IL-21 Receptor Is Critical for the Development of Memory B Cell Responses

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IL-21 Receptor Is Critical for the Development of Memory B Cell Responses

Andrew L. Rankin,* Heather MacLeod,* Sean Keegan,* Tatyana Andreyeva,* Leslie Lowe,* Laird Bloom,† Mary Collins,* Cheryl Nickerson-Nutter,* Deborah Young,* and Heath Guay*

Development of long-term humoral immunity, characterized by the formation of long-lived plasma cells (PCs) in the bone marrow and memory B cells, is a critical component of protective immunity to pathogens, and as such it is the major goal of vaccination. However, the mechanisms involved in the generation of long-term humoral immunity remain poorly understood. In this study, we used IL-21R–deficient (IL-21R.KO) mice to examine the role of the IL-21 pathway in the development of the B cell memory response. Primary IgG serum Ab responses to the T cell-dependent Ag 4-hydroxy-3-nitrophenylacetyl (NP) hapten conjugated to chicken γ globulin were delayed in IL-21R.KO mice, but reached normal titers within 3 to 4 wk of immunization. IL-21R.KO mice formed germinal centers and generated normal numbers of PCs in their bone marrow. Additionally, memory B cell formation was similar in wild-type and IL-21R.KO mice. However, NP-specific memory B cells and PCs failed to expand following secondary immunization of IL-21R.KO mice, and consequently, secondary IgG Ab responses to NP hapten conjugated to chicken γ globulin were significantly impaired. These results identify the IL-21 pathway as a critical component of the memory B cell response. 

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The development of Ab responses occurs in distinct stages and anatomical sites. Upon encountering cognate Ag, B cells can become activated and mature into isotype-switched short-lived plasma cells (PCs) at extrafollicular sites to generate primary Ab responses to either T cell-independent or T cell-dependent (TD) Ags (1, 2). In response to TD Ags, B cells can also form germinal centers (GCs) in the primary follicle with CD4+ T follicular helper (TFH) cells and follicular dendritic cells (3–6). B cells undergo a dynamic selection process in the GCs, during which they somatically mutate their Ig V region genes to generate high-affinity memory B cells and long-lived PCs that give rise to long-term serum Ab titers (3–6). The B cell memory response, characterized by the formation of long-lived PCs and quiescent memory B cells, is an important component of protective immunity to bacterial and viral pathogens (7, 8). Studies using mice with targeted gene interruptions have identified a number of molecules and signaling pathways that are necessary for the induction of Ab responses and the formation of GCs, but the molecular and cellular events that give rise to long-lived PCs, memory B cells, and the formation of anamnestic responses following re-exposure to Ag are not well understood.

IL-21 is a pleiotropic cytokine that binds to the family of receptors that share the common γ-chain, which includes the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (9). Production of IL-21 is restricted to NK cells and activated CD4+ T cells, including TFH cells (10). In some strains of autoimmune mice, IL-21 is also produced by extrafollicular Th cells that participate in autoantibody bodies (11, 12). In contrast, the receptor for IL-21 is expressed by various immune cells, including NK, T, and B cells, and signaling through IL-21R can play a role in the activation, expansion, differentiation, and survival of these cells (13). Notably, B cell development is normal in IL-21R–deficient (IL-21R.KO) mice; however, these mice have reduced IgG1 and elevated IgE serum Ab titers (14). Following immunization with TD Ags, IL-21R.KO mice have reduced Ag-specific IgG1 Ab titers and TFH cell numbers on days 7–10 of the primary immune response (14–16). Recent studies have shown that IL-21 plays an important role in the formation of GCs following immunization with TD Ags (17–20). In vitro, stimulation with IL-21 and anti-CD40 can induce the differentiation of naive and memory B cells into isotype-switched B cells (21–23). These studies have demonstrated a role for IL-21 in B cell isotype switching and the formation of early PC and GC responses, but whether IL-21 is necessary for the development of long-term humoral immunity is not known. In this study, using IL-21R.KO mice, we identify the IL-21 pathway as an essential component of the memory B cell response.

Materials and Methods

Mice and immunizations

IL-21R.KO mice were generated on the C57BL/6 background as previously described (24). Age- and sex-matched C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) and used as wild-type (WT) controls for IL-21R.KO mice. To induce primary Ab responses, mice were immunized i.p. with 100 µg 4-hydroxy-3-nitrophenylacetyl (NP)(36)-chicken γ globulin (CGG; Biosearch Technologies, Novato, CA) emulsified in CFA (MP Biomedicals, Solon, OH) or precipitated in alum (Pierce, Rockford, IL). To induce secondary responses, mice were rested for 1 to 2 mo post
primary immunization and then immunized i.p. with 50 µg NP(36)-CGG in sterile PBS. Mice were used according to protocols approved by the Pfizer Animal Care and Use Committee.

**NP-specific ELISAs**

NP-specific ELISAs were done as previously described (25, 26). Briefly, for IgM and IgG ELISAs, plates were coated overnight with 20 µg/ml NP(18)-BSA (Biosearch Technologies). The following day, serum samples were added, and bound Ab was detected using HRP-conjugated goat Abs directed against mouse IgM, IgG1, IgG2a, or IgG2b (Southern Biotechnol- ogy Associates, Birmingham, AL). Plates were developed using 3,3′,5′-tetramethylbenzidine, and reactions were stopped with 2 N sulfuric acid. Absorbances were read at 450 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA), and the data were analyzed using SoftMax Pro software. Arbitrary units of Ab were determined using standard control serum pooled from NP(18)-CGG–immunized C57BL/6 mice. To measure NP-specific IgE titers, the protocol described above was adapted as follows. ELISA plates were coated overnight with 20 µg/ml NP(15)-OVA (Biosearch Technologies). Bound serum Abs were detected using HRP-conjugated goat–anti-mouse IgE Ab (Bethyl Laboratories, Montgomery, TX). Because a positive control anti-NP IgE Ab was not available for this assay, an anti-OVA IgE mAb was used as an IgE standard (clone F-95, Chondrex, Redmond, WA). The presence of NP on NP-OVA conjugates was verified by detecting NP-specific IgG1 Abs in serum (clone E-G5, Chondrex, Redmond, WA). The presence of NP on NP-OVA conjugates was verified by detecting NP-specific IgG1 Abs in serum obtained from NP-CGG–immunized, but not naïve, mice (data not shown). Total IgE levels were measured using the Mouse IgE ELISA Quantitation Set according to the manufacturer’s directions (Bethyl Laboratories).

**ELISPOTS**

NP-specific Ab-secreting cells (ASCs) were measured by ELISPOTS as previously described (25, 27). Briefly, Multiscreen HA plates (Millipore, Bedford, MA) were coated with 100 µl (20 µg/ml) NP(18)-BSA (Biosearch Technologies) overnight at 4˚C and blocked the next day with RPMI 1640 plus 10% FBS. Bound Ab was detected using 100 µl alkaline phosphatase-conjugated goat Abs specific for either mouse IgM or IgG (Southern Biotechnology Associates). Plates were developed with NBT/BCIP (Pierce) according to the manufacturer’s protocol. Spots were counted manually using a dissection microscope.

**Flow cytometry**

To examine the expression of cell-surface markers by flow cytometry, splenocytes were treated with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO) and then washed with FACS buffer (PBS plus 0.5% BSA). Cells were treated with purified anti-CD16/32 (Fc block; clone 2.4G2, BD Pharmingen, San Diego, CA) and then stained with Abs diluted in FACS buffer for 30 min at 4˚C. Abs used in these studies included FITC anti-T and -B cell-activating Ag “GL7” (clone GL7), FITC-anti-CD4 (clone GK1.5), FITC-anti-CD8 (clone 11-26c.2a), FITC-anti-FcεRI (clone MAR-1), FITC-anti-CD123 (clone 5B11), PE-anti-IgE (clone 23G3), PerCP-Cy5.5-anti-CD3 (clone 145-2C11), PerCP-Cy5.5-anti-CD4 (clone RM4), PerCP-Cy5.5-anti-CD8a (clone 53-6.7), PerCP-Cy5.5-anti-F4/80 (clone BM8), PerCP-Cy5.5-anti-Gr1 (clone Gr1), PE-anti-Fas (clone Jo2), allophycocy- nin-anti-IgG1 (clone X56), allophycocyanin-anti-IgD (clone MAR-1), FITC-anti-CD3 (clone CD38), and FITC-anti-CD4 (clone CD49e). All Abs were purchased from BD Pharmingen except those directed against F4/80, IgE, FcεRI, CD123 (eBioscience, San Diego, CA), Alexa 700-anti-CD19 (clone 6D5), FITC-anti-ICOS (clone C398.4A), Pacific Blue-anti-CD4 (clone RM4-5), Alexa 700-anti-B220 (clone RA3-6B2), purified anti-CXCR5 (clone 2G8), Alexa 700-anti-CD38 (clone 90), FITC-anti-ICOS (clone C398.4A), Pacific Blue-anti-CD4 (clone RM4-5), Alexa 700-anti-B220 (clone RA3-6B2), PE-anti-programmed cell death-1 (clone PD1), and CD138 (clone 281-2). The following Abs were also used: FITC–anti-CD3 (clone 145-2C11), PerCP-Cy5.5–anti-CD4 (clone GK1.5), FITC–anti-CD8 (clone 145-2C11), PerCP-Cy7–anti-CD8 (clone 281-2), Alexa 700–anti-CD4 (clone MAR-1), FITC–anti-F4/80 (clone MAR-1), FITC–anti-CD123 (clone 5B11), PE–anti-IgE (clone 23G3), PerCP-Cy5.5–anti-CD3 (clone 145-2C11), PerCP-Cy5.5–anti-CD4 (clone RM4), PerCP-Cy5.5–anti-CD8a (clone 53-6.7), PerCP-Cy5.5–anti-F4/80 (clone BM8), PerCP-Cy5.5–anti-Gr1 (clone Gr1), PE–anti-Fas (clone Jo2), allophycocyanin–anti-IgG1 (clone X56), allophycocyanin–anti-IgD (clone MAR-1), FITC–anti-CD3 (clone CD38), and FITC–anti-CD4 (clone CD49e). All Abs were purchased from BD Pharmingen except those directed against F4/80, IgE, FcεRI, CD123 (eBioscience, San Diego, CA), Alexa 700-anti-CD19 (clone 6D5), FITC-anti-ICOS (clone C398.4A), Pacific Blue-anti-CD4 (clone RM4-5), Alexa 700-anti-B220 (clone RA3-6B2), purified anti-CXCR5 (clone 2G8), Alexa 700-anti-CD38 (clone 90), FITC–anti-ICOS (clone C398.4A), Pacific Blue–anti-CD4 (clone RM4-5), Alexa 700-anti-B220 (clone RA3-6B2), PE–anti-programmed cell death-1 (clone PD1), and CD138 (clone 281-2).

**Results**

Early, but not late, primary TD Ab responses are diminished in IL-21R.KO mice

To determine the role of IL-21 in the formation of B cell responses, we examined the ability of IL-21R.KO mice to generate NP-specific Ab responses to the TD Ag NP-CGG. WT mice generated both NP-specific IgM and IgG serum Ab responses to NP-CGG within 7 d of immunization, which peaked at day 14 of the response. IL-21R.KO mice generated similar magnitude NP-specific IgM serum Ab titers as WT mice (Fig. 1A). In contrast, NP-specific serum IgG responses were reduced at days 7 and 14 in IL-21R.KO mice (Fig. 1A), consistent with previous reports demonstrating a role for IL-21 in B cell isotype switching to IgG and differentiation to PCs (14, 21, 23). Surprisingly though, NP-specific Ab titers were of similar magnitude in IL-21R.KO mice and WT mice at days 21 and 35 of the primary response to NP-CGG (Fig. 1A) and persisted at similar levels in both IL-21R.KO and WT mice until the latest time point analyzed at 2 mo (data not shown). NP-specific IgG Ab responses in C57BL/6 mice are predominantly IgG1, but also contain IgG2b and IgG2c isotypes (Fig. 1B). Consistent with the total IgG responses we measured in WT and IL-21R.KO mice, NP-specific IgG1, IgG2b, and IgG2c titers were reduced in IL-21R.KO mice at day 7 of the primary response (32,491 versus 4,299; 474 versus 73; and 308 versus 35 U/ml; p < 0.05, respectively), but were similar in IL-21R.KO and WT mice at day 27 (69,960 versus 131,623; 897 versus 505; and 856 versus 795; all p > 0.05, respectively) (Fig. 1B) of the primary response to NP-CGG. Together, these data suggest that IL-21 is not required for long-term serum Ab responses but is necessary for the formation of early short-lived primary Ab responses to NP-CGG.

**IL-21R.KO mice generate normal numbers of NP-specific IgG bone marrow PCs**

Long-term humoral immunity is characterized by the formation of long-lived PCs that reside in the bone marrow (BM) and the development of quiescent recirculating memory B cells, which rapidly give rise to robust Ab responses following secondary exposure to their cognate Ag (2). To determine if IL-21R.KO mice generated long-term humoral immunity to NP, we first examined BM from IL-21R.KO and WT mice for the presence of NP-specific BM PCs. 1.5 mo following immunization. Consistent with the formation of isotype-switched B cell memory, BM from WT mice contained IgG (Fig. 1C), but very few IgM NP-specific ASCs (data not shown). Additionally, NP-specific IgM and IgG ASCs were present in similar numbers in BM of IL-21R.KO mice 1.5 mo following immunization with NP-CGG (14.6 versus 19.5 ACS/10^6 lymphocytes, respectively; Fig. 1C), indicating that IL-21R.KO is not required for the generation of ASCs in the BM.

**Secondary Ab responses are diminished in IL-21R.KO mice**

To determine if IL-21 may contribute to the B cell memory response, we next examined the ability of IL-21R.KO mice to generate secondary Ab responses to NP-CGG. To this end, we measured the NP-specific ASC responses in the spleen of WT and IL-21R.KO mice 3 and 5 d post secondary challenge with NP-CGG. NP-specific IgG ASCs were detected in large numbers in the spleens of WT mice at 3 and 5 d post secondary immunization, consistent with the rapid activation of memory B cells and generation of a robust IgG response upon re-encountering cognate Ag (Fig. 2 and data not shown). By contrast, NP-specific IgG ASCs were significantly reduced in spleens of IL-21R.KO mice at both 3 and 5 d post secondary immunization (Fig. 2 and data not shown). The IL-21 pathway has been shown to play a key role in isotype switching to IgG (21, 23), and this was also observed in NP-specific IgG ASC responses in the spleens of the IL-21R.KO mice could be accounted for by non-IgG NP-specific responses in these mice. Therefore, we also examined NP-specific IgM and IgE responses.
in IL-21R.KO mice 3 d post secondary challenge with NP-CGG. Consistent with the formation of isotype-switched memory, NP-specific IgM ASCs were ∼6-fold lower than IgG responses in WT mice 3 d post secondary immunization, and these responses were even further reduced in IL-21R.KO mice (Fig. 2). Interestingly, IgE ASC responses were significantly increased in IL-21R.KO mice when compared with WT mice (Fig. 2). Importantly however, the magnitude of the NP-specific IgE response in IL-21R.KO mice was 200-fold lower than that observed for the IgG responses measured in WT spleens (Fig. 2). Consequently, NP-specific IgE was undetectable in serum obtained from WT and IL-21R.KO mice by ELISA, although total IgE levels were elevated in IL-21R.KO mice, as has been reported previously (Supplemental Fig. 1A,1B) (14, 20). Thus, the absence of NP-specific IgE responses in IL-21R.KO mice was not compensated for by a net increase in either IgM or IgG responses. These data indicate that the IL-21 pathway is required for the generation of memory B cell responses.

**IL-21R.KO mice form GC responses**

The development of long-lived PCs and memory B cells primarily occurs within GCs and peaks at day 10–14 of the primary immune response to NP-CGG (30, 31). Although IL-21R.KO mice generated NP-specific IgG BM PCs, it is possible that the inability of IL-21R.KO mice to generate secondary Ab responses could be due to a defect in GC formation in these mice, which would subsequently affect the development of memory B cells. For example, mice deficient in Lyn kinase or lymphotoxin-α can generate high-affinity somatically mutated PCs in the absence of GC formation (32, 33). The ability of these mice to generate memory B cells was not explored. Multiple groups have recently reported that IL-21 is required for the generation and/or maintenance of CD4+ TFH cells to some T-dependent Ags (15, 16, 20). Accordingly, it is possible that the development of TFH cells, and subsequently GCs, is not completely impaired but rather delayed in IL-21R.KO mice. Therefore, we next assessed the ability of IL-21R.KO mice to generate TFH, GCs, and memory B cells following primary immunization with NP-CGG. TFH cells can be identified by flow cytometry based on their distinct phenotype—namely, B220−CD4+CXCR5+PD1+. Spleens obtained from immunized WT and IL-21R.KO mice contained B220−CD4+CXCR5+PD1+ cells (Fig. 3A). These cells also expressed high levels of ICOS, consistent with the cell-surface phenotype reported for TFH CD4+ T cells (Supplemental Fig. 2) (34). Interestingly, spleens from naïve IL-21R.KO mice contained significantly fewer TFH cells than those

**FIGURE 1.** IL-21R is necessary for the generation of early, but not late, primary Ab responses. A and B, WT and IL-21R.KO mice were immunized i.p. with 100 μg NP-CGG emulsified in CFA. NP-specific serum Ab responses were examined weekly by ELISA. Symbols indicate the average of four mice per group. Error bars indicate the SEM. C, NP-specific IgG ASCs were measured in the BM of WT and IL-21R.KO mice ∼1.5 mo postimmunization by ELISPOT. Plots show ≥8 mice per group, and error bars indicate the SEM. Data are representative of three independent experiments. *p < 0.05, Student t test.
**FIGURE 2.** IL-21R.KO mice do not generate secondary Ab responses. WT and IL-21R.KO mice were challenged i.p. with 50 μg NP-CGG in PBS 1.5–2 mo post primary immunization. Three days postchallenge, NP-specific IgM, IgG, and IgE ASCs in the spleen were measured by ELISPOT. Plots show ≥8 mice per group with data pooled from two independent experiments. Inset shows IgE ASCs on a magnified scale. Error bars indicate the SEM. The p values were calculated using Student t test.

obtained from naive WT mice (Fig. 3B). Nevertheless, both WT and IL-21R.KO mice had similar numbers of TFH for the 3 wk following primary immunization with NP-CGG (Fig. 3B). Thus, these results indicate that signaling through IL-21R is not required for TFH generation and that TFH cells are maintained through the peak of the NP-CGG GC response independent of signals through IL-21R.

We next assessed the ability of IL-21R.KO mice to generate GC B cells following immunization with NP-CGG. NP-specific GC B cells can be identified by flow cytometry based on the cell-surface phenotype CD4+ B220+GL7hi FashiNIP+. WT and IL-21R.KO mice immunized with NP-CGG generated a prominent B220+ GL7hiFashi population of GC B cells by day 7 postimmunization that contained both NIP+ and class-switched IgG1+NIP+ GC B cells (Fig. 4A, 4B). The absolute numbers of both NIP+ and class-switched IgG1+NIP+ GC B cells were significantly reduced in IL-21R.KO mice at 7 d postimmunization (Fig. 4C, 4D).

However, by day 14, spleens from WT and IL-21R.KO mice contained similar numbers of NIP+ and IgG1+NIP+ GC B cells (Fig. 4C, 4D). This pattern was maintained until day 35, when we observed a significant decrease in the absolute number of total NIP+ GC B cells in spleens obtained from IL-21R.KO mice. It is noteworthy that the absolute number of total NIP+ GC B cells present in spleens obtained from both WT and IL-21R.KO mice at this time point had returned to baseline numbers measured in naive mice. Importantly, the absolute number of IgG1+NIP+ B cells was similar in spleens obtained from WT and IL-21R.KO mice at day 35 postimmunization with NP-CGG. These observations mirror our findings that class-switched NP-specific IgG1 Abs are significantly reduced in the serum of IL-21R.KO mice early during the primary response, but recover to similar levels observed in WT mice within 3 wk postimmunization (Fig. 1B, 1C).

**Impaired accumulation of Ag-experienced memory B cells and PCs in IL-21R.KO mice during the recall response**

Because IL-21R.KO mice developed GCs that contained normal numbers of class-switched IgG1+NIP+ B cells, we next examined the characteristics of the memory B cell response following secondary immunization with NP-CGG. Ag-experienced memory B cells can be identified by flow cytometry as CD4−CD25−Foxp3−CXCR5+PD1+ cells (35, 36). By contrast, NIP+B220+CD138− cells are readily identifiable in spleens obtained from WT and IL-21R.KO mice by day 35 postimmunization (Fig. 5A). By contrast, NIP+B220+CD138− PCs were rare, constituting ∼3% of splenic NIP+Dump− PCs in both strains of mice at this time point. Three days post secondary immunization, NIP+B220+CD138− PCs were clearly present in spleens obtained from NP-CGG–primed WT mice (Fig. 5B). Conversely, splenic NIP+ PCs were infrequently observed in IL-21R.KO animals following secondary immunization (Fig. 5B, 5C). In addition, very few NIP+ cells in the IL-21R.KO mice expressed IgG3 d post secondary immunization, further corroborating the dramatic reduction in splenic IgG Ab secreting
FIGURE 4. IL-21R.KO mice develop GC B cells. GC responses were examined in the spleens of WT and IL-21R.KO mice 7 d post primary immunization with NP-CGG. A, Contour plots show the levels of expression of GL7 and Fas on B220<sup>+</sup>CD3<sup>+</sup> gated cells from WT (top panel) and IL-21R.KO mice (KO; bottom panel) with the average frequency of GL7<sup>hi</sup> Fas<sup>hi</sup> cells from five mice per group indicated. B, Contour plots show the frequency of NIP<sup>+</sup>B220<sup>+</sup>GL7<sup>+</sup>Fas<sup>+</sup> cells present in spleens obtained from naive (left panels) or immunized (center panels) WT (top panels) or IL-21R.KO mice (KO; bottom panels). The percentage of IgG1<sup>+</sup> B cells within the NIP<sup>+</sup> B220<sup>+</sup> GL7<sup>hi</sup> Fas<sup>hi</sup> population is shown (right panels) for WT (top panels) and IL-21R.KO mice (KO; bottom panels). Graphs show the absolute number of NIP<sup>+</sup>B220<sup>+</sup>Fas<sup>+</sup>B220<sup>+</sup> (C) and IgG1<sup>+</sup>NIP<sup>+</sup> GL7<sup>+</sup>Fas<sup>+</sup>B220<sup>+</sup> cells (D) in spleens obtained from WT (open circles) and IL-21R.KO mice (KO; filled squares) at the time points indicated. Data points indicate the average value obtained from five mice per group with SEM shown. Data are representative of three independent experiments. *p < 0.02; **p < 0.008, Mann–Whitney U test.

cells observed at this time point by ELISPOT (Fig. 2). Similar results were also observed 5 d (data not shown) post secondary challenge with NP-CGG.

We next assessed the absolute numbers of NIP<sup>+</sup>B220<sup>+</sup>CD138<sup>-</sup> memory B cells and NIP<sup>+</sup>B220<sup>+</sup>CD138<sup>-</sup>PCs in spleens from IL-21R.KO and WT mice. We included CD38 staining to exclude residual CD38<sup>+</sup> germinal B cells from our analysis of the NIP<sup>+</sup> memory B cell pool (37). Thirty-five days post primary immunization, the absolute number of NIP<sup>+</sup> memory B cells was similar in spleens obtained from IL-21R.KO and WT mice, indicating that signals through the IL-21R are not required for generation of memory B cells following primary immunization with NP-CGG (Fig. 5C). Three days post secondary immunization, NIP<sup>+</sup> memory B cells numbers had increased by >2-fold, and NIP<sup>+</sup>B220<sup>low</sup> CD138<sup>-</sup>PC numbers increased by >4-fold in WT type mice following secondary challenge. Conversely, memory B cell and PC numbers remained unchanged in spleens of IL-21R.KO mice (Fig. 5C). Thus, these results indicate that signals through the IL-21R are required for accumulation of memory B cells and Ag-experienced PCs following secondary immunization with NP-CGG.

Discussion

Little is known about the factors and processes that promote expansion of Ag-experienced plasma and memory B cell pools during B cell memory responses. Our data identify IL-21 as a key contributor to this process by demonstrating that the IL-21 pathway is essential for the accumulation of Ag-experienced memory B cells and PCs following secondary immunization.

It is not clear how IL-21 augments the memory B cell response. CD4<sup>+</sup> TFH cells play a critical role in promoting GC-dependent Ab responses, and IL-21 can impact the development and/or maintenance of TFH cells (15, 16, 20, 38). However, this requirement is not absolute and appears sensitive to the time-point(s) examined and immunogen used (17–19). In our studies, we found that the generation and maintenance of TFH cell numbers was similar in IL-21R.KO and WT mice following immunization with NP-CGG, indicating that IL-21R was not required for these processes during the time points examined. In contrast with TFH cell development, we found that day 7 GC formation and early Ab production to the T-dependent Ag NP-CGG were dependent on IL-21R. These observations are similar to initial studies reported by Ozaki et al. (14) as well as others published while this manuscript was under review (17–20). A time-course analysis performed following primary immunization revealed that the absolute number of Ag-specific GC B cells was similar in WT and IL-21R.KO mice from day 14–28. We did observe significantly reduced numbers of total NIP<sup>+</sup> GC B cells at day 35 postimmunization of IL-21R.KO mice. However, NIP<sup>+</sup> GC B cell numbers had returned to their respective background levels (as measured in naive mice) by this time point, which were significantly reduced in naive IL-21R.KO mice compared with naive WT animals. This suggests that the reduced numbers of total NIP<sup>+</sup> GC B cells observed at day 35 reflect basal differences in ongoing GC responses to environmental Ags and that signals delivered via the IL-21R are not required for maintenance of GCs. By contrast, a recent report from Zotos et al. (17) describes premature dissolution of GCs in IL-21/ R–deficient mice by day 28 in response to immunization with NP-keyhole limpet hemocyanin (KLH) in alum. Because different experimental systems were employed by the two groups [NP(36)-CGG in CFA versus NP(17)-KLH in alum], collectively, these results demonstrate that IL-21 is not required but may contribute to the maintenance of long-term GC response in a manner dictated by contextual clues, such as the immunogen, strength of signal, and/or adjuvant(s) encountered. In this regard, it is noteworthy that IL-21R.KO mice immunized with virus-like particles that lack TLR7 ligands exhibit impaired GC formation that could be normalized by retaining TLR7-stimulating bacterial RNA in the
virus-like particle inoculum (19). Maintenance of GCs, however, was not reported. The memory B cell response to the T-dependent Ag NP-CGG develops via a GC-dependent process culminating in the formation of Ag-experienced memory B cells and PCs (31, 39). Class-switched NIP +IgG1+ GC B cells were present in normal numbers from days 14–35 in NP-CGG–immunized IL-21R.KO mice, further highlighting that signals delivered via IL-21R are not required for GC maintenance. Interestingly, this population of GC B cells resembles a recently described PNA +IgG1+ GC B cell population that is destined to enter the memory pool (40). Consistent with this observation, Ag-specific memory B cell and PC numbers were similar 35 d postimmunization in spleens obtained from both WT and IL-21R.KO mice, thus indicating that IL-21R is not required for the formation of Ag-specific memory B cells. IL-21R.KO mice immunized with NP-KLH in alum were recently reported to contain significantly increased numbers of memory B cells at day 28 (17). We sought to examine whether the reported difference was adjuvant dependent and found that memory B cell formation was similar in WT and IL-21R.KO mice immunized with NP-CGG in CFA or alum (Supplemental Fig. 3). Within the same series of studies, IL-21R–deficient mice were also reported to have

**FIGURE 5.** IL-21R is required for the memory B cell response. WT and IL-21R.KO mice were challenged i.p. with 50 μg NP-CGG in PBS ~1–1.5 mo post primary immunization. Contour plots show the gating scheme used to identify NIP + memory B cells and PCs at 35 d post primary immunization (A) and 3 d post secondary challenge (B) by flow cytometry. To identify NIP-binding B cells, lymphocytes were first gated on Dump − negative cells (first column) and then gated on NP +IgD − cells (second column). NP-binding cells were further subdivided into three distinct populations based on their expression of B220 and CD138 to identify PCs and memory B cell populations (third column). The histogram shows the expression of IgG1 on Dump − NIP + IgD − cells (fourth column). Percentages indicate the mean value obtained from five mice per group with a representative plot presented. C, Graphs show the absolute numbers of NIP +IgD − Dump −B220 −CD138 −CD38 − memory B cells (left panel) and NIP +IgD −Dump −B220 −CD138 + PCs (right panel) in the spleen at 0 and 3 d post secondary challenge with NP-CGG. Bars show the average value obtained from ≥15 mice per group, and error bars show the SEM. **p < 0.005, Mann-Whitney U test.
activation of a normal memory B cell response; however, quantitative analysis to support this assertion was not provided (17). Collectively, these results suggest that experimental differences in the immunogens used [NP(36)-CGG versus NP(17)-KLH] may affect memory B cell formation via their impact on strength/avidity of BCR signaling and/or quality of T help. Although it is unclear why we did not observe increased numbers of memory B cells when IL-21R.KO mice were immunized with NP-CGG in alum, these findings further highlight that IL-21 is not required for the formation of memory B cells. Most importantly, however, our studies demonstrate that IL-21R is required for the memory B cell response because Ag-specific memory B cells, PCs, and plasmablasts failed to accumulate following secondary immunization, and consequently, class-switched Ab responses were significantly reduced. Our studies suggest that signals delivered via the IL-21R may directly support the proliferation of memory B cells as well as their differentiation into PCs and/or plasmablasts upon reencountering cognate Ag. This later point is supported by data demonstrating that human memory B cells can differentiate into PCs in vitro upon coculture with anti-CD40 and IL-21 in the presence or absence of BCR cross-linking (12, 23). Alternatively, IL-21 may affect the differentiation program of B cells during the primary response or the quality of T cell help available, both of which could indirectly impact the characteristics of memory B cell response (41, 42). Future studies will aim to determine the mechanism by which IL-21 augments the memory B cell response.

It is also important to note that the aforementioned study by Ozaki et al. (14) demonstrated that IL-21 is important for isotype switching to several subsets of IgG and suggested that IL-21 is the master regulator of switching to IgG1. However, that study only examined Ab responses to TD Ags (including NP-CGG) up to 10 d postimmunization (14). We also found that NP-specific IgG1, -2b, and -2c Ab responses to NP were reduced in IL-21R.KO mice on days 7 and 14 following immunization. However, these Ab responses were of similar magnitude in IL-21R.KO mice when compared with WT mice at later time points during the primary immune response. NP-specific IgG1+ GC B cells were also present in similar numbers in IL-21R.KO and WT mice from days 14–35 postimmunization. These findings indicate that the requirement for IL-21 for isotype switching to IgG is temporal, with B cells being more heavily dependent on IL-21 for isotype switching early during the primary immune response. Moreover, our findings demonstrate that IL-21 is not a master regulator of switching to IgG1. Instead, additional signals, such as those driven by IL-4, may also drive isotype switching to IgG1 (43). Collectively, our results show that IL-21 plays multiple distinct roles throughout the course of a TD Ab response and identify a previously unknown requirement for the IL-21 pathway in the generation of B cell memory responses.

Finally, blockade of the IL-21 pathway has been suggested as a therapeutic strategy for the treatment of autoimmune diseases, such as systemic lupus erythematosus (36–38). Treatment of lupus-prone MRL-Fas<sup>−/−</sup> and BXSB-Yaa mice with soluble IL-21.RFc protein, which neutralizes the IL-21 cytokine, reduced IgG autoantibody titers and ameliorated disease in these mice (36, 37). Moreover, IL-21R−/− deficient MRL-Fas<sup>−/−</sup> (A. Rankin, H. Guay, D. Herber, T. Andreyeva, Y. Carrier, M. Senices, N. Stedman, M. Ryan, L. Bloom, Q. Medley, M. Collins, C. Nickerson-Nutter, D. Young, and K. Dunussi-Jamopoulos, submitted for publication) and BXSB-Yaa mice do not develop disease, and IgG autoantibodies are undetectable in these animals (38). In contrast, when we measured Ab responses to the foreign Ag NP-CGG, we found that early, but not late, primary IgG Ab responses were diminished in IL-21R.KO mice (Fig. 1). These findings suggest that blockade of the IL-21 pathway could effectively reduce autoantibody titers, whereas only minimally affecting the development of IgG Ab titers to foreign Ags, as is the goal of vaccination. Thus, our findings may have relevance to research exploring blockade of the IL-21 pathway as a treatment for autoimmune disease in the clinic.

References
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