

IL-21 regulates germinal center B cell differentiation and proliferation through a B cell–intrinsic mechanism

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Germinal centers (GCs) are sites of B cell proliferation, somatic hypermutation, and selection of variants with improved affinity for antigen. Long-lived memory B cells and plasma cells are also generated in GCs, although how B cell differentiation in GCs is regulated is unclear. IL-21, secreted by T follicular helper cells, is important for adaptive immune responses, although there are conflicting reports on its target cells and mode of action in vivo. We show that the absence of IL-21 signaling profoundly affects the B cell response to protein antigen, reducing splenic and bone marrow plasma cell formation and GC persistence and function, influencing their proliferation, transition into memory B cells, and affinity maturation. Using bone marrow chimeras, we show that these activities are primarily a result of CD3-expressing cells producing IL-21 that acts directly on B cells. Molecularly, IL-21 maintains expression of Bcl-6 in GC B cells. The absence of IL-21 or IL-21 receptor does not abrogate the appearance of T cells in GCs or the appearance of CD4 T cells with a follicular helper phenotype. IL-21 thus controls fate choices of GC B cells directly.

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Abbreviations used: AID, activation-induced cytidine deaminase; ASC, antibody-secreting cell; cDNA, complementary DNA; GC, germinal center; mRNA, messenger RNA; SHM, somatic hypermutation; TD, T cell dependent; Tfh, T follicular helper.

The immunological memory that develops during T cell–dependent (TD) immune responses comprises populations of plasma cells and recirculating antigen-experienced B and T lymphocytes (Tarlinton, 2006). Two compartments of humoral memory, plasma cells and memory B cells, are generated in germinal centers (GCs) that develop within the secondary lymphoid organs during TD responses (Tarlinton, 2006). Although composed primarily of B lymphocytes, GCs contain small numbers of CD4⁺ T cells, dendritic cells, and macrophages and develop in association with antigen localized on the surface of follicular dendritic cells (Haberman and Shlomchik, 2003; Allen et al., 2007). After a period of B cell proliferation, several processes are initiated within the GC that affect affinity maturation whereby the mean binding affinity of antigen-specific antibody increases as a function of time (MacLennan, 1994;

Allen et al., 2007). Affinity maturation is driven in large part by the somatic hypermutation (SHM) of the immunoglobulin V genes of proliferating GC B cells, a process which is mediated by the enzyme activation-induced cytidine deaminase (AID). B cells expressing antigen receptors of improved affinity, usually as a result of SHM, are preserved preferentially. Iterations of proliferation, mutation, and avidity-based selection improve the mean affinity of the responding B cell population (MacLennan, 1994; Allen et al., 2007).

Normally, in an immune response to a protein antigen the vast majority of memory B cells and bone marrow plasma cells arise from the somatically diversified affinity-matured

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population of GC B cells (Tarlinton, 2006). It is inferred that avidity for antigen is a major determinant in plasma cell differentiation of GC B cells, whereas memory B cell formation is more influenced by survival (Lanzavecchia and Sallusto, 2002; Phan et al., 2006; Tarlinton, 2006). It also appears that both types of post-GC B cell are produced throughout the GC reaction rather than being released into the circulation in a single event (Blink et al., 2005). The persistence and continued activity of GC, which is indicated by the continued production of plasma cells and memory B cells and the increasing frequency of V gene mutation, implies that a proportion of GC B cells remain within the GC and undergo additional rounds of proliferation, mutation, and selection (MacLennan, 1994; Allen et al., 2007). B cells within GC therefore have several possible fates: death, division with or without SHM, or differentiation into either the memory B cell or plasma cell compartments.

GC persistence, development, and function absolutely require CD4⁺ T cells. T cells activated by antigen-presenting dendritic cells migrate into the B cell area in part as a result of their up-regulation of CXCR5, a chemokine receptor normally restricted to B cells (Allen et al., 2007). Indeed, the expression of CXCR5 contributes to the definition of what are now called T follicular helper (Tfh) cells (Vinuesa et al., 2005). In addition to CXCR5, Tfh cells are distinguished from other CD4 T cells by their elevated expression of ICOS and CD40L (Vinuesa et al., 2005), both of which are critical for the initiation and maintenance of the GC (Tarlinton, 2006). Intriguingly, up-regulation of many of the molecules that define the Tfh phenotype appears to be mediated by Bcl-6, which is required for their development in a cell-intrinsic manner (Johnston et al., 2009). Tfh cells are also enriched for secretion of IL-21 (Chtanova et al., 2004; Nurieva et al., 2008) and IL-4 (Reinhardt et al., 2009). IL-21 is associated with growth and differentiation of many types of lymphocytes, including B and T cells (Ettinger et al., 2008). The effects of IL-21 on B cells vary depending on the context. In vivo, IL-21R deficiency leads to a state of pan-hypogammaglobulinaemia while promoting high titers of IgE (Ozaki et al., 2002). In vitro, IL-21 has been shown to increase both Blimp-1 and Bcl-6 in B cells (Ozaki et al., 2004; Arguni et al., 2006), suggesting an ability for IL-21 to influence multiple aspects of B cell differentiation. Recent data support the notion that IL-21 has a critical, possibly obligatory, role in the development of Tfh cells and, through this, on the formation of GC (Nurieva et al., 2008; Vogelzang et al., 2008), whereas other data suggest a less universal association (Linterman et al., 2009). IL-21 has also been shown to augment the formation of Th17 cells (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007), which have been shown to both secrete IL-21 and promote GC formation in a mouse model of autoimmunity (Hsu et al., 2008), strengthening the view that the effects of IL-21 on GC activity are T cell mediated. An earlier study, however, using IL-21R-deficient mice reported GC and memory development to be normal (Ozaki et al., 2002), raising uncertainty as to exactly what the

requirement of IL-21 may be in the GC reaction, on which cell types it may act, and what its activities might be. This uncertainty has been heightened by recent publications suggesting that IL-4 is a key mediator of Tfh activity (King and Mohrs, 2009; Reinhardt et al., 2009).

Given the multitude of potential roles for IL-21 on lymphocyte behavior (Ettinger et al., 2008), we wished to assess the development of a humoral immune response in mice lacking IL-21 or the IL-21R. These experiments confirmed a role for IL-21 in the formation of plasma cells, contradicted a mandatory autocrine role for IL-21 in Tfh development or function, and revealed a previously undefined role for this cytokine in the GC reaction and the regulation of their output. These actions of IL-21 on B cells were direct, as they were replicated by the selective absence of the IL-21R on B cells and not on T cells, suggesting that the major activity of IL-21 in the GC is on B cells and is not to establish or maintain cells of a Tfh phenotype.

RESULTS

IL-21 is required for normal plasma cell formation

Previous experiments have shown that IL-21 can act as a potent inducer of immunoglobulin class switch recombination and B cell differentiation in both human and mouse B cells (Ettinger et al., 2008). We therefore examined the development and localization of antibody-secreting cells (ASCs) in spleen and bone marrow of IL-21R^{-/-} and IL-21^{-/-} mice after immunization with a TD antigen delivered i.p. in alum and compared this with the response of C57BL/6 (control) mice. 7 d after immunization, the frequency of antigen-specific IgG1 ASC in the spleens of control mice was significantly higher than in either IL-21R^{-/-} or IL-21^{-/-} mice (Fig. 1 A, left). These IgG1 ASCs were located in and around the bridging channels in spleen sections of day-7 immunized mice, although at reduced frequency in mutant spleens (Fig. 1 B and not depicted). The frequency of NP-specific IgG1 ASC in the spleens of all strains declined between days 7 and 28, with approximately two- to threefold more remaining in the spleens of control mice compared with IL-21R^{-/-} and IL-21^{-/-} mice at day 28. We also examined IgE production in response to immunization and, as expected from previous studies (Ozaki et al., 2002), found this to be elevated (Fig. S1). Affinity maturation among splenic ASC was assessed by determining the proportion of secreting antibody capable of binding a low-level haptenation plate coat (Roes and Rajewsky, 1993). At day 28, the proportion of NP-specific ASC that was of high affinity in the spleens of IL-21^{-/-} and IL-21R^{-/-} mice was significantly reduced compared with control mice (Fig. 1 A, right), suggesting a defect in affinity maturation.

To assess GC-derived ASC, we examined the frequency of NP-specific IgG1 ASC in the bone marrow (Fig. 1 C), a site enriched in GC-derived plasma cells (Smith et al., 1997; Takahashi et al., 1998). The mean frequency of NP-specific IgG1 ASC in the knockout mice was less than in control mice at both 14 and 28 d after immunization, reaching statistical significance at the later time. The proportion of NP-specific ASC secreting high-affinity IgG1, however, was significantly

lower in the bone marrow of IL-21^{-/-} and IL-21R^{-/-} mice than in C57BL/6 at both day 14 and day 28 after immunization (Fig. 1 C, right). Thus, IL-21 has a role in two aspects of ASC development after immunization: in the expansion of early extrafollicular ASC in spleen and in the production of high-affinity ASC resident in the bone marrow.

A GC abnormality in the absence of IL-21 signaling

The reduced frequency and affinity of NP-specific bone marrow plasma cells indicated a possible GC abnormality in the immune response of IL-21^{-/-} and IL-21R^{-/-} mice. We therefore investigated GC formation in spleen by immunizing mice as before and determining both the distribution of

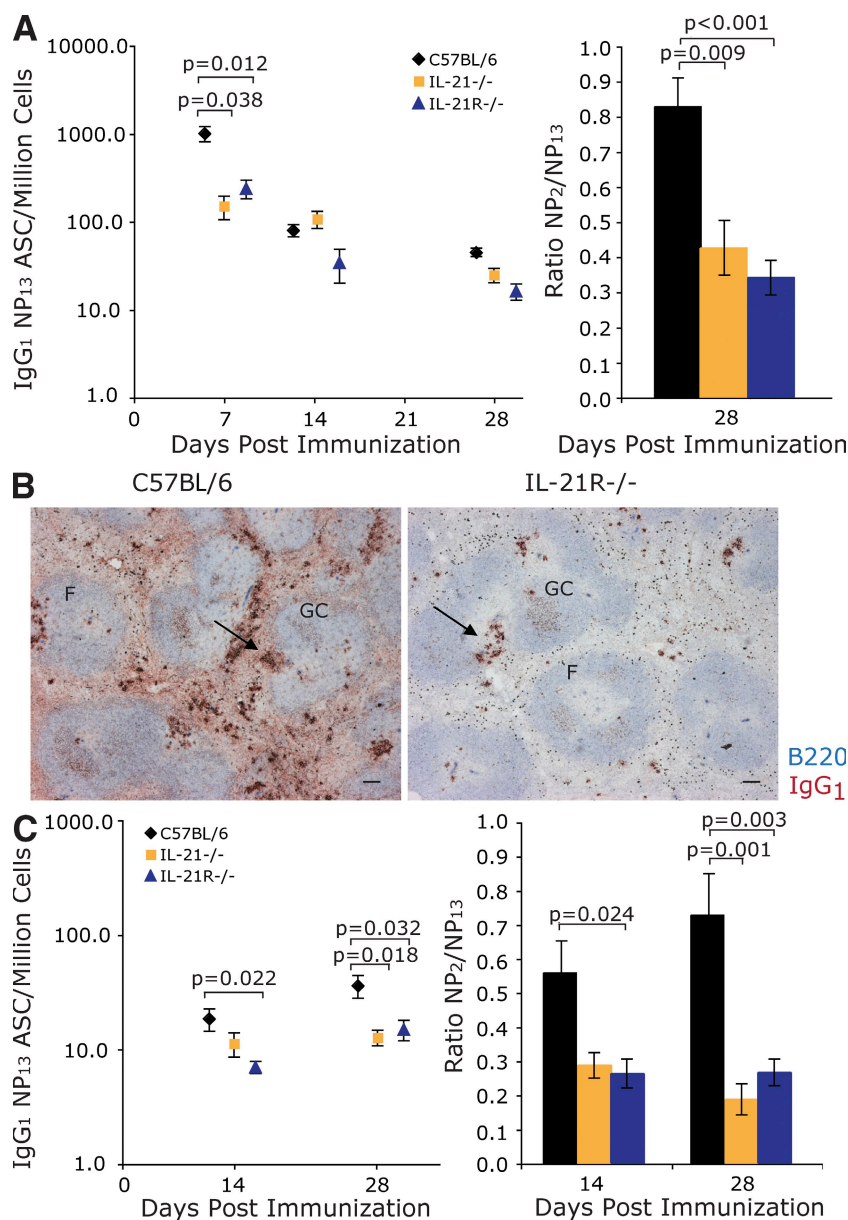


Figure 1. Loss of IL-21 or IL-21R affects plasma cell formation after TD immunization. (A) The frequency of NP-specific IgG1 ASC among splenocytes from IL-21^{-/-}, IL-21R^{-/-}, and C57BL/6 mice, immunized i.p. with NP-KLH in alum, was assessed by ELISpot at the indicated times after immunization. The results are presented as ASC per million splenocytes (left). At day 28, the ratio of high-affinity (NP₂ binding) to total (NP₁₃ binding) IgG1 NP-specific ASC was determined using differentially haptenated plate coats (right). (B) Immunohistochemical staining of spleen sections from C57BL/6 and IL-21R^{-/-} mice at day 7 after immunization. Blue is B220, identifying B cell follicles (F), and red is IgG1, revealing foci of plasma cells (arrows) in C57BL/6 spleens with few in IL-21R^{-/-} spleens. IgG1 staining in follicles marks presumptive immune complexes within GCs. Bars, 100 μ m. (C) After immunization, NP-specific IgG1 ASCs appear in the bone marrow of both C57BL/6 and IL-21/IL-21R^{-/-} mice but accumulate only in controls (left), whereas affinity maturation also occurs only in control mice (right) as measured by ELISpot using differentially haptenated plate coats. Results are derived from at least three experiments with 4–10 mice per time point per genotype. Error bars for ELISpot frequency (left) and ratio (right) are SEM. Differences were examined using a Student's *t* test and only marked if significant.

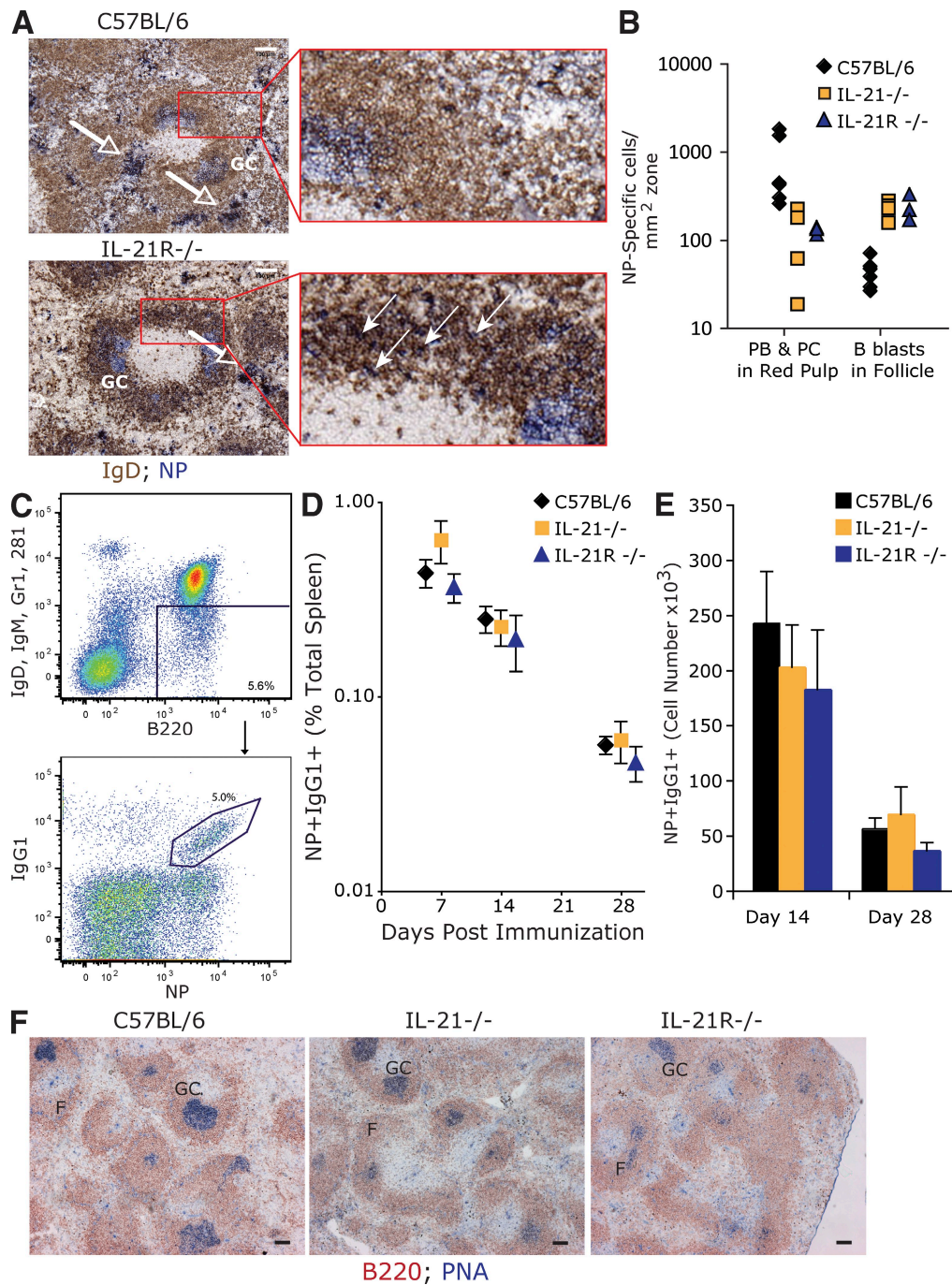


Figure 2. Intrafollicular response in IL-21^{-/-} and IL-21R^{-/-} mice. (A) Representative spleen histology staining revealing the localization of NP-reactive cells (blue) 7 d after immunization in control and IL-21R^{-/-} mice. B cells follicles were stained with IgD (brown). GCs are indicated, as are foci of ASC (open arrows). NP-reactive B cells in follicles are shown by closed arrows in expanded region. Bars, 100 μ m. (B) Quantification of the histology staining shown in A from C57BL/6, IL-21^{-/-}, and IL-21R^{-/-} mice of NP-specific plasmablasts (PBs) and plasma cells (PCs) in the red pulp and NP-specific B cell blasts in the follicles. (C) Flow cytometric scheme for the analysis of antigen-specific B cells in the spleens of NP immunized mice. Spleen cells were partitioned into isotype-switched B cells, and the percentage of those cells that were IgG1⁺ and NP binding was determined to calculate the fraction of total spleen involved in the B cell response. Values shown are the percentage of events displayed and not overall splenic frequencies. (D) Frequency distribution of antigen-reactive B cells in the spleen after immunization of C57BL/6, IL-21^{-/-} and IL-21R^{-/-} mice. (E) Total spleen cell number of antigen-reactive B cells in C57BL/6, IL-21^{-/-}, and IL-21R^{-/-} mice after immunization at the times indicated. Data in D and E are representative of at least three independent experiments of between 5 and 11 mice at each time point. Error bars are \pm SEM. Differences were examined using a Student's *t* test and only marked if significant. (F) Representative appearance of GC in the spleen by immunohistochemistry, 14 d after immunization, from C57BL/6, IL-21^{-/-}, and IL-21R^{-/-} mice. GC was revealed with PNA (blue). Follicles (F) revealed with B220 (red). Bars, 100 μ m.

antigen-specific cells by immunohistochemistry and their frequency by flow cytometry (Fig. 2). Resolving the location and nature of NP-reactive B cells revealed substantial differences between knockout and control mice. Although NP-specific cells were identified as ASC in bridging channels and in GC within follicles in spleens of all mice 7 d after immunization, there was an unusual accumulation of NP-binding B cell blasts in the follicular mantles of the knockout strains (Fig. 2, A and B; and Fig. S2 B). Parenthetically, this staining revealed NP-specific cells irrespective of isotype, showing

that the deficiency in ASC in the knockout spleens shown in Fig. 1 is a defect in development and not in isotype switching. NP staining also revealed an unexpected role for IL-21 in migration of antigen-activated B cells early in the immune response. Despite their aberrant localization, the frequency and total number of NP-specific B cells in spleens of immunized IL-21^{-/-} and IL-21R^{-/-} mice at day 7 were approximately equal to those in control mice (Fig. 2, C–E). In fact the representation of NP⁺IgG1⁺ B cells in the spleens of the different groups was similar at all times examined, up

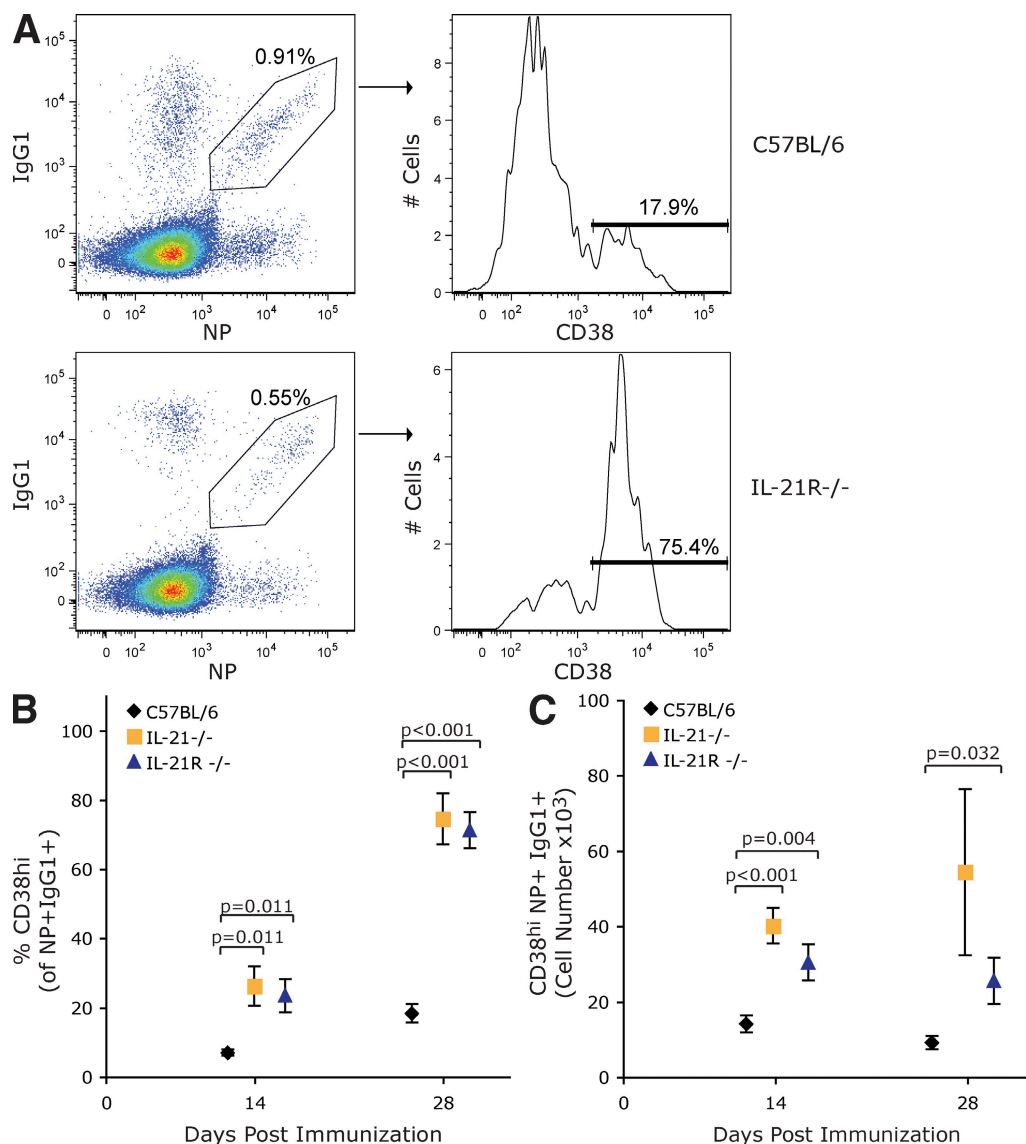


Figure 3. Accelerated formation of memory B cell compartment in IL-21^{-/-} and IL-21R^{-/-} Deficient Mice. (A) Representative flow cytometric plots resolving GC and memory compartments among NP-specific B cells in spleens of C57BL/6 and IL-21R^{-/-} mice 28 d after immunization. Splenocytes, previously gated to include only isotype-switched B cells, were assessed for expression of IgG1 and binding of NP (left). Expression of CD38 on NP⁺IgG1⁺ B cells resolved memory (CD38^{hi}) and GC (CD38^{lo}) B cells (right). The percentages are the proportion of displayed events falling within the indicated NP⁺IgG1⁺ and CD38^{hi} gates. (B) Proportion of NP-specific B cells with a memory phenotype at times indicated after immunization of C57BL/6, IL-21^{-/-}, and IL-21R^{-/-} mice. (C) Absolute numbers of NP-specific IgG1 memory B cells in spleens of C57BL/6, IL-21^{-/-}, and IL-21R^{-/-} mice at the indicated times after immunization. Symbols in B and C indicate the mean of between 5 and 11 mice at each time point, \pm SEM, and derived from at least three independent experiments. Differences were examined using a Student's *t* test and only marked if significant.

Table I. Distribution of V_H gene somatic mutation in IL-21^{-/-} and IL-21R^{-/-} memory B cells

Genotype	VH186.2 ⁻	VH186.2 ⁺ a	Unmutated	Position 33 mutation ^b	V_H mutation frequency	Excluding germline sequences	
						Position 33 W→L ^b	V_H mutation frequency
			% (number)	% (number)		%	
C57BL/6	4	24	8 (2)	54 (13)	5.1 ± 3.1	59	5.55 ± 2.8
IL-21 ^{-/-}	1	12	42 (5)	8 (1) [#]	1.7 ± 2.5**	14 [#]	2.86 ± 2.8 [#]
IL-21R ^{-/-}	3	28	61 (17)	7 (2) [*]	1.0 ± 1.9**	18 [#]	2.64 ± 2.2**

The V_H mutation frequency is shown as mean mutation per V_H gene. The V_H mutation frequency ranges are the following: C57BL/6, 0–8; IL-21^{-/-}, 0–9; IL-21R^{-/-}, 0–7.

[#], $P < 0.05$; ^{*}, $P < 0.005$; ^{**}, $P < 0.001$.

^aSequences from single cell PCR were analyzed exactly as previously described (Blink et al., 2005) to define VH186.2 and mutations.

^bPercentage of sequences containing mutations that results in an amino acid replacement of tryptophan with leucine at position 33.

to day 53 (Fig. 2, D and E; and not depicted). Despite the early differences in NP-specific B cell location, GC histology in day-14 immunized knockout and control mice was very similar (Fig. 2 F), but by day 28 after immunization, GC in the IL-21/IL-21R^{-/-} spleens were markedly smaller than those in controls (Fig. S2, C and D).

The discrepancy between histologically reduced GC at day 28 yet similar numbers of NP-reactive B cells in the spleen was reconciled by resolving NP-specific IgG1⁺ B cells into GC and memory compartments using expression of CD38 (Oliver et al., 1997; Ridderstad and Tarlinton, 1998) measured by flow cytometry (Fig. 3 A). These analyses revealed the proportion of NP-specific B cells with a memory phenotype to increase in control mice from <10% at day 14 to ~20% at day 28 (Fig. 3 B), which is consistent with other examples of this immunization protocol (Takahashi et al., 2001). Memory B cell representation in the IL-21 and IL-21R knockout spleens, which was already significantly increased at day 14 compared with controls, reached >70% of antigen-specific B cells by day 28 (Fig. 3 B). The absolute number of NP⁺IgG1⁺ memory B cells in the knockout mice was significantly different from controls at days 14 (IL-21 and IL-21R) and 28 (IL-21R), despite not increasing over that period (Fig. 3 C). Thus, the increasing representation of memory B cells seen by flow cytometry in immunized IL-21 and IL-21R knockout mice was a result of the accelerated loss of GC B cells, as indicated by histology. Importantly, the memory B cells formed in the knockout strains persisted and were functional, in that boosting at day 53 with soluble antigen increased the frequency of ASC (Fig. S3). The formation of GC in the absence of IL-21R or IL-21 supports a previous study (Ozaki et al., 2002) and contradicts others (Nurieva et al., 2008; Vogelzang et al., 2008), whereas the subsequent premature dissolution reveals a unique role for IL-21 signaling in the maintenance of GC. In considering whether other potential Tfh cytokines had effects similar to those of IL-21, we immunized IL-4-deficient mice. These mice showed proportions of GC and memory B cells similar to controls at all time points measured, albeit at significantly reduced frequencies (Fig. S4), suggesting distinct roles for IL-21 and IL-4 in the GC (Reinhardt et al., 2009).

Tfh development in IL-21-deficient mice

The abnormal development of ASC and GC in IL-21^{-/-} and IL-21R^{-/-} mice raised the question of the nature of the T cell help in these mice (Nurieva et al., 2008; Vogelzang et al., 2008). We examined spleens of immunized control, IL-21^{-/-}, and IL-21R^{-/-} mice for CD4⁺ T cells coexpressing CXCR5 and PD1, a combination of markers which is widely used in identifying Tfh cells (Vinuesa et al., 2005), and found such cells in all groups, albeit at somewhat variable frequency at the time points assessed (Fig. 4, A and B). Interpreting the frequency of Tfh in IL-21 knockout mice, however, is complicated by the GC B cell phenotype in these mice and the possibility that Tfh development, persistence, or localization is influenced by the presence of GC B cells. We therefore used histology to determine if CD3⁺ cells were in fact located within the GC of IL-21-deficient mice at day 14. Such cells were clearly visible and appeared to be distributed equivalently with those in controls (Fig. 4 C). These results suggest that although IL-21 may have a role in Tfh expansion or persistence, it is not required for their appearance, localization, or ability to support GC formation.

Attenuated V gene mutation and selection in the absence of IL-21

To determine if the premature dissolution of GC and expansion of memory B cells in mice deficient for IL-21 or IL-21R might alter the incidence of SHM among those memory B cells, single NP-specific IgG1⁺ memory B cells (B220⁺NP⁺IgG1⁺CD38^{hi}) were isolated from the spleens of IL-21^{-/-}, IL-21R^{-/-}, and control mice 28 d after immunization. RNA was extracted from these single cells, complementary DNA (cDNA) synthesized, and rearrangements involving VH186.2 and Cγ1 amplified by PCR, this being the dominant rearrangement in the response of C57BL/6 mice to NP (Cumano and Rajewsky, 1985). Comparing the sequence of these amplified transcripts to the germline VH186.2 sequence revealed a significant reduction in the proportion of knockout NP-specific memory B cells carrying mutated V_H genes, in the mean frequency of SHM, and in the proportion of V_H 186.2 genes carrying the affinity enhancing exchange of tryptophan for leucine at position 33 (Table I). The significance of the

reduced frequency of SHM or representation of tryptophan at V_H position 33 in the knockout memory B cells was not reversed by excluding germline sequences from the analysis (Table I). It appeared, therefore, that memory B cells in the IL-21^{-/-} and IL-21R^{-/-} mice had not undergone as many rounds of mutation and selection as those in controls. The groups did not differ significantly in the degree of either

enrichment for, or selection against, amino acid replacements in the CDRs and the framework regions, respectively (unpublished data). Reduced affinity maturation in the memory compartment of IL-21R^{-/-} mice was also apparent in the lower frequency of high-affinity ASC produced after boost immunization (Fig. S3). Thus, immunization of IL-21^{-/-} and IL-21R^{-/-} mice produced memory B cell compartments that

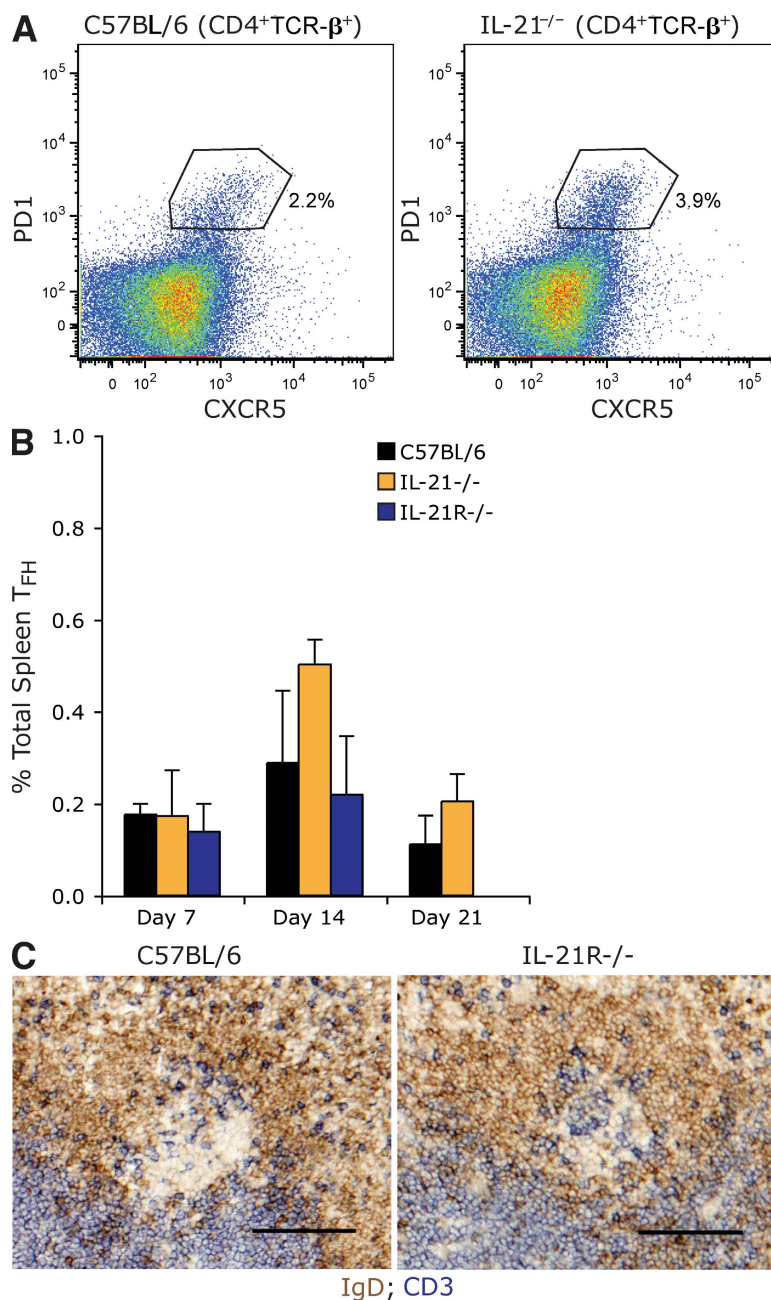


Figure 4. IL-21 is not essential for Tfh cell development. (A) Representative flow cytometry gating for identifying Tfh cells among splenocytes. CD4⁺TCR-β⁺ cells were assessed for expression of PD1 and CXCR5 to resolve Tfh cells in C57BL/6 and IL-21^{-/-} mice. Percentages are of the CD4⁺TCR-β⁺ population. (B) Frequency of Tfh cells (CD4⁺TCR-β⁺PD1⁺CXCR5⁺) in C57BL/6, IL-21^{-/-}, and IL-21R^{-/-} mice at times indicated after immunization. Values are mean frequency of two to six mice per time point from at least two experiments and error bars are ± SD. (C) Immunohistochemical staining of CD3⁺ cells (blue) in the GCs of the spleens from day 14 immunized C57BL/6 and IL-21R^{-/-} mice. The example shown is representative of at least four mice from two experiments for each genotype. B cell follicles were revealed with IgD (brown). Bars, 100 μm.

were less mutated and less stringently selected for affinity than controls, a difference which may contribute to the reduced affinity maturation among plasma cells (Fig. 1).

IL-21 acts directly on B cells

The abnormalities in the immune response of intact IL-21^{-/-} and IL-21R^{-/-} mice did not distinguish between IL-21 acting directly on the B cells or having an effect via, for example, T_H or T_H17 cells, both of which are known to respond to and produce IL-21 and both of which are implicated in GC development (Hsu et al., 2008; Nurieva et al., 2008; Vogelzang et al., 2008). To resolve the impact of IL-21 on B cells and T cells, a series of chimeric mice were made by reconstituting irradiated IL-21R^{-/-} or IL-21^{-/-} recipients with mixtures of donor bone marrow (Fig. 5, A and D). In the first series, IL-21^{-/-} and IL-21R^{-/-} recipients were reconstituted with a 4:1 mixture of CD3 ϵ ^{-/-} and Ly5.1, CD3 ϵ ^{-/-} and IL-21^{-/-}, or CD3 ϵ ^{-/-} and IL-21R^{-/-} bone marrow such that CD3 T cells in these mice were Ly5.1 WT, unable to secrete IL-21, or lacked the IL-21R, whereas the vast majority of other leukocytes were WT. 8 wk after reconstitution, mice were immunized with NP-KLH in alum and the frequency of NP-specific B cells was determined at days 14 and 28 of the response (Fig. 5 B). Although the overall B cell response to NP was similar in all groups, declining by an equivalent degree between days 14 and 28, the distribution of NP-specific B cells into the GC and memory compartments was strikingly different. As in the intact IL-21-deficient mice (Fig. 2), there was a near threefold increase in the representation of memory B cells at day 28 compared with controls in the CD3 ϵ ^{-/-}/IL-21^{-/-} chimeras in which the T cells were unable to secrete IL-21. The CD3 ϵ ^{-/-}/IL-21R^{-/-} group, in which T cells lacked the IL-21R, showed a slight, albeit significant, increase over controls (Fig. 5 C). Despite the ability of NKT cells to secrete IL-21 (Harada et al., 2006), they were ruled out as the source of IL-21 through analysis of TCR α 18^{-/-} immunized mice (Fig. S5).

In separate experiments, irradiated mice were reconstituted with a 1:1 mixture of Ly5.1 control and Ly5.2 IL-21R-deficient bone marrow and were thus chimeric for IL-21R^{+/+} (Ly5.1) and IL-21R^{-/-} (Ly5.2) lymphocytes (Fig. 5 D). These mice were immunized and the relative contribution of the two types of B cells to the immune response was determined by flow cytometry (Fig. 5 E). Control and IL-21R^{-/-} B cells were present among the total NP-reactive IgG1⁺ B cell population at days 14 and 28 at near 50:50 ratios, reflecting the chimerization of naive B cells. The proportion of NP⁺IgG1⁺ B cells with a CD38^{hi} memory phenotype, however, was near threefold increased among the IL-21R^{-/-} B cells at day 28, compared with the control IL-21R^{+/+} NP⁺IgG1⁺ B cells in the same spleens (Fig. 5 F). Thus, the loss of GC B cells seen in intact IL-21R^{-/-} mice was replicated in mixed chimeras in which the B cells lacked the IL-21R but not in chimeras in which T cells were IL-21R deficient. These chimera experiments show conclusively that the GC phenotype apparent in intact IL-21^{-/-} and IL-21R^{-/-} mice is the result of a failure

of CD3⁺ cells to secrete IL-21 and a failure of GC B cells to bind the IL-21, respectively. Collectively these data show that IL-21 mediates its GC effects by acting directly on B cells and not via a T cell subset.

Maintenance of B cell proliferation in GC requires IL-21R

We next determined if the loss of GC B cells in the absence of IL-21R was associated with altered proliferation by quantifying the fraction of such B cells in cell cycle at varying times after immunization. Control and IL-21R^{-/-} mice, immunized with NP-KLH in alum 7, 14, or 21 d earlier, were sacrificed and the isotype switched, and NP-binding immunoglobulin light chain lambda (λ_1 ⁺) B cells were purified from spleen by cell sorting after magnetic enrichment for λ_1 ⁺ B cells (Fig. 6 A), which together with V_H186.2 comprises the canonical anti-NP BCR in C57BL/6 mice (Cumano and Rajewsky, 1985). The representation of λ_1 ⁺ B cells among splenocytes from control and IL-21R^{-/-} mice at these times ranged from 2.0 \pm 0.2 to 1.6 \pm 0.4% (mean \pm SEM) with no significant differences. NP-specific GC (CD38^{lo}) B cells were identified by flow cytometry as indicated (Fig. 6 A) and the proportion of these cells in cell cycle (DNA content > 2n) determined by staining the nuclei with propidium iodide. There was no significant difference in the proportion of antigen-specific GC B cells in cell cycle in IL-21^{-/-} compared with control mice at day 7 (Fig. 6 B). By day 14, however, a significant difference was apparent between the two groups with a mean of <20% of IL-21^{-/-} GC B cells in cell cycle compared with \sim 40% in controls (Fig. 6 B). This difference persisted through to day 21 (Fig. 6 B). Diminished proliferation of IL-21R-deficient GC B cells was observed using the alternative approach of measuring BrdU incorporation into antigen-specific B cells at days 7, 14, and 28 after immunization (Fig. S6). Thus, in the absence of IL-21 signaling, GC B cell proliferation was curtailed significantly at later time points.

DISCUSSION

This paper confirms a requirement for IL-21 in the efficient formation of early ASC (Ozaki et al., 2002; Ettinger et al., 2008; Odegard et al., 2008) and extends these results to show an important role for this cytokine in the persistence of GC. This function of IL-21 was revealed by the accelerated resolution of the GC reaction with a concomitant premature formation of an antigen-specific memory B cell compartment in mice lacking either IL-21R or IL-21. This effect was, importantly, mediated by IL-21 acting directly on GC B cells and was not replicated by loss of IL-4. The accelerated dissolution of GC in the absence of IL-21 binding to its receptor also affects the bone marrow ASC compartment, which is not unexpected given that these cells also originate in the GC. As a consequence when compared with controls, the bone marrow plasma cell compartment was underpopulated and significantly reduced in affinity maturation in the mutant mice, which is consistent with its formation having been prematurely curtailed. Analysis of GC B cell proliferation at different time points in the immune response in IL-21 mutant

mice revealed it to be initially normal but to then diminish significantly in a later stage of the response. Collectively, these data provide a unique insight into the means by which T cells sustain and regulate GC B cell behavior.

Memory in the absence of IL-21

An intriguing aspect of the formation of B cell memory in the absence of IL-21 is the prevalence of unmutated V_H gene sequences among the memory B cells, raising the possibility

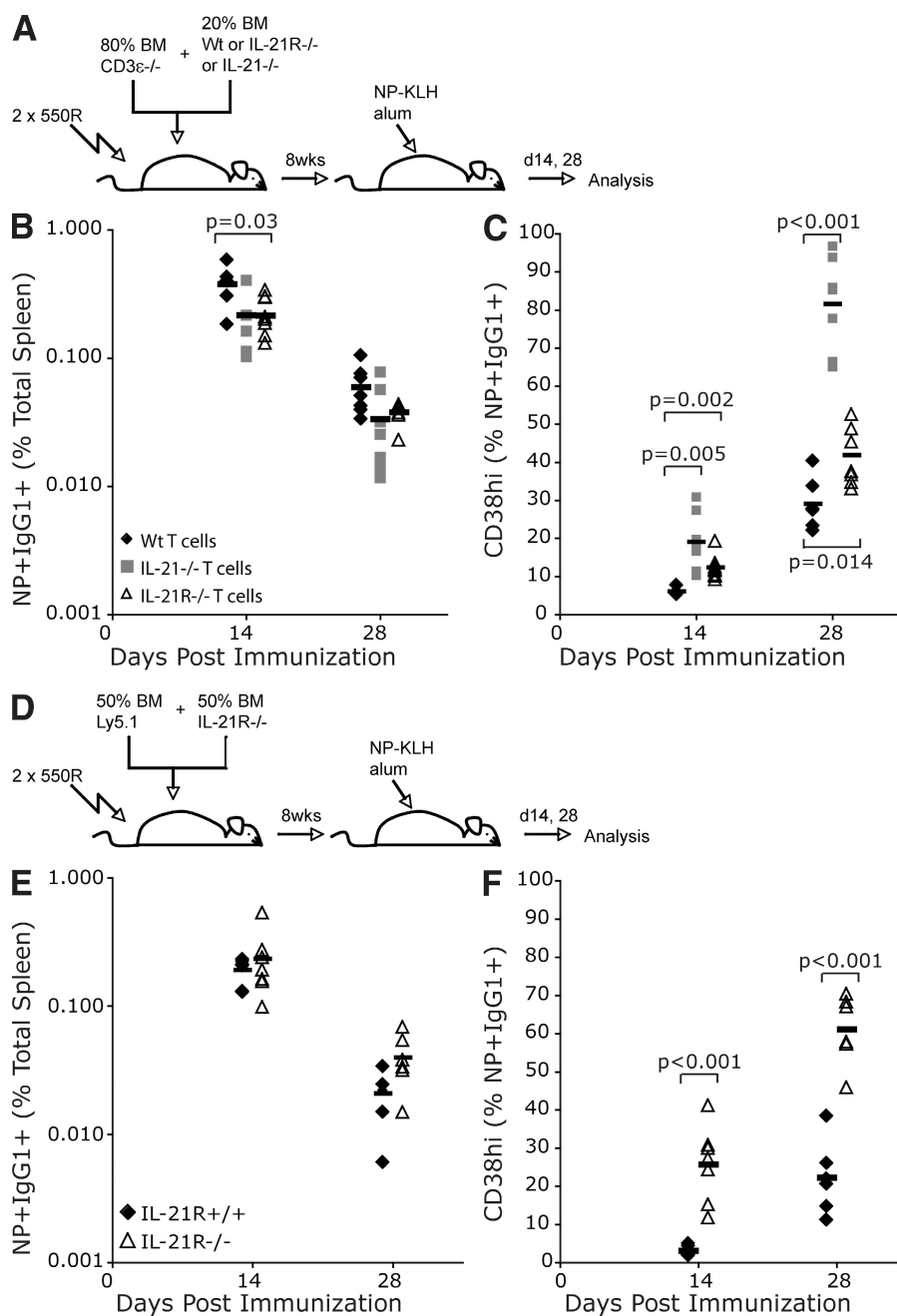


Figure 5. Radiation chimeras show IL-21 acts directly on B cells. (A) Production of chimeric mice with IL-21 or IL-21R deficiency restricted to the T cells. Irradiated recipients were reconstituted with a 1:4 ratio of IL-21R^{-/-} and CD3^{-/-}, IL-21^{-/-} and CD3^{-/-}, or WT and CD3^{-/-} bone marrows and subsequently immunized with NP-KLH in alum. (B) Symbols depict the frequency of NP+IgG1⁺ B cells in individual chimeric mice at the indicated time in the presence of WT T cells, IL-21^{-/-} T cells, and IL-21R^{-/-} T cells. Mean is indicated by the bar and data are from two independent experiments. (C) The proportion of memory cells among the NP+IgG1⁺ B populations shown in B. (D) Production of chimeric mice reconstituted with a 1:1 ratio of C57BL/6 (Ly5.1) and IL-21R^{-/-} (Ly5.2) bone marrow cells. (E) Frequency of NP+IgG1⁺ B cells in individual mice, partitioned according to Ly5 allotype (IL-21R status) at the indicated times with mean shown by the bar. Data are from two independent experiments. (F) The proportion of the NP-specific B cells shown in E with a CD38^{hi} memory phenotype. Significant differences were determined by a Student's *t* test and are indicated.

that these B cells never entered into or remained within the GC for any extended period. When coupled with the early appearance of memory phenotype B cells and the mislocalization of antigen-specific B cells in the mantle zone early in the response, the lack of mutations may indicate an alternate route for memory formation for a portion of the responding B cells. Germline isotype-switched memory B cell compartments have been reported in mice reconstituted with Bcl-6-deficient bone marrow (Toyama et al., 2002) and in mice treated shortly after immunization with an ICOS antagonistic antibody (Inamine et al., 2005). In both cases, the germline memory was considered to have arisen from antigen-driven B cell expansion outside GCs. It is intriguing to speculate that the NP⁺IgG1⁺ B cells carrying unmutated V_H genes at day 28, seen in both the IL-21 and IL-21R mutant mice, are derived from the NP⁺ cells located in the mantle zone early in the response, cells which were Bcl6 negative (unpublished data). Why, in the absence of IL-21, a fraction of reactive B cells are mislocalized is an intriguing question. B cells exposed to antigen initially

migrate to the T cell border, where they receive signals from antigen-activated CD4 T cells that promote their further migration to either the bridging channels as plasmablasts or their reentry to the follicles for the formation of GC (Batista and Harwood, 2009). Recently, the orphan G protein-coupled receptor Ebi2 (also called GPR183) has been shown to have an important role in this bifurcation, as its up-regulation is associated with extrafollicular plasmablast formation and down-regulation with B cell localization on follicular dendritic cells (Gatto et al., 2009). Interestingly, Ebi2 is repressed by Bcl6 (Shaffer et al., 2001), itself a target of IL-21 in B cells (Ozaki et al., 2004; Arguni et al., 2006). It may be that early in an immune response, the absence of IL-21 reduces the prevalence of Ebi2 up-regulation among antigen-reactive B cells, leaving such cells in the follicles with the appearance of non-GC-derived memory. Consistent with that, GC B cells from IL-21 deficient mice contain less Bcl6 messenger RNA (mRNA) and, in vitro, exposure to IL-21 reduces Ebi2 mRNA in CD40L-stimulated B cells (Fig. S6).

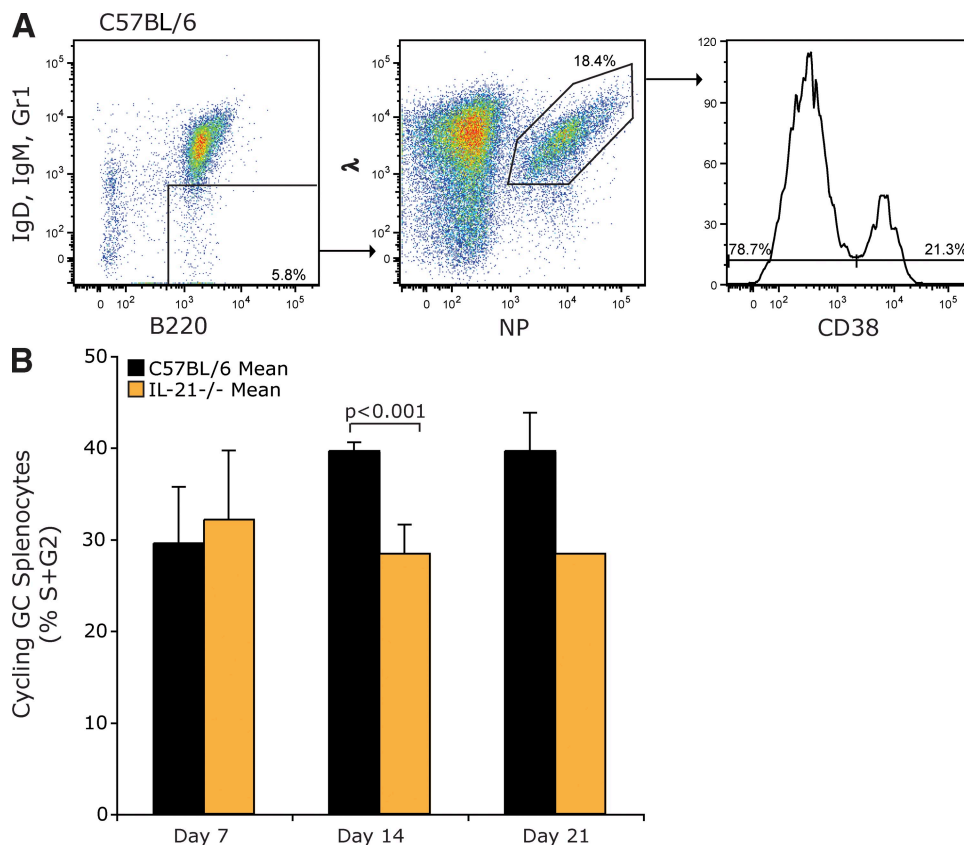


Figure 6. Loss of IL-21 signaling reduces B cell proliferation in GCs. (A) Representative flow cytometric plots for the identification and isolation of NP-specific B cells within the spleens of immunized mice. Spleen cells were enriched for Ig lambda (λ_1) expression using magnetic separation then fractionated into isotype switched B cells and the percent of Ig λ ⁺ NP binding among those cells determined. Expression of CD38 was used to differentiate germinal GC (CD38^{lo}) from memory B cells (CD38^{hi}) and gates for sorting NP-specific GC B cells set accordingly. (B) Proportion of GC B cells (NP⁺ λ ⁺CD38^{lo}) cells in (S + G2) phase in C57BL/6 and IL-21^{-/-} mice at times indicated. Values are derived from two experiments and are the mean of between three and eight mice (\pm SD) at each time. Significant difference at day 14 was determined by a Student's *t* test. Day-21 data for IL-21-deficient mice are from a pool of three animals because low numbers of GC B cells prevented individual analysis.

GC activity incorporating the role of IL-21 in B cell behavior

Data presented in this paper are consistent with IL-21 having a key role in maintaining GC B cell proliferation and regulating differentiation. Within the GC, B cell proliferation and differentiation are typically assigned to distinct regions and distinct cell types, dark and light zones, and centroblasts and centrocytes, respectively. There are relatively few known molecules that distinguish between these zones and cell types. Bcl6 is highest in centroblasts, which are proliferating and mutating, and lowest in centrocytes (Falini et al., 2000; Cattoretti et al., 2006). IRF4, induced by CD40 stimulation in GC B cells and able to directly repress *Bcl-6* transcription (Saito et al., 2007), is apparently restricted to centrocytes in established GC (Cattoretti et al., 2006). Although such differences between GC cell types presumably facilitate the unique biological activities within each, it is somewhat less clear what drives the interchange between centroblast and centrocyte. Centrocytes are considered the GC B cells that undergo differentiation, being out of cell cycle and in proximity to CD4⁺ T cells, which deliver the stimuli that promote survival, proliferation, and differentiation. The enhanced memory formation, diminished GC B cell proliferation, and premature GC dissolution observed in the IL-21 mutants may indicate a role for IL-21 in promoting the transition of centrocytes to centroblasts. Using CXCR4 expression as a marker, we found normal proportions of centroblasts and centrocytes among GC B cells in IL-21 mutants at all times examined (unpublished data), which argues against this transition being blocked in the absence of IL-21. Similarly, we did not observe increased apoptosis among GC B cells in our analysis of cell cycle distribution. Our data support at a minimum the conclusion that IL-21 acts to enhance GC B cell proliferation, which is consistent with IL-21's reported activity on B cells in vitro (Ettinger et al., 2008). The role of IL-21 in the generation of plasma cells is not yet resolved. The extrafollicular plasma cell deficiency could be the cumulative effect of reduced proliferation, differentiation, as previously reported (Odegard et al., 2008), or even altered migration. Bone marrow plasma cell defects could be accounted for by the premature termination of the GC reaction rather than defective differentiation or selection by themselves.

IL-21 and the development of Tfh

An alternative explanation for the GC defect in IL-21 deficient mice is that IL-21 is required for the induction or function of a CD4 T cell subset that is essential for GC maintenance or activity. IL-21 has been shown through in vitro experiments to be capable of generating Th17 cells, which have been shown to secrete IL-21 along with IL-17 (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). Although there is uncertainty about the stringency of the requirement for IL-21 in the formation of Th17 in vivo (Coquet et al., 2008; Sonderegger et al., 2008), it is possible that this T cell subset may influence GC function, as IL-17 has been shown to influence GC B cell activity in at least one mouse strain (Hsu et al., 2008). Of greater relevance are studies describing

a requirement for IL-21 in the formation of CD4⁺ CXCR5⁺ Tfh cells (Nurieva et al., 2008; Vogelzang et al., 2008), identified as being the T cells within the GC (Vinuesa et al., 2005). These studies show that in the absence of IL-21 or its receptor, development of Tfh is diminished and GC do not arise after immunization. Interestingly, the original study on IL-21R^{-/-} mice (Ozaki et al., 2002) detected GC after immunization and we found CD4⁺ T cells with the phenotype of Tfh in mice incapable of IL-21 signaling (Fig. 4). Two possible reasons for the discrepancy between our GC results and those published earlier are differences in the formulation of antigen (alum in our case rather than sheep red blood cells or CFA) and kinetics. The fact that we observe Tfh development in the absence of IL-21 shows clearly that any dependency is not universal. We also find Tfh and GC in IL-21-deficient mice infected with influenza virus (unpublished data), as have others using different viruses (Yi et al., 2009). We also find that the B cell response in IL-21R^{-/-} mice immunized with sheep red blood cells shows accelerated resolution of GC, as reported in this paper with alum (unpublished data), indicating that time is a critical variable in these analyses and that the phenotype revealed here required the identification and tracking antigen-specific B cells. Thus, although IL-21 does not act on Tfh development, differentiation, or activation, it may act on their expansion or persistence and has a critical role in regulating GC B cells.

In this paper we identify IL-21 as a T cell-derived factor that is required for the maintenance of GC. It would seem a logical extension of the results presented in this paper that naturally occurring variation in either the production of IL-21 or sensitivity to its binding may alter the outcomes of immunization and immune responses. Heightened IL-21 signaling on B cells may be associated with prolonged periods within the GC with a concomitant extended period of exposure to AID activity, which may lead to the accumulation of excessive somatic mutations, some of which may contribute to transformation (Jankovic et al., 2007). Additionally, a surfeit of IL-21 signaling may inhibit differentiation within the GC leading to diminished memory. Conversely, too little IL-21 signaling may replicate aspects of the situation described in this paper in which memory may be formed but with inadequate affinity maturation. Regulating IL-21 production and signaling, therefore, will be crucial in achieving an appropriate level of affinity maturation in immune memory compartments.

MATERIALS AND METHODS

Mice, immunization, and tissue recovery. All mutant mice were 8–12 generations backcrossed onto the C57BL/6 background. IL-21 receptor-deficient (IL-21R^{-/-}; provided by W. Leonard, National Heart, Lung and Blood Institute, Bethesda, MD), IL-21 ligand-deficient (IL-21^{-/-}; provided by ZymoGenetics Inc., Seattle, WA), and IL-4 ligand-deficient (IL-4^{-/-}; The Jackson Laboratory) mice were bred and maintained in the specific pathogen-free facilities of the Peter MacCallum Cancer Centre (Melbourne, Australia). TCR α 18^{-/-} mice (Cui et al., 1997) were maintained at University of Melbourne. C57BL/6 mice used in immunization cohorts were similarly housed. Reconstitution experiments used donor bone marrow from T cell-deficient (CD3 ϵ ^{-/-}; Malissen et al., 1995) and Ly5.1

congenic strains, all bred and maintained at the Walter and Eliza Hall Institute for Medical Research (WEHI), mixed in the indicated ratios with IL-21⁻ or IL-21R-deficient bone marrow. Immunizations were performed with the hapten 4(hydroxy-3-nitrophenyl)acetyl coupled to the protein Keyhole Limpet Haemocyanin (NP-KLH) at a molar ratio of ~17:1. 100 µg of antigen was precipitated on alum and delivered by i.p. injection as previously described (Blink et al., 2005). At the indicated times after immunization, mice were sacrificed, spleens and femurs removed, and single cell suspensions prepared for analysis as previously described (Blink et al., 2005). All procedures involving animals were approved by the Animal Ethics Committee of WEHI.

Antibodies, flow cytometry, and immunohistochemistry. All antibodies used in this work were purified and conjugated in house unless otherwise indicated. Antibodies used were the following: B220 (RA3-6B2; QDot655), B220 (RA3-6B2; PE-Cy7; BD), IgM (331.12; Alexa Fluor 680), IgD (11/26C; Alexa Fluor 680), Gr1 (8C5; Alexa Fluor 680), CD138 (281; Alexa Fluor 680), IgG1 (X56; Allophycocyanin; BD), CD38 (NIMR5; FITC, biotin), FAS/CD95 (Jo2; biotin; BD), PNA (FITC; Vector Laboratories), FcγRII/III (24G2; supernatant), GL7 (GL7; supernatant), PD-1, ICOS, and CD4. Antibody-stained lymphocyte populations were analyzed using an LSR II flow cytometer (BD) with at least 100,000 viable cells, defined as negative for propidium iodide uptake, analyzed before setting electronic gates on B220⁺(IgM, IgD, Gr1)⁻ cells, and collecting an enriched file of 50,000 events where possible. Antigen-specific B cells, identified as IgG1⁺ NP-binding, were further analyzed for expression of CD38 as indicated. Immunohistochemistry used, as indicated in the relevant figures, tissue samples frozen in OCT (Tissue-Tek; Sakura). Sections were cut using a microtome (Leica) at -20°C, set at 7 µm thickness, and then stained as previously described (Toellner et al., 1996; Tucker et al., 2007) using the reagents indicated in the relevant figures. Antibodies, with their specificities and conjugations, were the following: B220 (RA3-6B2; unlabeled or biotin), mouse anti-rat Igκ (HRP; BD), and streptavidin alkaline phosphatase (SouthernBiotech). Substrates were AEC (SK 4200; Vector Laboratories), Fast Blue (SK 5300; Vector Laboratories), and DAB + Chromogen (K3468; Dako). Detection of NP-specific B cells in frozen sections was done exactly as previously described (Toellner et al., 1996).

ELISpot and ELISA. For ELISpots, spleen or bone marrow cells were added to a 96-well cellulose ester membrane plate (MAHAS4510; Millipore) coated with 20 µg/ml of either NP₂ or NP₁₃-BSA and incubated for at least 20 h at 37°C and 10% CO₂. Anti-NP IgG₁ was shown using goat anti-mouse IgG₁ conjugated to HRP (1070-05; SouthernBiotech) and visualized with substrate 3-amino-9-ethylCarbazole (A-5754; Sigma-Aldrich). Spots were counted using an automated reader (AID ELISpot Reader System, software version 4). Blood was collected and serum separated at times indicated for ELISA. 96-well plates were coated with NP₁₃-BSA or rat anti-mouse IgE and diluted serum was incubated for at least 20 h at room temperature. NP and total IgE were detected with goat anti-mouse IgG₁-HRP and goat anti-mouse IgE-HRP, respectively (SouthernBiotech) and visualized with ABTS substrate (2,2'-Azinobis (3-ethylbenzthiazoline Sulfonic Acid) Diammonium salt; A-1888; Sigma-Aldrich).

Analysis of SHM in antigen-specific B cells. 28 d after immunization, NP-specific IgG1⁺CD38^{hi} memory B cells in spleen were identified using the staining protocol described above on a FACS-Aria (BD) equipped for single cell deposition. Single NP⁺IgG1⁺ memory B cells were sorted directly into lysis solution and stored at -70°C until processed. The composition of the lysis solution and all procedures in isolating RNA, synthesizing cDNA, amplifying and sequencing of V_H186.2-Cγ1 rearrangements were as described (Blink et al., 2005). V, D, and J_H segments were identified by comparison with germline sequences, as were the location of CDR1 and CDR2 within the V_H gene and the distribution and frequency of mutations.

Proliferation of GC B cells. At the times indicated, NP⁺CD38^{lo} GC B cells in spleen were identified using a modification of the staining protocol described in the previous section and sorted on a FACS-Aria (BD). Anti-IgG₁ staining was replaced with anti-Igλ staining (Jc5; biotin) allowing magnetic bead pre-enrichment of Igλ-expressing B cells using anti-biotin microbeads and separation of labeled cells over a MACS LS column, according to the manufacturer's protocol (Miltenyi Biotec). Streptavidin-Cy5 (SouthernBiotech) was used for staining of Igλ⁺ cells for FACS sorting. Between 25,000 and 100,000 GC B cells were sorted per mouse, washed, and resuspended in 200 µl of PI Buffer (0.1% sodium acetate with 0.2% Triton X-100, 10 µg/ml RNase A, and 50 µg/ml propidium iodide). Cells were vortexed twice for 10 s, incubated at room temperature for 20 min in the dark, and then analyzed on a Canto II or LSR II flow cytometer (BD) with a minimum of 5,000 events recorded. FlowJo version 8.8.2 cell cycle analysis software (Tree Star, Inc.) was used to analyze data.

Online supplemental material. Fig. S1 shows the total serum IgE levels and NP-specific IgE levels in C57BL/6, IL-21^{-/-}, and IL-21R^{-/-} mice after immunization. Fig. S2 demonstrates the effect of IL-21 and IL-21R signaling on NP⁺ B cell localization. Fig. S3 shows memory B cell recall responses in the absence of IL-21 signaling. Fig. S4 compares WT mice and IL-4^{-/-} mice in their GC and memory B cell responses to a TD antigen. Fig. S5 examines the role IL-21 secretion by NKT cells in the formation of GCs and memory B cells. Fig. S6 shows that the loss of IL-21R reduces B cell proliferation in vivo and that IL-21 reduces Ebi2 mRNA expression in B cells in vitro. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091777/DC1>.

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