IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses

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During T cell–dependent responses, B cells can either differentiate extral follicularly into short-lived plasma cells or enter follicles to form germinal centers (GCs). Interactions with T follicular helper (Tfh) cells are required for GC formation and for selection of somatically mutated GC B cells. Interleukin (IL)-21 has been reported to play a role in Tfh cell formation and in B cell growth, survival, and isotype switching. To date, it is unclear whether the effect of IL-21 on GC formation is predominantly a consequence of this cytokine acting directly on the Tfh cells or if IL-21 directly influences GC B cells. We show that IL-21 acts in a B cell–intrinsic fashion to control GC B cell formation. Mixed bone marrow chimeras identified a significant B cell–autonomous effect of IL-21 receptor (R) signaling throughout all stages of the GC response. IL-21 deficiency profoundly impaired affinity maturation and reduced the proportion of IgG1+ GC B cells but did not affect formation of early memory B cells. IL-21R was required on GC B cells for maximal expression of Bcl-6. In contrast to the requirement for IL-21 in the follicular response to sheep red blood cells, a purely extral follicular antibody response to Salmonella dominated by IgG2a was intact in the absence of IL-21.

IL-21 belongs to a family of cytokines that bind the common cytokine receptor y chain in conjunction with additional receptor subunits; IL-21 exclusively binds IL-21R (Spolski and Leonard, 2008). CD4+ T cells and NKT cells produce IL-21 (Parrish-Novak et al., 2000; Coquet et al., 2007). Within the CD4+ T cell subset, IL-21 is expressed at the highest levels by T follicular helper (Tfh) cells and Th17 cells (Chetanova et al., 2004; Nurieva et al., 2007; Bauquet et al., 2009). Its receptor is expressed on T cells, B cells, NK cells, macrophages, and DCs (Spolski and Leonard, 2008). IL-21 promotes antibody production, plasma cell differentiation, and switching to IgG1 in the context of thymus-dependent (TD) responses (Kasaian et al., 2002; Ozaki et al., 2002, 2004; Pène et al., 2004; Ettinger et al., 2005; Kuchen et al., 2007; Avery et al., 2008; Spolski and Leonard, 2008; Vogelzang et al., 2008). A requirement of IL-21 for Tfh cell differentiation has also been reported (Nurieva et al., 2008, 2009; Vogelzang et al., 2008); in naive T cells, IL-21 leads to up-regulation of Bcl-6, the transcriptional regulator of Tfh cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). The defect in Tfh cell formation in the absence of IL-21 signaling has been proposed to explain the reduced numbers of germinal center (GC) B cells in the absence of IL-21 (Nurieva et al., 2008; Vogelzang et al., 2008).

After binding protein antigen and receiving cognate help from T cells, B cells can differentiate along the extral follicular pathway, generating short-lived plasma cells that produce low affinity antibodies, or can enter the follicles and give rise to GCs (MacLennan et al., 2003).
Within the GCs, B cells undergo somatic hypermutation, and those acquiring higher affinity for the immunizing antigen receive selection signals from Tfh cells. Selected cells emerge from the GCs as memory B cells or long-lived antibody-secreting plasma cells. Tfh and GC B cells have a mutual influence on each other: Tfh cells need B cell–derived signals for their optimal development (Nurieva et al., 2008) and Tfh cells are also essential for GC formation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Nevertheless, normal GC induction with a paradoxical accumulation of IgG memory B cells was initially reported in Il21r−/− mice (Ettinger et al., 2008). We also recently reported that IL-21 deficiency did not reduce spontaneous GCs, Tfh cells, or autoimmunity in a lupus-prone mouse (Linterman et al., 2009b).

Given this evidence of the effects of IL-21 on B and T cells, we asked whether the reported effects of IL-21 on GCs may not only be secondary to the effects of IL-21 on Tfh cell formation but may also result from a direct influence on GC B cells. In this report, we show that IL-21 exerts direct effects on GC B cells, suggesting that the reduction of GC B cells is not only a consequence of reduced Tfh cells. Furthermore, we show that IL-21 is required for optimal affinity maturation and maximal expression of the GC B cell transcriptional regulator, Bcl-6.

RESULTS AND DISCUSSION

IL-21-deficient mice form reduced GCs that bind peanut agglutinin (PNA) weakly

To assess the role of IL-21 signaling in both GC and Tfh cell formation, we compared the responses of Il21r+/+ and Il21r−/− mice after TD immunization with sheep RBCs (SRBCs). Analysis of basal serum IgGs revealed the reported reduction in serum IgG1 and elevated IgE titers in the absence of IL-21 (Fig. 1, A and B; Ozaki et al., 2002). The percentage of GC B cells identified by expression of GL-7 and Fas (CD95) was measured by flow cytometric analysis of splenocytes before or on days 6, 8, and 14 after immunization. Without immunization, there were fewer background Tfh cells in Il21r−/− compared with Il21r+/+ mice (Fig. 2 A). We observed normal generation of Tfh cells in Il21r−/− mice, with equivalent proportions on day 6 after immunization compared with Il21r+/+ mice (Fig. 2 A). Although formation was normal, there was a more rapid decline in Tfh cell numbers in Il21r−/− mice (Fig. 2 A). There was a slight decrease in the total number of CD4+ cells in the absence of IL-21 (Fig. S1 C), although this was not statistically significant.

T cells could be easily identified in the GCs of IL-21–deficient mice 8 d after immunization (Fig. 2 B). In the absence of IL-21, there were more T cells per 1 mm² of GC area than in IL-21–sufficient mice (166 vs. 104 CD3+ cells/mm², respectively; P = 0.03), and on average there were slightly more T cells per GC in Il21r−/− compared with Il21r+/+ mice (22 ± 15 vs. 16 ± 14, respectively; P = 0.2). Despite this increase in the number of follicular T cells per GC, the total number of T cells found in follicles per square millimeter of spleen area was reduced in IL-21–deficient mice (97 ± 44 vs. 126 ± 34 per section; P = 0.05), consistent with the results obtained by flow cytometric staining on day 8. This is explained by the smaller size of GCs in IL-21–deficient mice. Collectively, these data indicate that IL-21 is not required for T cell formation but contributes to Tfh cell maintenance.

We also investigated the possibility that GC B cell and/or Tfh cell proliferation might be reduced in the absence of IL-21. Enumeration of Ki-67+ cells among CD4+CXCR5 high PD-1+ Tfh cells did not reveal any differences in mice deficient for IL-21 (Fig. S2 A). Likewise, there was no difference in the proportion of Ki-67+ cells among B220+GL-7+Fas+ GC cells in the absence of IL-21 (Fig. S2 B).

IL-21R signals in B cells are required for GC formation

To investigate whether IL-21R signaling in the B cells themselves is required for GC formation, we generated Il21r+/+/ Il21r−/− mixed bone marrow chimeras. Subletally irradiated Rag1−/− mice were reconstituted with a 1:1 mix of either CD45.1 Il21r+/+/CD45.2 Il21r−/− or CD45.1 Il21r+/+ CD45.2 Il21r−/− bone marrow. 8 wk later, chimeric mice were immunized with SRBCs and the percentage of B220+ cells, GL-7+Fas+B220+ GC B cells, CD4+ T cells, and CXCR5 high PD-1 high CD4+ Tfh cells derived from the CD45.2

To investigate whether IL-21R signaling in the B cells themselves is required for GC formation, we generated Il21r+/+ and Il21r−/− mice did not reveal statistically significant increases in the amount of ST3Gal and ST6Gal mRNA expression between both groups of mice (Fig. S1 B), suggesting that changes in these two enzymes are not responsible for reduced PNA binding in the absence of IL-21.

Tfh cells form but decline faster in the absence of IL-21

We investigated the kinetics of Tfh cell formation after SRBC immunization in IL-21–deficient mice by enumerating CXCR5 high PD-1 high CD4+ Tfh cells by flow cytometry on days 0, 6, 8, and 14 after SRBC immunization. In the absence of immunization, there were fewer background Tfh cells in Il21r−/− compared with Il21r+/+ mice (Fig. 2 A). We observed normal generation of Tfh cells in Il21r−/− mice, with equivalent proportions on day 6 after immunization compared with Il21r+/+ mice (Fig. 2 A). Although formation was normal, there was a more rapid decline in Tfh cell numbers in Il21r−/− mice (Fig. 2 A). There was a slight decrease in the total number of CD4+ cells in the absence of IL-21 (Fig. S1 C), although this was not statistically significant.

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Brief Definitive Report

The function of common γ chain cytokines is modified by the context in which they are delivered (Rochman et al., 2009), and there is evidence suggesting that the magnitude of Tfh cell populations is influenced by the types of antigen, adjuvant, and avidity of TCR (Ansel et al., 1999; Malherbe et al., 2008; Fazilleau et al., 2009). It is therefore likely that weaker immunization strategies, e.g., smaller doses of SRBCs or cells with decreased immunogenicity (i.e., less fresh), may increase the dependence on IL-21 and may induce a faster decline in GC and Tfh cells. It is also possible that the differences reported in this paper are caused by the genetic background of the mice used. Former studies used Il21−/− mice either on a mixed 129xB6 F1 background or on a 129xB6 background backcrossed seven generations to B6. The mice used in this study were also generated on the 129xB6 background and backcrossed 10–12 generations to B6, and as such, would be expected to have a maximum of 0.1% 129 genome. IL-2 plays an essential role in T cell expansion and is tightly linked to the IL-21 locus. Our analysis of polymorphic markers between 129 and C57BL/6 revealed that the IL-2 locus is also derived from the 129 background in our N10 backcrossed mice (unpublished data), excluding this as a modifier of the Tfh cell phenotype in this study.

Given the described ability of IL-6 to induce Bcl-6 (Nurieva et al., 2009), the modest effect of IL-21R signaling on Tfh cell formation is also probably explained by functional redundancy between IL-21 and IL-6. As has been described for

Figure 1. IL-21–deficient mice form detectable GCs after immunization, but the kinetics of the GC is altered. (A and B) ELISA analysis of total serum IgG1 (A) and IgE (B) in unimmunized mice of the indicated genotypes. (C) Flow cytometric contour plots and graphical analysis of GL-7+Fas+ GC cells gated on live B220+ lymphocytes from Il21+/+ and Il21−/− mice at the indicated time points after SRBC immunization. Each symbol represents one mouse. (D) Photomicrographs of spleen sections stained for IgD (brown) and PNA (blue) from Il21+/+ and Il21−/− mice 8 d after immunization. Data are representative of two independent experiments (n ≥ 4 per group). Bars, 200 µm. (E) Flow cytometric histograms and graphical analysis of PNA binding on GL-7+Fas+B220+ GC B cells from Il21+/+ and Il21−/− mice 7 d after SRBC immunization. Statistically significant differences are indicated (*, P ≤ 0.05; **, P ≤ 0.01). Data are representative of two independent experiments, each symbol represents one mouse, and tops of bars are drawn through the median values. MFI, mean fluorescence intensity; ns, not significant.
Th17 cell formation, it is possible that a role for IL-21 would become more apparent in IL-6–deficient mice (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007; Coquet et al., 2008; Sonderegger et al., 2008). Furthermore, other cytokines may also contribute to Tfh cell formation. In humans, IL-12 can induce human T cells to differentiate into IL-21–producing Tfh-like cells in vitro (Schmitt et al., 2009).

Given the reports of reduced IgG1 responses in immunized IL-21–deficient mice (Ozaki et al., 2002), we investigated whether the decline in GC B cells was mainly caused by loss of IgG1+ GC B cells. After SRBC immunization, ∼20% of GC B cells within the CD45.2 II21r+/− compartment are IgG1+. This proportion was only slightly reduced among the II21r−/− GC cells (unpublished data). However, analysis of the proportion of GC B cells and IgG1+ GC B cells of CD45.2 origin in each chimera revealed no parallel decline in IL-21R–deficient GC B cells and IgG1+ GC B cells 6 d after SRBC immunization (Fig. 3, C and D). IgG1+ IL-21R–deficient GC B cells were virtually absent in unimmunized mice. This suggests that IL-21 deficiency profoundly, but not exclusively, affects Ig switching to IgG1 and/or maintenance of IgG1+ GC B cells.

IL-21 is required for optimal affinity maturation but not for early memory (EM) B cell formation

To investigate how IL-21 affects the process of affinity maturation and formation of EM B cells, CD45.1 SWHEL transgenic B cells bearing a rearranged hen egg lysozyme (HEL)–specific VD10 element targeted into the H chain locus combined with an HEL–specific κ L chain transgene (Phan et al., 2003) were adoptively transferred into IL-21–deficient or –sufficient CD45.2 recipients. The recipients were then immunized with SRBCs conjugated with HEL protein bearing three substitutions (HEL3X), which binds the Ig receptors on SWHEL B cells with substantially lower affinity compared with native HEL, enabling affinity maturation to proceed normally (Phan et al., 2003; Paus et al., 2006). Flow cytometric staining with nanomolar concentrations of HEL3X can directly enumerate high affinity IgG+ variants (Phan et al., 2003; Paus et al., 2006); quantification of the proportion of HEL3Xhi-binding B cells by flow cytometry closely reflects the acquisition of mutations that confer higher affinity for the immunizing HEL3X (Phan et al., 2003; Paus et al., 2006; Randall et al., 2009). In HEL3X SRBC–immunized IL-21–deficient recipients, there was a 72% reduction in HEL3Xhi-binding GC B cells (P = 0.035) 10 d

Figure 2. IL-21–deficient mice form Tfh cells after immunization, but their maintenance is impaired. (A) Flow cytometric contour plots and graphical analysis of CXCR5+PD-1+ Tfh cells gated on CD4+ B220− live lymphocytes from II21+/+ and II21−/− mice at the indicated time points after SRBC immunization (percentages are shown). (B) Photomicrographs of spleen sections taken from II21+/+ (top) and II21−/− (bottom) mice 8 d after immunization with SRBCs. In all panels, IgD is stained in brown; blue color stains indicate PNA binding (left), CD3 (middle), and PD-1 (right). Bars, 200 µm. Statistically significant differences are indicated (*, P ≤ 0.05). Data are representative of two independent experiments, each symbol represents one mouse, and tops of bars are drawn through the median values. ns, not significant.
We also observed a higher ratio of Tfh cells to HEL-binding donor derived GC B cells (Fig. 4 C). This is in line with our earlier observation of smaller GCs and an increased

Figure 3. IL-21R expression is required on both GC B cells and Tfh cells for their maintenance but is dispensable for their formation. (A and B) Gating strategy (A) and bar graphs (B) of mixed bone marrow chimeras containing a 1:1 ratio of control Il21r+/+CD45.1/Ii21r+/+CD45.2 or Il21r+/+CD45.1/Ii21r−/−CD45.2 bone marrow. Spleens from unimmunized mice (A; and B, top) or from mice immunized with SRBCs 6 or 14 d previously (B, middle and bottom) were analyzed by flow cytometry for the percentage of B220+ B cells that are CD45.2+ (B220+), the percentage of GL-7+Fas+ GC cells among B220+ cells that are CD45.2+ (GC cells), the percentage of CD4+ Th cells that are CD45.2+ (CD4+), and the percentage of CXCR5highPD-1high Tfh cells among CD4+ cells that are CD45.2+ (Tfh cells). Each bar represents a single recipient mouse; individual mice have been numbered and placed in the same order in each of the plots. Statistically significant decreases are indicated (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001). Data are representative of two independent experiments. (C and D) Gating strategy (C) and dot plots (D) of the proportion of B220+ cells, GC (B220+Fas+GL-7+), and IgG1+ GC (B220+Fas+GL-7+IgG1+) cells derived from the CD45.2 compartment of mixed chimeras reconstituted with a 1:1 ratio of either CD45.1 Il21r+/+CD45.2 Il21r+/+ (left) or CD45.1 Il21r+/+CD45.2 Il21r−/− (right) bone marrow before (C; and D, top) or 6 d after SRBC immunization (D, bottom). In D, each number represents a single recipient mouse from two separate groups of the chimeric mice indicated above the left and right panels.
number of Th cells per GC. It has been speculated that reduced competition for Th cells may lower the stringency of the selection process (Allen et al., 2007).

Memory B cells can form independently of the GC response (Blink et al., 2005; Inamine et al., 2005; Chan et al., 2009). After adoptive transfer of SW<sub>HEL</sub> B cells and HEL<sup>3X</sup> SRBC immunization, donor-derived EM B cells can be identified on the basis of their B220<sup>hi</sup> BCR<sup>hi</sup> phenotype; these cells express low levels of GL-7 and Fas, and high levels of CD38 (Chan et al., 2009). Most of these EM B cells have been reported to be unswitched and of low affinity, and have undergone limited division, suggesting that they have left the response before GC formation (Chan et al., 2009). We investigated the dependence of these memory cells on IL-21. Although GC B cells (BCR<sup>hi</sup>) were reduced both in total numbers and as a proportion of HEL-binding donor cells in the spleens of IL-21-deficient recipients, the proportion of EM B cells (BCR<sup>lo</sup>) out of all HEL-binding donor cells remained comparable (P = 0.786; Fig. 4, D–G), as did total EM B cell numbers (not depicted).

IL-21 acts directly in B cells to regulate Bcl-6 expression in GC cells

Bcl-6 is essential for the generation of the GC response (Dent et al., 1997). We have recently shown that Bcl-6 acts in a gene dose-dependent manner: halving the gene dose of Bcl-6 reduced GCs after SRBC immunization by nearly 50% (Linterman et al., 2009b). In addition, previous work has demonstrated that unmutated memory B cells can form in the absence of Bcl-6 (Toyama et al., 2002) and that IL-21 induces Bcl-6 expression in CD4<sup>+</sup> T cells (Nurieva et al., 2009). Thus, we speculated that the mechanism by which IL-21 contributes to GC formation and affinity maturation might be through induction or maintenance of Bcl-6 in GC B cells.

To test this hypothesis, we quantified Bcl-6 protein levels by flow cytometry in mixed CD45.1 Il21<sup>+/+</sup>/CD45.2 Il21<sup>−/−</sup> chimeric mice 6 d after SRBC immunization. As expected, Bcl-6 was up-regulated in GC B cells compared with non-GC B cells (Fig. 5, A and B). Strikingly, in every mouse analyzed, GC B cells lacking IL-21R expression showed a significant reduction (P = 0.002) in Bcl-6 levels compared with Il21<sup>+/+</sup> GC B cells in the same mouse.
(Fig. 5, B and C). This suggests that IL-21 contributes to GC B cell formation by signaling directly in B cells to induce maximal expression of Bcl-6. There are multiple ways by which Bcl-6 defects may impair affinity maturation: by decreasing the efficiency of somatic hypermutation through direct or indirect effects on AID expression; by promoting early differentiation and exit of GC B cells from the GCs, preventing them from undergoing successive rounds of mutation; or by reducing the ability of GC B cells to interact and elicit survival signals from follicular DCs or Tfh cells.

**IL-21 is not required for pathogen clearance or extracellular humoral immunity after *Salmonella* infection**

Previous reports have revealed that IL-21 can support plasma cell differentiation from naive B cells in vitro (Ettinger et al., 2007). In vivo, large numbers of BLIMP-1+ plasma cells are found in IL-21 transgenic mice (Ozaki et al., 2004), but this study could not differentiate whether the plasma cells were of follicular (GC) or extracellular origin. IL-21–producing T cells have been reported to colocalize with extracellular plasma cell foci in the autoimmune *lpr* mouse strain (Odegard et al., 2008). To investigate whether IL-21 is required for an extracellular response against an infectious pathogen, we chose *Salmonella* infection as a model. The initial humoral response to *Salmonella* species infection requires CD4+ T cell help, and the early phase of the antibody response is derived purely from extracellular plasma cells; GCs do not form until at least 5 wk after infection (Hess et al., 1996; Yrlid and Wick, 2000; Cunningham et al., 2007). *Il21*+/+ and *Il21*−/− mice were infected with live attenuated *Salmonella enterica* serovar Dublin strain SL5631, which also elicits a CD28-dependent antibody response derived exclusively from extracellular foci and dominated by IgG2a production (Linterman et al., 2009a). Consistent with previous reports, GC responses were absent 12 d after *Salmonella* infection (Fig. S4). There was no difference in the amount of *Salmonella*-specific IgM, IgG2a, IgG2b, or IgG3 between *Il21*+/+ and *Il21*−/− mice (Fig. 6 A). Bacterial clearance was also intact in the absence of IL-21 (Fig. 6, B and C). These data demonstrate that normal extracellular antibody responses can be achieved in the absence of IL-21.

Evaluation of antibody responses to other Th1 pathogens such as *Toxoplasma gondii* have shown reduced antibody titers at day 100 after infection in *Il21r*−/− mice (Ozaki et al., 2002). These antibodies most likely derive from GCs, given that extracellular plasma cells are short lived and the half-life of IgGs is <3 wk. Also, in contrast to the response to *Salmonella*, both extracellular foci and GCs with proliferating centroblasts can be observed during *T. gondii* infection (Rifaat et al., 1981). After immunization with ovalbumin or KLH, antigen-specific IgG1 levels in *Il21r*−/− mice were lower than wild type, and antigen-specific IgE was produced at much higher titers (Ozaki et al., 2002). In addition, there was a specific reduction in IgG1+ plasma cells (Ettinger et al., 2008). Collectively, this evidence suggests that IL-21 is dispensable for non-IgG1 extracellular antibody but it is still possible that IL-21 is required for IgG1+ extracellular responses.

This report provides further evidence of the complex biological functions of IL-21 in immune responses, demonstrating direct effects on GC B cells that regulate GC initiation,

![Figure 5. Lack of IL-21R signaling reduces the expression of Bcl-6 in GC B cells.](image-url)

(A) Flow cytometric contour plots indicating the gating strategy for non-GC and GC B220+ cells (left) and BCL-6 expression on GL-7+ (middle) and Fas+ (right) B cells. (B and C) Histogram overlays (B) and bar graphs (C) showing the fluorescence intensity of BCL-6 staining in GC and non-GC B cells as gated in A derived from the CD45.1 *Il21r*+ or CD45.2 *Il21r*− compartment of CD45.1 *Il21r*+CD45.2 *Il21r*− mixed bone marrow chimeras 6 d after SRBC immunization. The horizontal dashed line highlights the median levels of Bcl-6 found in non-GC B cells. Each set of two bars represents the data from the CD45.1 and CD45.2 compartments of a single mouse. MFI, mean fluorescence intensity.
long-term maintenance, production of GC IgG1+ B cells, and affinity maturation. Our data underscore the accumulating evidence of shared signaling pathways in T and B cells to regulate follicular responses: in both GC and Tfh cells, IL-21 regulates expression of the transcriptional regulator that directs their lineage commitment, Bcl-6. The data presented in this paper have implications for immunotherapy: dampening IL-21 signaling may be useful to diminish production of high affinity autoantibodies, and IL-21 may be useful in vaccine development for its ability to promote long-lived and high affinity–matured antibody responses.

MATERIALS AND METHODS

Mice and immunizations. C57BL/6 (B6) and Il21−/− mice were housed in specific pathogen-free conditions at the Australian National University (ANU) Bioscience Facility. Il21−/− mice were housed under specific pathogen-free conditions at the Peter MacCallum Cancer Center. Il21−/− mice were generated at Lexicon Genetics (now Lexicon Pharmaceuticals) and provided by ZymoGenetics, Inc. These mice were backcrossed 12 generations onto the C57BL/6 background. Primers used to amplify polymorphisms that distinguish between the 129 and C57BL/6 genetic backgrounds of the interval comprising the Il2 gene in Il21−/− mice were provided by C. King (Garvan Institute of Medical Research, Sydney, Australia). Il2−/− mice were provided by W. Leonard (National Institutes of Health, Bethesda, MD) and were backcrossed 12 generations onto the C57BL/6 background. SWHEL mice carry a Vk10 κ L chain transgene and a knocked-in VH10 H chain in place of the JH segments of the endogenous IgH gene that encodes a high affinity antibody for HEL (Phan et al., 2003). All animal procedures were approved by the ANU Animal Ethics and Experimentation Committee.

To generate TD GC responses, 8–12-wk-old mice were immunized intraperitoneally with 2 × 10^9 SRBCs (IMVS Veterinary Services).

Adoptive transfer of SWHEL splenocytes. 10^6 CD45.1 SWHEL B cells and 2 × 10^8 SRBCs (IMVS Veterinary Services) conjugated to low affinity HEL3X antigen were adoptively transferred into Il21+/+ or Il21−/− (CD45.2) mice via intravenous injection, as described previously (Paus et al., 2006).

Figure 6. Lack of IL-21 does not alter Salmonella pathogen clearance or production of anti–Salmonella humoral immunity. (A) ELISA analysis of the titers of Salmonella-specific IgM, IgG1, IgG2a, IgG2b, and IgG3 in sera from Il21+/+ (white bars) and Il21−/− (black bars) mice 12 d after Salmonella infection. (B and C) Dot plots of spleen weights (B) and the number of bacteria per liver (C) in Il21+/+ and Il21−/− mice 12 d after infection with Salmonella. Data are representative of two independent experiments. Each symbol represents one mouse and tops of bars are drawn through the median values. nd, not detected.

Bacteria and inoculation. S. enterica serovar Dublin strain SL5631 (Segall and Lindberg, 1991) was grown in Luria-Bertani medium overnight. Mice were inoculated with 5 × 10^5 CFUs from a log-phase culture administered intraperitoneally in PBS. Liver bacterial load was measured 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 streptavidin conjugates for flow cytometry were from BD except where otherwise indicated: anti–mouse B220-PerCP, CD4-PerCP, inducible T cell co-stimulator–FITC (eBioscience), GL-7–FITC, Fas–PE, CXCR5–biotin, PD-1–PE (eBioscience), PNA–biotin (Vector Laboratories) CD8–allophycocyanin (APC), streptavidin–PerCP-Cy5.5, streptavidin–PE-Cy7, CD38–PE, CD45.2–PerCP-Cy5.5, IgG1–PE, Ki-67–A647, HyHEL9–Alexa Fluor 647, B220–APC-Cy7, CD45.1–Pacific blue, Ki-67–Alexa Fluor 647, and Bcl-6 (Santa Cruz Biotechnology, Inc.) conjugated to Alexa Fluor 647 using an antibody conjugation kit (Invitrogen). For immunohistochemistry, the primary antibodies and reagents used were sheep anti–mouse IgD (The Binding Site), biotinylated anti–mouse CD3 (BD), and PNA–biotin (Vector Laboratories); the secondary antibodies used were rat anti–goat IgG and streptavidin–alkaline phosphatase (Vector laboratories); and the tertiary antibody used was rabbit anti–rat horseradish peroxidase (HRP; Dako).
Bone marrow chimeras. Recipient C57BL/6-CD45.1 RAG1−/− mice were sublethally irradiated with 1,000 rad and reconstituted via intravenous injection with 2 × 10^6 donor bone marrow–derived hematopoietic stem cells.

Cell isolation, culture, and stimulation. Single-cell suspensions were prepared from spleens of immunized mice by sieving and gentle pipetting through 70-µm nylon mesh filters (Falcon; BD). Splenocytes were prepared from spleens of immunized mice by sieving and gentle pipetting. Cell isolation, culture, and stimulation.

Flow cytometry. For surface staining, single-cell suspensions were prepared as described in the previous section, and cells were maintained in the dark at 4°C throughout the experiment. Cells were washed twice in ice-cold FACS buffer (2% FCS, 0.1% NaN₃ in PBS), and incubated with each antibody and conjugate layer for 30 min and washed thoroughly with FACS buffer between each layer. Intracellular staining used the Cytofix/Cytoperp kit (BD) according to the manufacturer’s instructions. For detection of Bcl-6 expression and HEL-binding B cells, HyHEL-9 and rabbit polyclonal anti-Bcl-6 were conjugated to Alexa Fluor 647 (Invitrogen) using an antibody labeling kit (Invitrogen). Flow cytometers (FACS Calibur and LSR II; BD) and software (CellQuest or FACS Diva, respectively; BD) were used for the acquisition of flow cytometric data, and FlowJo software (Tree Star, Inc.) was used for analysis.

ELISA. Anti-Salmonella IgM, IgG1, IgG2a, IgG2b, and IgG3 (Southern-Biotech) were detected in plasma from blood taken at days 7 and 12 by ELISA. Fig. S1 shows photomicrographs of spleen sections from B21−/− or B21+/+ mice after SRBC immunization stained with IgD and PNA, and compares expression levels of ST3Gal I, ST6Gal I, and the housekeeping gene actin. Primers specific for ST3Gal I, ST6Gal I, and the housekeeping gene-specific fold change, normalized toβ-actin, was calculated using the 2^-ΔΔCt method.

Statistical analysis. All data were analyzed using the nonparametric Mann-Whitney U test, excluding bone marrow chimera experiments in Fig. 3 and Fig. 5, which were analyzed using a paired Student’s t test. All statistical analyses were performed with Prism software (version 5; GraphPad Software, Inc.).

Online supplemental material. Fig. S1 shows photomicrographs of spleen sections from B21−/− or B21+/+ mice after SRBC immunization stained with IgD and PNA, and compares expression levels of ST3Gal I and ST6Gal I from GC cells in immunized B21−/− or B21+/+ mice and the total number of CD4 + cells in the same mice. Fig. S2 depicts the proportion of Th and GC cells that express K67 from B21−/− and B21+/+ mice after immunization. Fig. S3 presents an alternative way of analyzing the bone marrow chimera data from Fig. 3. Fig. S4 shows the proportion of B cells that have a GC phenotype 12 d after infecting B21−/− and B21+/+ mice with Salmonella. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091738/DC1.

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