). C57BL/6 B cells carrying a "knocked-in" B cell receptor specific for hen egg lysozyme (HEL) were injected into the tail vein of recipient mice together with SRBCs conjugated to HEL^{2x}, and the HEL-specific germinal center response by the donor cells was assessed 5 days after immunization (Figure 3A). In recipients with normal Roquin, the absence of CD28 diminished the germinal center response by SW$_{HEL}$ B cells to ~2% of that in CD28-sufficient hosts, confirming that this process is normally CD28 dependent. By contrast, CD28-deficient recipients with mutant Roquin were capable of supporting the formation of immunogen-specific germinal center B cells equally to *Rc3h1*$^{+/+}$*Cd28*$^{+/+}$, *Rc3h1*$^{san/san}$*Cd28*$^{+/+}$, and *Rc3h1*$^{san/san}$ mice (Figure 3B). Taken together, these results indicate that *Rc3h1*$^{san/san}$ can rescue the germinal center response in CD28-deficient animals through a B cell-extrinsic mechanism.

The Sanroque Mutation in Roquin Restores the Number of Tfh Cells in CD28-Deficient Mice

The absence of germinal centers in *Cd28*$^{-/-}$ mice is linked with deficient T cell priming and the absence of differentiated helper CD4$^+$ T cells, including Tfh cells (Walker et al., 1999). Tfh cells provide selection signals to somatically mutated germinal center B cells that have increased their affinity for the immunizing antigen, signals that cause them to differentiate into long-lived antibody-secreting plasma cells or memory B cells. Tfh cells are also required for maintaining germinal center reactions, possibly through enabling the recycling of selected germinal center B cells to undergo further rounds of division and somatic hypermutation (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000; Vinuesa et al., 2005b).

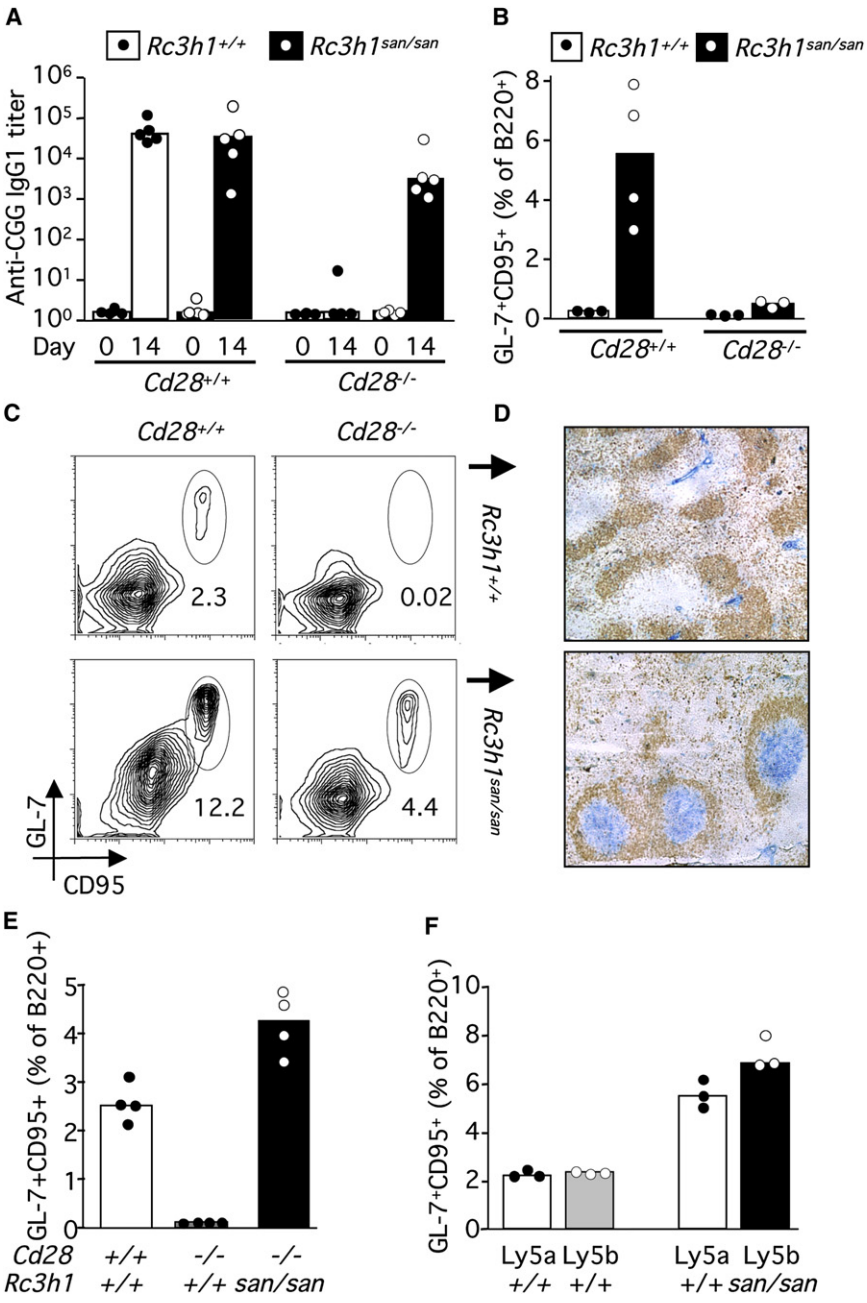

Figure 2. *Rc3h1*$^{san/san}$ Rescues Germinal Center Responses in *Cd28*$^{-/-}$ Mice

(A) Anti-CGG IgG1 antibody titers 0 and 14 days after immunization measured by ELISA in *Cd28*$^{+/+}$ or *Cd28*$^{-/-}$ mice that are either *Rc3h1*$^{+/+}$ (white bars) or *Rc3h1*$^{san/san}$ (black bars).

(B) Germinal center B cells (B220^{+} GL-7^{+} CD95^{+}) expressed as a percentage of total B220^{+} cells from unimmunized *Cd28*$^{+/+}$ or *Cd28*$^{-/-}$ mice that are either *Rc3h1*$^{+/+}$(white bars) or *Rc3h1*$^{san/san}$ (black bars).

(C) Flow-cytometric contour plots showing GL-7 versus CD95 staining gated on B220^{+} cells from mice with the indicated genotypes 8 days after SRBC immunization. Oval gates show the germinal center B cell subset (GL-7^{+} CD95^{+}).

(D) Photomicrographs of frozen spleen sections stained with the germinal center marker PNA (blue) and IgD (brown) in *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ (top) and *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ (bottom) 8 days after SRBC immunization.

(E) Bar graph showing the percentage of germinal center B cells gated with the gates shown in (C) in mice with the indicated genotypes 8 days after SRBC immunization.

(F) Germinal center B cell percentages from 1:1 bone marrow chimeras 8 days after SRBC immunization. The left panel shows results for three recipients reconstituted 12 weeks earlier with a 1:1 mix of *Rc3h1*$^{+/+}$.Ly5a and *Rc3h1*$^{+/+}$.Ly5b bone marrow; the right panel shows results for three recipients reconstituted 12 weeks earlier with a 1:1 mix of *Rc3h1*$^{+/+}$.Ly5a and *Rc3h1*$^{san/san}$.Ly5b bone marrow. In all bar graphs, each circle represents one mouse, and bars represent the median values for each group. Data are representative of two (A) or four (B–F) experiments.

We assessed Tfh numbers by enumerating CXCR5^{+} CD4^{+} cells that also expressed the inhibitory receptor PD-1. Human Tfh cells are known to express high amounts of PD-1 mRNA (Chtanova et al., 2004). Several groups, including our own, have identified Tfh cells in mice in the past, only on the basis of CXCR5 expression on CD4^{+} T cells. Nevertheless, discrimination of Tfh cells on the basis of CXCR5 staining alone is unsatisfactory because of the relatively low expression of this chemokine receptor on T cells and the nonselective induction of CXCR5 in vitro in all primed T cells. Analysis of follicular T cells by immunohistochemistry revealed that PD-1 is in fact the best marker when used alone for specifically identifying germinal center T cells, with minimal PD-1 staining observed anywhere else in the spleen (Figure 4A) (Haynes et al., 2007). In fact, most TCRβ^{+} T cells in germinal centers are PD1^{+}. Also, combining CXCR5 with PD-1 provides better discrimination of this subset by flow cytometry than using CXCR5 alone (Figure 4B).

Unimmunized *Rc3h1*$^{san/san}$ mice accumulate Tfh cells; this is partially corrected in CD28-deficient *Rc3h1*$^{san/san}$ mice, which have background Tfh cell levels comparable to those of wild-type mice, although these are still 4-fold higher than those seen in *Cd28*$^{-/-}$ mice (Figure 4C). This subset expands even further after immunization in both *Rc3h1*$^{san/san}$ mice and control littermates (Figure 4D). To determine whether the *Rc3h1*$^{san/san}$ mutation could correct the deficiency in Tfh cells observed in *Cd28*$^{-/-}$ mice, we assessed the number and percentage of splenic Tfh cells (CD4^{+}CXCR5^{+}PD-1^{+}) by flow cytometry 8 days after SRBC immunization. Both the percentage and absolute number of Tfh cells were restored in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice to levels comparable to those of wild-type mice (Figures 4D and 4E). These data show that *Rc3h1*$^{san/san}$ can restore Tfh cell formation in CD28-deficient animals.

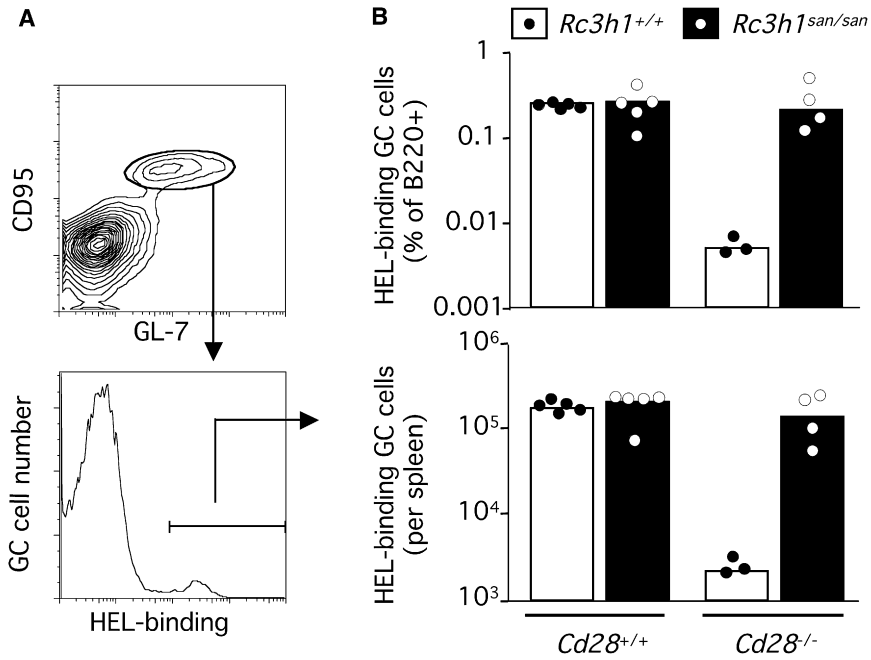

Figure 3. *Rc3h1*$^{san/san}$ Restoration of the Germinal Center Response in *Cd28*$^{-/-}$ Mice Is B Cell Extrinsic

(A) Gating strategy used for identifying the HEL-specific germinal center response from transferred SW_{HEL} B cells. Cells in the top panel have been gated on a live lymphocyte gate and on B220.

(B) Bar graphs showing HEL-specific donor-derived germinal center B cells as a proportion of B220$^+$ cells (top panel) or the total number per spleen (bottom panel) 5.5 days after immunization with HEL^{2x} SRBCs in mice with the indicated genotypes. Each symbol represents one mouse. Bars represent the median values in each group. Data are representative of two experiments.

Rc3h1$^{san/san}$ Breaks Compartmentalization between CD28L and ICOSL in Germinal Center Responses

We speculated that Roquin ensures the compartmentalization between CD28 ligands and ICOS ligands. Nevertheless, an alternative second hypothesis explaining the observed results could be that *Rc3h1*$^{san/san}$ dysregulates signaling downstream from CD28; in other words, *Rc3h1*$^{san/san}$ may allow for signaling through the CD28 pathway in the absence of CD28. If this were the case, removing ICOSL would not have an effect. To test this hypothesis, we generated *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ mice. The SRBC-induced germinal center response in these triple-loci-altered animals was 75% reduced compared to that in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice, indicating that the rescue of the germinal center response is largely dependent on intact signaling through ICOS-ICOSL and that a response that is normally ICOSL independent has become ICOSL dependent in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice. *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ mice were still capable of forming a small number of GL-7$^+$ CD95$^+$ B220$^+$ cells (Figure 4F), suggesting that ICOS- and CD28-independent pathways also contribute to the *Rc3h1*$^{san/san}$-mediated rescue of the germinal center response in CD28-deficient mice. We next assessed the contribution of signaling through ICOS to the correction of Tfh cell numbers in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice. Similar to the germinal center B cell response, the percentage and number of Tfh cells was 4-fold lower in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ mice 8 days after SRBC immunization (Figure 4G). These findings support the idea that *Rc3h1*$^{san/san}$ breaks down the compartmentalization of signals through CD28 and ICOS and excludes the hypothesis that *Rc3h1*$^{san/sa}$ allows signaling through the CD28 pathway in the absence of ICOS.

We also considered the possibility that a defect in cytotoxic T lymphocyte antigen-4 (CTLA-4) signaling may contribute to the observed enhancement of germinal center reactions. Mice transgenic for a soluble CTLA-4-immunoglobin (Ig) fusion protein have been shown to form germinal centers when immunized in conjunction with a CD28 agonist. However, these germinal centers do not appear to involute, with the response still being present 40 days after immunization, indicating that CTLA-4 signaling helps terminate germinal center reactions (Walker et al., 2003). We investigated whether *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice show prolonged germinal center reactions typical of defective CTLA-4 signaling. *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ germinal centers peaked at approximately day 8 after SRBC immunization and, like controls, were already reduced at day 14, and only background numbers of cells were present 30 days after immunization (data not shown). This suggests that CTLA-4 is functioning normally in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice to induce the involution of germinal center reactions. CTLA-4 expression was also slightly elevated in activated *Roquin*$^{san/san}$ CD4$^+$ T cells and in *Rc3h1*$^{san/san}$ Treg cells (Figure S1 available online). Taken together, these data indicate that the *Rc3h1*$^{san/san}$-dependent rescue of germinal center and Tfh cell formation in CD28-deficient mice relies on signaling through the ICOS-ICOSL pathway.

Rc3h1$^{san/san}$ Breaks Down CD28L-ICOSL Discrimination to *Salmonella* Infection

To assess whether *Rc3h1*$^{san/san}$ can also break the compartmentalization of CD28 and ICOS in responses to infectious agents, we immunized mice with live attenuated *Salmonella enterica* serovar Dublin strain SL5631. The response to *Salmonella* has been shown to be critically dependent on CD4$^+$ T cells (Hess et al., 1996; Yrlid and Wick, 2000). Furthermore, CD28 costimulation appears to be essential for T helper 1 (Th1) cell-mediated bacteria clearance and intact IgG2a^b production (McSorley and Jenkins, 2000; Mittrucker et al., 1999). Although antibody production does not contribute to the resolution of a primary infection, it has been shown to be essential to protect against *Salmonella* reinfection (McSorley and Jenkins, 2000). An attractive feature of this model is that the IgG2a^b plasma cell response during the first weeks after the infection is exclusively extrafollicular, and germinal centers do not form until after 5 weeks postinfection (Cunningham et al., 2007). This also allows us to test whether Roquin plays a role in ensuring the

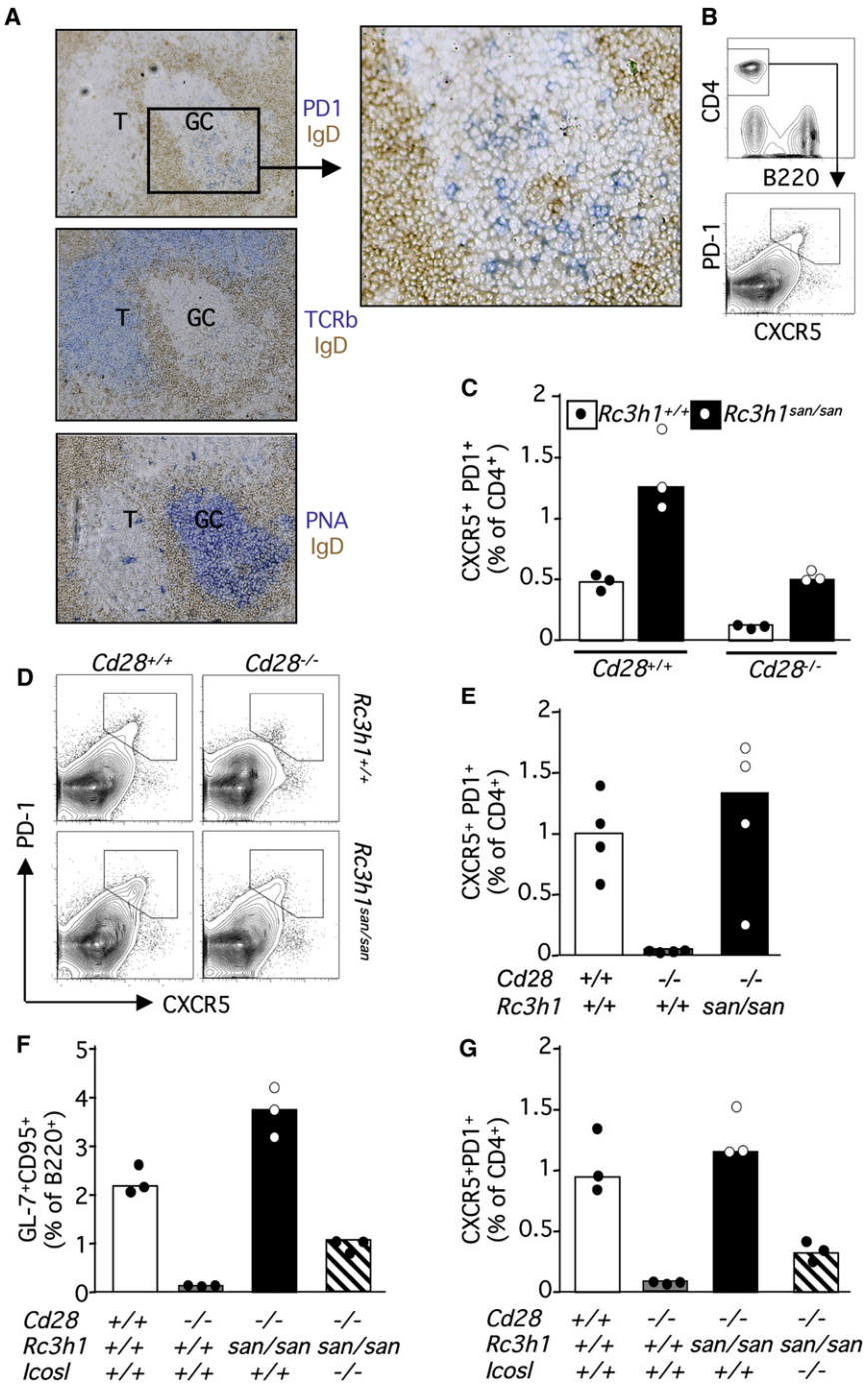

Figure 4. Restoration of Tfh Cell Formation and Germinal Centers in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ Mice Requires Signaling through the ICOS-ICOSL Pathway

(A) Photomicrographs of frozen spleen sections stained with IgD (brown), PD-1 (blue, top panel), TCRβ (blue, middle panel), and PNA (blue, bottom panel).

(B) Gating strategy for CD4$^+$B220$^-$CXCR5$^+$PD1$^+$ Tfh cells.

(C) Bar graphs show CD4$^+$CXCR5$^+$PD-1$^+$ (Tfh) cells as a percentage of splenic CD4$^+$ T cells assessed by flow cytometry from unimmunized mice with the indicated genotypes.

(D) Representative flow-cytometric contour plots showing PD-1 versus CXCR5 staining of mice with the indicated genotypes 8 days after immunization with SRBCs. Gates have been drawn around the CXCR5^{hi} PD1^{hi} population representing Tfh cells. A stringent cell-gating strategy allowed exclusion of dead cells, nonlymphoid cells, B cells, B:T cell duplets, and autofluorescent cells (Figure 1B).

(E) Bar graphs show CD4$^+$CXCR5$^+$PD-1$^+$ (Tfh) cells gated as shown in (B) as a percentage of splenic CD4$^+$ T cells from mice with the indicated genotypes 8 days after SRBC immunization.

(F) Percentage of germinal center B cells in mice with the indicated genotypes 8 days after SRBC immunization. Observed differences between all the genotype groups reached statistical significance of $p = 0.05$.

(G) Percentage of Tfh cells in mice with the indicated genotypes 8 days after SRBC immunization. Observed differences between all the genotype groups reached statistical significance of $p < 0.01$, with the exceptions of *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ versus *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ and *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ versus *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$, in which significance reached $p < 0.001$, and of *Roquin*$^{+/+}$ *Cd28*$^{+/+}$ versus *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$, in which no significant difference was observed. Data are representative of four experiments.

compartmentalization between CD28 ligands and ICOS ligands during extrafollicular antibody responses.

Twelve days after *Salmonella* infection, CD28-deficient mice had more than ten times higher bacterial counts in the liver (Figure 5A), and despite having intact anti-*Salmonella* IgM production (Figure 5B), they were unable to produce any *Salmonella*-specific IgG2a^b (Figure 5C) antibodies. Immunohistochemistry confirms previous work showing that this response is occurring outside the B cell follicle and that the number of CD138$^+$ cells is greatly reduced in CD28-deficient mice (Figure 5D). These defective responses were completely rescued in *Cd28*$^{-/-}$ mice homozygous for the *san* allele of *Rc3h1*. Again, this rescue was shown to be dependent on ICOSL substituting for CD28 ligands because bacterial counts and *Salmonella*-specific IgG2a^b titers in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ mice were comparable to those of *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ *Icosl*$^{+/+}$ mice (Figures 5A–5D).

A third alternative hypothesis that could explain the observed rescue is that even in the presence of intact CD28L signaling, there might be a role for ICOSL in the response. If this were the case, ICOSL deficiency would confer a phenotype in CD28$^{+/+}$ mice. To investigate this possibility, we also assessed the response in ICOSL-deficient mice. Unlike the defective responses observed in CD28$^{-/-}$ mice, these responses were mostly intact in ICOSL$^{-/-}$ mice, confirming that ICOSL does

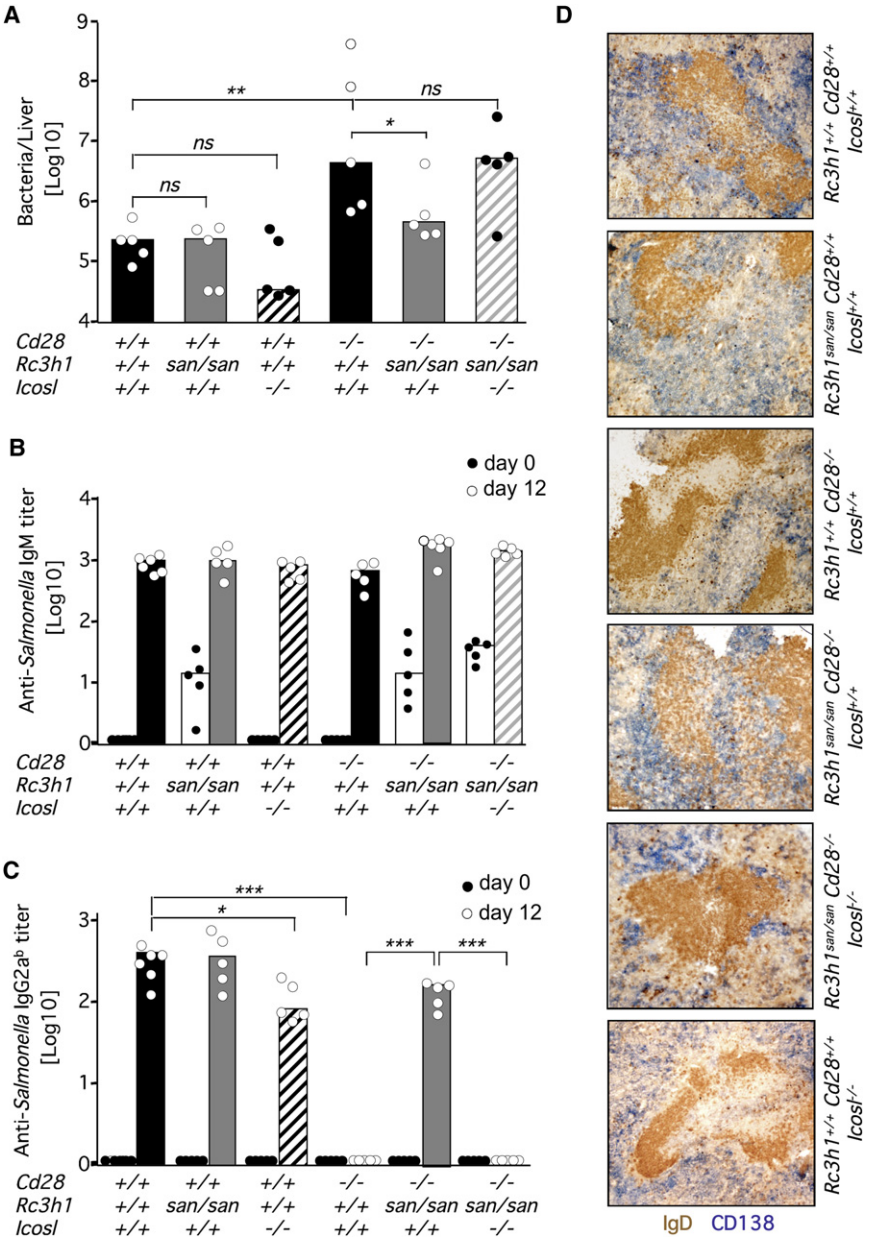

Figure 5. *Rc3h1*$^{san/san}$ Corrects the Pathogen-Clearance Defect and the IgG2a^b Response in CD28-Deficient Mice Inoculated with *Salmonella* via Signaling through ICOSL

(A) Number of colony-forming units in the liver of mice of the indicated genotypes on day 12 after inoculation. Each symbol represents one mouse (ns = not significant; *p < 0.01, **p < 0.001, and ***p = 0.0001).

(B) Anti-*Salmonella* IgM titer as determined by ELISA at day 0 and day 12 after infection. Each symbol represents one mouse.

(C) Anti-*Salmonella* IgG2a^b titer as determined by ELISA on day 0 and day 12 after inoculation. Each symbol represents one mouse.

(D) Photomicrographs of frozen spleen sections stained with IgD (brown) and CD138 (blue) from mice of the genotypes indicated on day 12 after infection. Images are representative of five mice per group.

not play a major role in the extrafollicular IgG2a^b response and the T cell response that mediates bacterial clearance in CD28$^{+/+}$ animals. Together, these results show that Roquin also ensures the compartmentalization between CD28 ligands and ICOS ligands during T cell-mediated responses to pathogens, including extrafollicular antibody responses.

The Rc3h1$^{san/san}$ Allele Restores Homeostasis of Peripheral Treg Cells in Cd28$^{-/-}$ Mice

Having established that the critical function of CD28 in the establishment of adaptive immunity can become CD28 independent in the presence of Roquin$^{san/san}$, we investigated whether this also applied to the other major role of CD28: the support of Treg cell development and homeostasis. Mice deficient in CD28 have a >80% decrease in the numbers of thymic and peripheral CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (Salomon et al., 2000; Tai et al., 2005; Tang et al., 2003), whereas ICOS deficiency has only a minor impact on peripheral steady-state Treg cell numbers (Burmeister et al., 2008), and this impact appears to be age related: By 9 weeks of age, we did not observe any difference in the percentage of circulating Treg cells in mice lacking ICOS expression (Figure 6A). By 18 weeks of age, ICOS-deficient mice had a ~20% reduction in spleen and peripheral blood Treg cells (Figure S2).

As reported for the other CD28-dependent responses (germinal center and Tfh cell formation), *Rc3h1*$^{san/san}$ mice also show a peripheral expansion of Treg cells, both as a proportion CD4$^+$ splenocytes and in total numbers (Vinuesa et al., 2005a) (Figure 6B). To determine whether homozygosity for the *san* allele of *Rc3h1* can restore Treg cell numbers in the absence of CD28, we compared the percentage and total number of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in the spleens of *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice with those found in CD28-sufficient mice. Both the percentage and number of splenic Treg cells in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ and wild-type mice were comparable and more than four times greater than those found in mice lacking CD28 (Figures 6D and 6D).

To determine whether the restoration of peripheral Treg numbers in *Cd28*$^{-/-}$ mice is dependent on signaling through the ICOS-ICOSL pathway, we analyzed the percentage and number of splenic Treg cells in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ mice. In these mice, both the percentage and total number of splenic Treg cells were less than half those of ICOSL-sufficient mice, although they were still higher than those of CD28-deficient mice (Figure 6E).

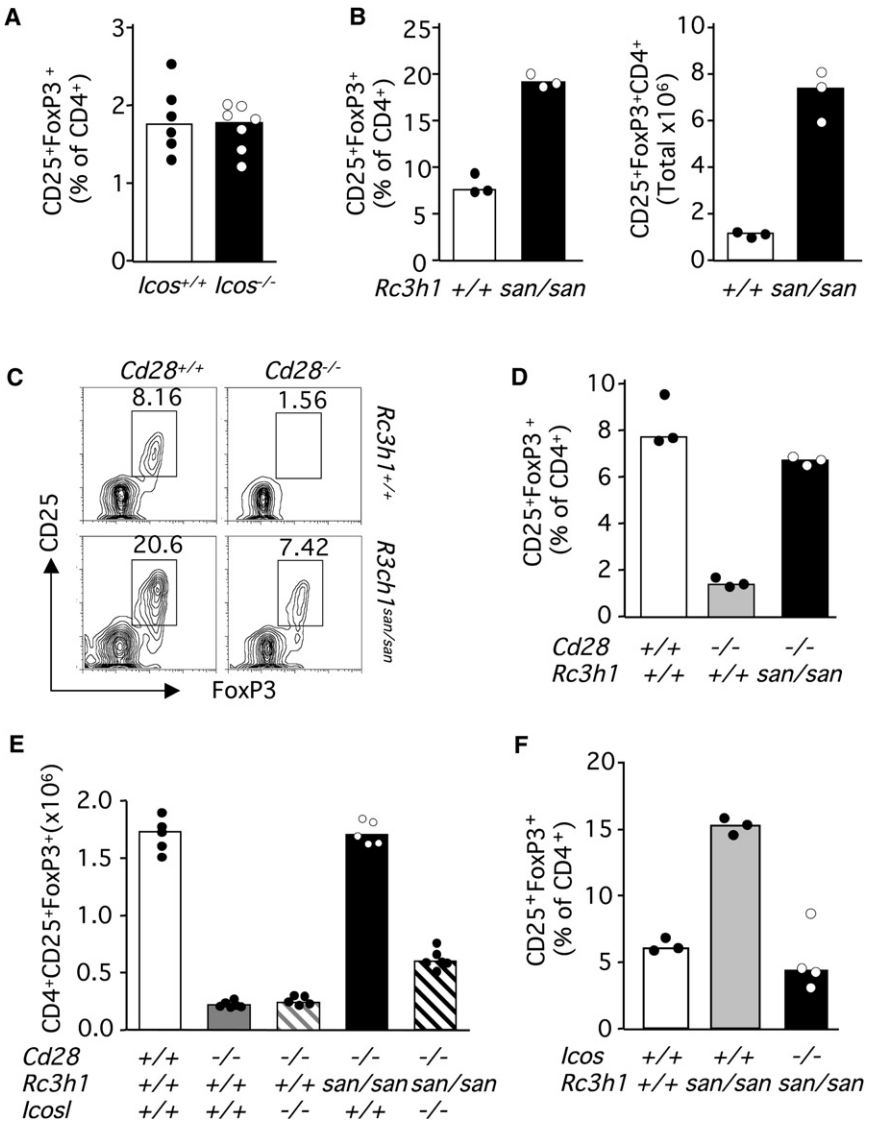

Figure 6. Loss of Peripheral Treg Cells Caused by *Cd28* Deficiency Is Rescued by *Rc3h1*$^{san/san}$ Because of Overexpression of Icos

(A) Percentage of peripheral blood CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, determined by flow cytometry, from 9- to 10-week-old wild-type and *Icos*-deficient mice.

(B) Bar graphs show splenic CD4$^+$CD25$^+$FoxP3$^+$ (Treg) cells as a percentage of splenic CD4$^+$ T cells (left) or in total numbers assessed by flow cytometry from unimmunized 10-week-old mice with the indicated genotypes.

(C) Representative flow-cytometric contour plots showing CD25 versus FoxP3 staining gated on live CD4$^+$ T cells from the spleens of unimmunized mice. Gates have been drawn around the CD25$^+$ FoxP3$^+$ population representing Treg cells.

(D) Percentage of splenic Treg cells in 10-week-old mice with the indicated genotypes gated as shown in (C) as determined by flow cytometry.

(E) Bar graphs show total number of splenic Treg cells in 10-week-old mice of the indicated genotypes as determined by flow cytometry. Statistical analyses were performed, and key comparisons are *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ *Icosl*$^{+/+}$ versus *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ ($p = 0.1067$) and *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{+/+}$ versus *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ ($p < 0.0001$).

(F) Bar graphs show percentage of splenic Treg cells in 10-week-old mice of the indicated genotype as determined by flow cytometry. Data are representative of five experiments.

The observation that there was no substantial difference between the number or percentage of peripheral Treg cells in *Cd28*$^{-/-}$ *Icosl*$^{+/+}$ mice and in *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ mice (Figure 6E) and even between those in 9-week-old *Cd28*$^{+/+}$ *Icos*$^{+/+}$ and in *Cd28*$^{+/+}$ *Icos*$^{-/-}$ mice (Figure 5A) indicates that in the presence of wild-type Roquin, ICOSL does not play an additional role to that of CD28 in peripheral Treg cell homeostasis of young mice. This illustrates again how a process that is normally ICOSL independent has become ICOSL dependent in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice. We next examined whether the expansion of Treg cells in *Rc3h1*$^{san/san}$ mice is due to aberrant signaling through ICOS by generating *Rc3h1*$^{san/san}$ mice deficient in ICOS. Loss of signaling through ICOS restored the percentage of splenic Treg cells in *Rc3h1*$^{san/san}$ mice to levels comparable to those of wild-type mice (Figure 6F), further supporting the existence of an ICOS-driven mechanism responsible for the increase in peripheral Treg cell numbers in *Rc3h1*$^{san/san}$ mice and their restoration in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice.

Rc3h1$^{san/san}$ Allows ICOS Signaling to Substitute for CD28 Signaling In Vivo for IL-2 Expression

Signaling through CD28 acts on the effector T cells of the immune system to produce IL-2, a crucial factor for Treg cell survival and the establishment of a full peripheral Treg cell repertoire (Fontenot et al., 2005; Tang et al., 2003). Although ICOS costimulation is a very poor inducer of IL-2 production (Dong et al., 2001; Riley et al., 2002), it is possible that ICOS overexpression can lead to sufficient IL-2 production to maintain peripheral Treg cell numbers in the absence of CD28. To test this possibility, we compared IL-2 mRNA expression during an in vivo T cell response to SRBCs in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice, *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ mice, and CD28-sufficient controls. Mice were immunized with SRBCs, and CD4$^+$ T cells were FACS sorted 3 days after injection. As previously reported, *Cd28*$^{-/-}$ mice produce less IL-2 mRNA than wild-type mice (Figure 7A). *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ mice were found to have approximately twice the amount of IL-2 mRNA, whereas *Roquin*$^{san/san}$ *Cd28*$^{-/-}$ mice had the same expression levels as wild-type *Rc3h1*$^{+/+}$ *Cd28*$^{+/+}$ mice (Figure 7A). These data support the hypothesis that overexpression of ICOS due to dysregulated Roquin contributes to the maintenance of the peripheral Treg cell pool in the absence of CD28 through restoring IL-2 production.

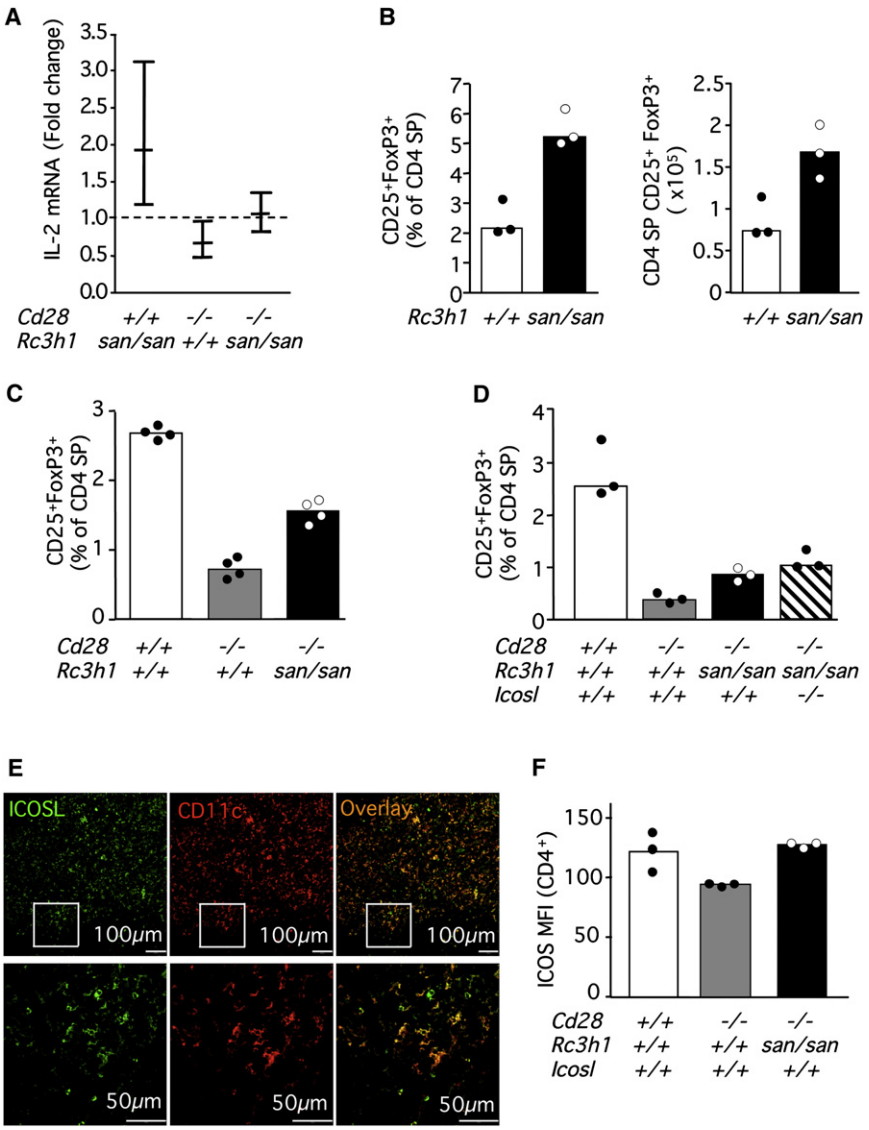

Figure 7. *Rc3h1*$^{san/san}$ Does Not Rescue the Defective Thymic Treg Cell Development in *Cd28*$^{-/-}$ Mice

(A) Fold change of IL-2 mRNA expression relative to that in wild-type C57BL/6 mice 3 days after SRBC immunization.

(B) Percentage and total number of thymic Treg cells (CD4 SP FoxP3^{+}) in 4-week-old unimmunized *Rc3h1*$^{+/+}$ (white bars) or *Rc3h1*$^{san/san}$ (black bars) mice.

(C) Percentage of CD4 SP thymocytes that express FoxP3 in mice with the indicated genotypes.

(D) Number of Treg cells in 8-week-old unimmunized mice with the indicated genotypes.

(E) Expression of CD11c (red) and ICOSL (green) on 8-week-old C57BL/6 thymi.

(F) MFI of ICOS on the surface of CD4 SP thymocytes in 8-week-old unimmunized mice with the indicated genotypes. In all graphs, each symbol represents one mouse, and bars are drawn through the median values in each group. Data are representative of three experiments.

Rc3h1$^{san/san}$ Cannot Rescue Thymic Treg Cell Development in Mice Deficient in CD28

CD28 has been shown to be essential for thymic Treg cell development through the induction of IL-2 and other IL-2-independent signaling events (Tai et al., 2005; Tang et al., 2003). To examine whether the rescue of peripheral Treg cells in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice is a consequence of restoring Treg cell development in the thymus, we assessed the number and percentage of CD4 single-postive (SP) FoxP3^{+}-expressing thymocytes in 4-week-old *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice. As shown for peripheral Treg cells, *Rc3h1*$^{san/san}$ mice have twice the number of Treg cells within the CD4 SP thymic compartment (Figure 7B). Nevertheless, unlike the effect of *Rc3h1*$^{san/san}$ on peripheral Treg cells, mutant Roquin could not restore thymic Treg cells in mice deficient in CD28 (Figure 7C), although a small increase was observed in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice as compared with *Rc3h1*$^{+/+}$ *Cd28*$^{-/-}$ mice. This small increase in thymic Treg cell numbers, due to mutant Roquin, was independent of ICOS signaling (Figure 7D), indicating a non-ICOS-related effect.

To investigate the cause for the failure of *Rc3h1*^{sansan} to rescue thymic Treg cell numbers in *Cd28*$^{-/-}$ mice, we first tested whether ICOSL was expressed in the thymus by confocal microscopy. Indeed, ICOSL is strongly expressed in the thymic medulla, with the majority of the expression colocalizing with CD11c^{+} cells (Figure 7E). The next obvious question is whether *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ thymocytes overexpress ICOS. We had previously observed that *Rc3h1*$^{san/san}$ CD4 SP thymocytes (with intact CD28 signaling) expressed slightly higher levels of ICOS (Vinuesa et al., 2005a). When thymocyte ICOS expression was assessed by flow cytometry, we found that the levels of ICOS on *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ thymocytes were comparable to those in wild-type mice (Figure 7F). This may explain why thymic Treg cells are not restored in *Rc3h1*$^{san/san}$ mice lacking CD28. Taken together, these results show that mutant Roquin, in the absence of CD28, does not cause ICOS overexpression in CD4 SP thymocytes or restore thymic Treg cells.

DISCUSSION

The question addressed by these studies was whether the evolution of two biochemically paralogous costimulatory receptors posed a potential for functional crosstalk and misregulation, given that one of their ligands is constitutively expressed in the absence of pathogen-associated molecular patterns. The results demonstrate that functional compartmentalization of CD28 and ICOS is readily broken down and is normally critical to ensure that the initiation of immune responses depends on a pathogen-induced "signal 2" through CD28. *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice form normal primary antibody responses, Tfh cells, large

germinal centers, intact extrafollicular plasma cell responses, and normal numbers of peripheral Treg cells—functions known to be critically dependent on intact signaling through CD28. Given that relatively little is understood about mechanisms controlling ICOS and ICOSL expression, there are likely to be a range of pathological and physiological circumstances in which this crosstalk comes into play.

Three alternative hypotheses could explain the rescue of responses in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ animals: First, *Rc3h1*$^{san/san}$ allows ICOSL to substitute for CD28 ligands, whereas in *Rc3h1*$^{+/+}$ T cells, these two potential costimulatory sources are distinguished and functionally compartmentalized so that ICOSL cannot substitute for CD28L. Second, Roquin may allow for signaling through the CD28 pathway in the absence of CD28. If this were the case, removing ICOSL would not have an effect, but we show profound abrogation of *Rc3h1*$^{san/san}$-mediated rescue in *Rc3h1*$^{san/san}$ mice doubly deficient in CD28 in all responses tested. Third, even in the presence of intact CD28L signaling, there might be a role for ICOSL in the response. If this were the case, ICOSL deficiency would confer a phenotype in *Cd28*$^{+/+}$ mice. We have shown two examples in which ICOSL does not play a role in the response of *Cd28*$^{+/+}$ animals: First, whereas *Cd28*$^{-/-}$ mice cannot mount a T cell-dependent antibody response to *Salmonella* or clear bacteria—both of these responses are dependent on Th1 cells—these responses are intact in ICOSL$^{-/-}$ mice. Second, peripheral Treg cell numbers are 80% lower in 9-week-old *Cd28*$^{-/-}$ mice, but they are normal in age-matched *Icos*$^{-/-}$ mice. Having excluded the latter two, only the first hypothesis is consistent with the results of the *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ *Icosl*$^{-/-}$ triple-mutant studies.

We have shown that this compartmentalization acts during immune responses to foreign antigens such as sheep red blood cells and, importantly, to the infectious agent *Salmonella*. The physiological counterpart of a challenge with antigen in the absence of CD28 ligands would be a situation in which the antigen is a self-antigen. The most obvious evolutionary advantage of functional compartmentalization of signals through CD28 and ICOS is the retaining of the need for microbial induction of costimulatory ligands at the time of T cell priming, because the constitutive and broad expression of ICOSL could bypass the need for danger signals to provide full costimulation. Our previous work has provided evidence that this sort of dysregulation can pose a real threat for autoimmunity: ICOS overexpression on naive T cells contributes to lupus manifestations in *Rc3h1*$^{san/san}$ mice (Yu et al., 2007).

A ''controlled'' breakdown of this compartmentalization, or what could also be seen as the ''reverse'' type of compartmentalization, i.e., making T cell costimulation in germinal centers exclusively dependent on ICOS, and not on CD28, may be equally important for physiological humoral immune responses. Processed microbial antigens can be trapped in an inert state in the form of immune complexes on the surface of follicular dendritic cells for months or years within germinal centers, and this source of antigen is probably important for the maintenance of germinal center reactions and ongoing affinity maturation and for the reactivation of memory B cells that may help maintain long-lived protective humoral immunity. Also, the switch from CD28 dependence to ICOS dependence that maintains Tfh cell help for germinal center B cells and Tfh cell survival is likely to also be critically important to prevent the emergence of high-affinity autoantibodies from germinal centers. It would be potentially dangerous to make this Tfh cell costimulation and subsequent selection of mutated germinal center B cells dependent on the presence of Toll-like receptor (TLR) ligands or other danger signals; it is well known that signals delivered by TLR ligands in conjunction with B cell receptor signals can lead to T cell-independent self-reactive B cell differentiation (Vos et al., 2000). The need for danger signals is even less desirable if we take into account the fact that the germinal center milieu is extremely rich in apoptotic cells that expose self-nuclear antigens on their surface and that a proportion of somatically mutated cells will have randomly acquired specificity against these self-antigens (Diamond et al., 1992).

Gene duplication followed by positive selection is not only an effective way to resolve adaptive conflicts through subfunctionalization as described above. It also drives the acquisition of unique functions by individual genomes: This process is termed ''neofunctionalization,'' and there is evidence to suggest that neofunctionalization has also driven the selection of the adaptive changes of *Icos*. In the context of evolution, the first time *Cd28*, *Ctla4*, and *Icos* are present as an immunological cluster in the same species is in chickens (Bernard et al., 2007), and this coincides with the appearance of germinal centers, which occur only in homeothermic vertebrates (birds and mammals). Although somatic hypermutation during immune responses is seen in ectotherms, which lack germinal centers, it is not accompanied by efficient improvement in overall antibody affinity, suggesting that affinity maturation depends on germinal centers for selection of mutated B cells by antigen-specific follicular T cells (Hsu, 1998). It seems, therefore, that the acquisition of novel functions by the *Cd28* paralog *Icos*, specifically its new role in the survival and B cell helper function of Tfh cells, opened up the possibility for affinity maturation and effective immunological memory to occur.

Although most of the rescue of responses normally dependent on CD28, caused by *Rc3h1*$^{san/san}$, requires signaling through ICOS, there appear to be small non-ICOS mediated contributions to Tfh cell development and germinal center formation in *Rc3h1*$^{san/san}$ mice lacking CD28. Our adoptive cell transfer of wild-type B cells and bone marrow chimera experiments have excluded a B cell-autonomous action of Roquin as an explanation the rescued germinal center response. We have shown previously that besides dysregulating ICOS expression *Rc3h1*$^{san/san}$ CD4^{+} cells have high mRNA expression of other key Tfh cell molecules, such as CD200, CXCR5, PD-1, CD84, Bcl6, and the cytokine IL-21 (Vinuesa et al., 2005a). It is possible that one or more of these factors are driving additional Tfh cell development in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ T cells and/or directly promoting germinal center responses, as has been shown in mice overexpressing IL-21 (Ozaki et al., 2004).

The germinal centers observed in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice are larger than those seen in wild-type mice, and this is reminiscent of the germinal centers induced in *Ctla4-Ig* transgenic mice treated transiently with CD28 for allowing initiation of T cell-dependent responses (Walker et al., 2003). In these mice, germinal center reactions do not appear to involute normally, and they display abundant Tfh cells expressing high amounts of ICOS that persist long after the CD28 mAb has been cleared,

suggesting that CTLA-4 negatively regulates germinal center longevity and Tfh cell numbers. Furthermore, a link between signaling through CTLA-4 and *Icos* mRNA repression has been suggested (Riley et al., 2001). Nevertheless, our results showing that germinal center longevity is not prolonged in *Rc3h1*$^{san/san}$ *Cd28*$^{+/+}$ nor *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice argues against the possibility that CTLA-4 exerts its repressive effect through Roquin.

Signals through CD28 also support generation of Treg cells within the thymus and peripheral Treg cell homeostasis (Tai et al., 2005; Tang et al., 2003). Although thymic Treg cell numbers are increased in *Rc3h1*$^{san/san}$ mice, we show that this increase is not dependent on ICOS signaling, which is not surprising given the finding that ICOS is not overexpressed on *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ CD4SP thymocytes and that CD28 deficiency alone partially corrects the excess of thymic Treg cells seen in *Rc3h1*$^{san/san}$ mice. Furthermore, the most likely reason why ICOSL cannot substitute for CD28L signals for Treg cell generation in the thymus in the presence of the *san* allele of *Rc3h1* is that the latter depends on a critical Lck-binding motif within the polyproline domain of CD28 that is absent in ICOS (Tai et al., 2005). In contrast with the thymic Treg cell deficiency, our data show that peripheral Treg cell numbers are restored in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice in an ICOS-dependent manner. This suggests that the smaller thymic Treg cell pool may be able to expand to fill the peripheral niches.

Our data showing that in the presence of defective Roquin, IL-2 production occurred normally in the absence of CD28 signaling suggests this is a consequence of ICOS overexpression and could contribute to the maintenance of peripheral Treg cells in the absence of the major costimulator. Expansion of naturally occurring thymic-derived Treg cells might not be the only mechanism that fills the peripheral Treg cell niches in *Rc3h1*$^{san/san}$ *Cd28*$^{-/-}$ mice. A non-mutually exclusive explanation may lie in a possible increased generation of "adaptive" Treg cells (aTreg cells) in the periphery, which could also be driven by excess ICOS signaling. Naive CD4^{+} CD25^{-} T cells stimulated with CD28 and TGFβ can become regulatory cells phenotypically identical to thymic-derived Treg cells (Liang et al., 2005).

In general, there is still little experimental evidence of the mechanisms by which gene duplicates are compartmentalized and regulated for enabling genetic novelty. Elegant work has dissected the molecular basis of the evolution of a bifunctional ancestor in the yeast galactose pathway into a pair of duplicate genes, *GAL1* and *GAL3*, that acquired critical coinducer and galactokinase subfunctions, respectively (Hittinger and Carroll, 2007). In this particular "genetic switch," adaptive changes in the promoter region that would have compromised one of the functions allowed a differentially regulated transcriptional response. Our data parallels these findings by identifying a molecule, Roquin, that can regulate the functional switch between two gene duplicates.

This regulation, unlike the transcriptionally regulated, inducible nature of ICOS (Tan et al., 2006), appears to hinge on evolutionary changes in the 3′UTR of *Icos* that allow a differentially regulated posttranscriptional induction of mRNA decay through Roquin-controlled microRNAs (Yu et al., 2007). Roquin appears to be constitutively expressed and constantly regulating ICOS in both naive and activated T cells, although its microRNA cofactor (miR-101) is downregulated in Tfh cells (Yu et al., 2007). Indeed, we show that ICOS is induced to the same extent (~4 fold) in both *Rc3h1*$^{san/san}$ effector and memory CD4^{+} T cells and controls.

In conclusion, our data reconciles how two related and functionally overlapping receptor-ligand pairs can coordinately initiate immune responses to protein antigens and select somatically derived high-affinity mutants without jeopardizing the maintenance of peripheral tolerance. This is achieved through Roquin's tight regulation of ICOS expression, which prevents ICOS from taking over the critical CD28 functions of initiating T cell help in the context of danger signals and determining the size of the peripheral Treg cell pool, but allows its prominent role in the selection of high-affinity germinal center B cell mutants independently of CD28 and danger signals and in the regulation of the size and longevity of germinal centers.

EXPERIMENTAL PROCEDURES

Mice and Immunizations

Rc3h1$^{san/san}$ C57BL/6 mice, crosses to *Icos*$^{-/-}$, *Icosl*$^{-/-}$, and *Cd28*$^{-/-}$ mice, and SW_{HEL} mice were housed in specific pathogen-free conditions at the Australian Phenomics Facility. SW_{HEL} mice have a VDJ region introduced by homologous recombination in the Ig H chain locus that, in combination with a transgene-encoded light chain, binds HEL with high affinity (Phan et al., 2003). All animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

For generating thymus-dependent responses for assessing specific IgG titers, mice were immunized intraperitoneally (i.p.) with 50 μg of alum-precipitated chicken gammaglobulin (CGG) plus 1 × 10^{8} heat killed *B. pertussis*. Where indicated, 8- to 12-week-old mice were immunized i.p. with 2 × 10^{9} SRBCs. For experiments involving SW_{HEL} mice, 1 × 10^{4} SW_{HEL} B cells were transferred into C57BL/6 recipients, which were immunized intravenously with 2 × 10^{8} SRBCs conjugated with mutant HEL, HEL^{2x} (Paus et al., 2006).

Bacteria and Inoculation

Salmonella enterica serovar Dublin strain SL5631 (Segall and Lindberg, 1991) was grown in Luria-Bertani medium overnight. Mice were inoculated with 5 × 10^{5} colony-forming units from a log-phase culture administered i.p. in phosphate-buffered saline (PBS). Bacterial load was measured in the liver of all mice at day 12 after infection by homogenizing organs, plating serial dilutions in PBS onto Luria-Bertani agar, and incubating at 37°C overnight.

Antibodies

Antibodies and strepatavidin conjugates for flow cytometry were from BD PharMingen except where otherwise indicated: anti-mouse B220-PerCP, CD4-PerCP, ICOS-PE (eBioscience), FoxP3 (eBioscience), GL-7-FITC, CD95-PE, CXCR5-biotin, PD-1-PE, CTLA-4-PE, CD25-APC, CD8-APC, and streptavidin-PerCP Cy5.5. Background ICOS staining was determined with stained cells from ICOS-deficient mice. For immunohistochemistry, the primary antibodies and reagents used were as follows: sheep anti-mouse IgD (The Binding Site), biotinylated anti-mouse TCRbeta (PharMingen), rat anti-mouse PD-1 (Biolegend), PNA-biotin (Vector Laboratories), and rat anti-mouse CD138 (BD PharMingen). Rabbit anti-rat horseradish peroxidase (HRP) (Dako) was used as a secondary antibody. For immunofluorescence, monoclonal Abs used were rat anti-mouse Icosl (clone MIL-666, generated by immunization of Lewis rats with an *Icosl* transfectant) and hamster anti-mouse CD11c-biotin (eBioscience). Anti-mouse ICOSL was detected with goat anti-rat FITC (Southern Biotechnology Associates), then rabbit anti-FITC (Sigma-Aldrich), then goat anti-rabbit-FITC (Southern Biotechnology Associates).

Cell Isolation, Culture, and Stimulation

For flow cytometry, single-cell suspensions were prepared from spleens and/or pooled lymph nodes (inguinal, axillary, subcapsular, cervical, mesenteric, and para-aortic) of unimmunized and/or immunized mice.

Flow Cytometry

Spleen and or lymph node cell suspensions were prepared by sieving and gentle pipetting. For surface staining, cells were maintained in the dark at 4°C throughout. Cells were washed twice in ice-cold FACS buffer (2% fetal calf serum, 0.1% NaN_3 in PBS), then incubated with each antibody and conjugate layer for 30 min and washed thoroughly with FACS buffer between each layer. Intracellular staining used Cytofix/Cytoperm Kit (BD Biosciences) following the manufacturer's instructions. For detection of HEL-binding B cells, HEL was conjugated to Alexa 647 (Molecular Probes). A FACSCalibur (Becton Dickinson) was used for the acquisition of flow-cytometric data, and Flowjo software was used for analysis.

Enzyme-Linked Immunosorbent Assay

Anti-*Salmonella* IgM, IgG1, $IgG2a^b$, IgG2b, and IgG3 (Southern Biotechnology Associates) were detected in plasma from blood taken at day 7 and day 12 by enzyme-linked immunosorbent assay (ELISA). 96-well ELISA plates (Nunc) were coated with SL5631 cell lysate. The lysate was prepared from an overnight culture with a French press. Protein concentration in the lysate was determined by Bradford assay, and each well was coated with 12.5 μg protein. For the CGG immunization experiment, sera from mice were collected 14 days after immunization with CGG and analyzed for αCGG-IgG1 antibodies with CGG (1.5 μg/ml)-coated 96-well plates. Serial serum dilutions were applied, immunoglobulin concentration was determined with HRP-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates), and the enzyme bound to plates was developed with Phosphatase Substrate tablets (Sigma S0942). Plates were read at 405 nm with a Thermomax Microplate Reader (Molecular Devices). The titers for serum samples were calculated as the log serum concentration required to achieve 50% maximum optical density.

Immunohistochemistry

5 μm acetone-fixed frozen sections of spleen were air dried and washed in 0.1 M Tris-buffered saline (TBS) (pH 7.6), and then primary antibodies were added in TBS and incubated for 45 min. After a further wash in TBS, secondary reagents that had been previously absorbed in 10% normal mouse serum were added to the sections for 45 min. When biotin-conjugated primary or secondary reagents were used, streptavidin alkaline phosphatase (Vector Laboratories) was added after a further wash in TBS and incubated for 20 min. HRP activity was detected with diaminobenzidine tetrahydrochloride solution (Sigma) and hydrogen peroxide. Alkaline phosphatase activity was detected with the AP-Substrate Kit III (SK-5300, Vector laboratories). Sections were viewed under an Olympus IX71 Microscope (Olympus).

Immunofluorescence

Tissue samples for immunofluorescence were embedded in Tissue-Tek OCT compound (Bayer Healthcare), and 6 μm thick sections were cut and fixed in acetone. Antibodies were added in PBS and incubated in the dark for 30 min. Biotinylated antibodies were detected with streptavidin-Alexa Fluor 555 (Invitrogen). Sections were mounted with Vectashield mounting medium (Vector Laboratories). Confocal images were obtained at room temperature on a LSM 510 Meta microscope (Zeiss) equipped with either a 10× or a 40× 1.4 N.A. water lens and 488 and 543 lasers with Zeiss LSM software.

Real-Time PCR

$CD4^+$ splenocytes from mice of the indicated genotypes 3 days after SRBC immunization were sorted, frozen in Trizol, RNA extracted, and cDNA made. Expression of IL-2 was determined with quantitative RT-PCR comparing IL-2 to two housekeeping genes, β-actin and β2M, with four biological replicates per strain and five technical replicates per sample and gene. Technical replicates were averaged, and data were analyzed by both randomly pairing mice in the control and experimental groups and averaging the cT of biological replicates. The same pattern of differential expression was observed with both analysis methods and with both housekeeping genes.

Statistical Analysis

Data were analyzed with a two-tailed Student's t test with Prism software.

SUPPLEMENTAL DATA

Supplemental Data include three figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00068-5.

ACKNOWLEDGMENTS

This work was funded by a Viertel Senior Medical Research Fellowship, National Health and Medical Research Council grants 316956 and 427620 to C.G.V. and C.C.G., and Juvenile Diabetes Research Foundation grant 1-2006-96 to C.G.V. and D.S. We thank M. Cook and A. Liston for critical review of the manuscript and helpful discussions.

Received: February 24, 2008
Revised: May 12, 2008
Accepted: December 18, 2008
Published online: February 12, 2009

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Note Added in Proof

In the version of this paper published online on February 12, the legend of Figure 3 contained descriptions for panels A, B, and C, although Figure 3 only had panels A and B. This legend now has been corrected online and in print.