The Journal of Immunology

IL-21 and IL-4 Collaborate To Shape T-Dependent Antibody Responses

Helen M. McGuire, Alexis Vogelzang, Joanna Warren, Claudia Loetsch, Karlo D. Natividad, Tyani D. Chan, Robert Brink, Marcel Batten and Cecile King

J Immunol published online 21 October 2015
http://www.jimmunol.org/content/early/2015/10/21/jimmunol.1501463

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published October 21, 2015, doi:10.4049/jimmunol.1501463

IL-21 and IL-4 Collaborate To Shape T-Dependent Antibody Responses

Helen M. McGuire,*,†,[1] Alexis Vogelzang,*,†,[1] Joanna Warren,* Claudia Loetsch,*,† Karlo D. Natividad,* Tyani D. Chan,*,† Robert Brink,*,† Marcel Batten,*,† and Cecile King*,†

The selection of affinity-matured Ab-producing B cells is supported by interactions with T follicular helper (Tfh) cells. In addition to cell surface–expressed molecules, cytokines produced by Tfh cells, such as IL-21 and IL-4, provide B cell helper signals. In this study, we analyze how the fitness of Th cells can influence Ab responses. To do this, we used a model in which IL-21R–sufficient (wild-type [WT]) and –deficient (*Il21r*$^{-/-}$) Ag-specific Tfh cells were used to help immunodeficient *Il21r*$^{-/-}$ B cells following T-dependent immunization. *Il21r*$^{-/-}$ B cells that had received help from WT Tfh cells, but not from *Il21r*$^{-/-}$ Tfh cells, generated affinity-matured Ab upon recall immunization. This effect was dependent on IL-4 produced in the primary response and associated with an increased fraction of memory B cells. *Il21r*$^{-/-}$ Tfh cells were distinguished from WT Tfh cells by a decreased frequency, reduced conjugate formation with B cells, increased expression of programmed cell death 1, and reduced production of IL-4. IL-21 also influenced responsiveness to IL-4 because expression of both membrane IL-4R and the IL-4–neutralizing soluble (s)IL-4R were reduced in *Il21r*$^{-/-}$ mice. Furthermore, the concentration of sIL-4R was found to correlate inversely with the amount of IgE in sera, such that the highest IgE levels were observed in *Il21r*$^{-/-}$ mice with the least sIL-4R. Taken together, these findings underscore the important collaboration between IL-4 and IL-21 in shaping T-dependent Ab responses. ***The Journal of Immunology*, 2015, 195: 000–000.**

T follicular helper (Tfh) cells are found within germinal centers (GC), specialized areas within secondary lymphoid organs where B cells proliferate to form high-affinity Abs in response to infection or immunization. Within the GC, Tfh cells facilitate the survival and differentiation of memory B cells and Ab-secreting plasma cells. Tfh cells are characterized by expression of the chemokine receptor CXCR5, programmed cell death 1 (PD-1), the transcriptional repressor B cell lymphoma 6 (Bcl6), and cytokines such as IL-21 that influence the differentiation of Ab-forming B cells (1–5).

Tfh cell–secreted IL-21 supports the differentiation and survival of B cells (6, 7). However, evidence for a CD4$^+$ T cell–intrinsic role for IL-21 has been controversial, with studies showing an important contribution of IL-21–responsive CD4$^+$ T cells (2, 8, 9) or an exclusively B cell–intrinsic role for IL-21 (10, 11). This variation may reflect context-dependent differences, such as the Ag or adjuvant, contribution of extrafollicular Ab production, and cytokine redundancy. In this regard, IL-6 has been shown to support Tfh cell differentiation in the absence of IL-21, and in the absence of both cytokines, Tfh cells are markedly reduced (12, 13).

In addition to IL-21, Tfh cells secrete other cytokines such as IL-4 that help define the Ab response (14–18). The α-chains of the receptors for IL-4 (IL-4Ra) and IL-21 (IL-21Ra) are neighboring genes separated by ∼37 Kb on chromosome 7 in mice and by a similar distance on chromosome 16 in humans. IL-4 and IL-21 have distinct receptor α-chains, but they share the common γ-chain for cell signaling (19–21). IL-21R is expressed on a wide range of immune and nonimmune cells (22, 23), which underlies the broad influence IL-21 has over a range of cell types during immune responses (24, 25). There are two forms of the IL-4: one is membrane bound, and the other is a soluble form (sIL-4R) released by proteolytic cleavage (26, 27). sIL-4R inhibits the biologic actions of IL-4 both in vitro and in vivo (28, 29). The important collective roles of IL-4 and IL-21 are illustrated by the finding that mice made genetically deficient in both *Il4* and *Il21r* exhibit a significantly more pronounced phenotype than the absence of either molecule alone, characterized by a severely impaired Ab response (16). Although their influence on Ab production is established, the kinetics and timing of the individual contributions of IL-4 and IL-21 in the GC reaction remain incompletely understood.

Th2 responses and the development of Th2-associated pathology are significantly reduced in the absence of IL-21–IL-21R signaling (30, 31). Whether IL-21 regulates IL-4–induced Th2 cell differentiation directly (32, 33) or amplifies Th2-driven responses by downregulating the expansion of IFN-γ–producing Th1 cells (33, 34) remains incompletely understood. Under Th2 polarizing conditions in vitro a significant number of IL-21$^+$ cells coexpress IL-4 (35), and a deficiency in the IL-21R is associated

*Department of Immunology, Garvan Institute of Medical Research, Darlinghurst, New South Wales 2010, Australia; and †St. Vincent's Clinical School, University of New South Wales, Sydney, New South Wales 2052, Australia

[1]H.M.M. and A.V. contributed equally to this work.

Received for publication June 29, 2015. Accepted for publication September 23, 2015.

This work was supported by National Health and Medical Research Council, Australia, Project Grants 596836 and 1029942 and a grant from the Rebecca L. Cooper Foundation.

The sequence presented in this article has been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank) under accession number BGT144240.

Address correspondence and reprint requests to Dr. Cecile King, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia. E-mail address: c.king@garvan.org.au

The online version of this article contains supplemental material.

Abbreviations used in this article: 129, 129/SvJ; alum, aluminum hydroxide; B6, C57BL/6; Bcl6, B cell lymphoma 6; BM, bone marrow; GC, germinal center; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetate; NP, 4-hydroxy-3-nitrophenyl acetyl; PD-1, programmed cell death 1; rm, recombinant murine; s, soluble; Tfh, T follicular helper; WT, wild-type.

with decreases in both serum IL-4 and IL-4R expression on macrophages following infection (31) and in allergic responses (36).

Paradoxically, despite reduced IL-4 detected in sera postinfection and reduced production of IgG1, *Il21r*$^{-/-}$ mice produce increased levels of the Ab isotype IgE in an IL-4–dependent manner (16). Although the role of IL-21 in IgE production from human B cells in vitro has generated conflicting findings (37–39), IgE is increased in sera of patients with loss-of-function mutations in the IL-21R gene (40). Switching to IgE may initiate within the GC, but IgE^{+} cells can undergo rapid differentiation into plasma cells and are mostly found outside the GC (41). Both GC IgG1^{+} and memory IgG1^{+} cells have been shown to undergo a secondary switch to IgE in a process that requires IL-4 and is inhibited by IL-21 (41). The mechanism for the inhibitory effect of IL-21 on IgE has been explored in several studies. IL-21 has been shown to downregulate IgE production from IL-4–stimulated B cells through the inhibition of germ line C(ε) transcription (32). Additionally, the transcriptional repressor Bcl6 negatively regulates the Iε promoter (42), and in this manner IL-21 may have a negative effect on IgE production by increasing the expression of Bcl6 in B cells (43).

In this study, we analyze the relationship between IL-21 and IL-4 in the generation of Ab-forming B cells in response to T-dependent Ag. IL-21 makes better Tfh cells, improving their frequency and ability to produce IL-4, which was important for optimal immunity. We also observed a previously unappreciated effect of IL-21R on the expression of both membrane-bound and soluble forms of IL-4R that may contribute to the role of IL-21 in the differential generation of IgG1- and IgE-producing B cells.

Materials and Methods

Mice

Il21r$^{-/-}$ mice were obtained from Dr. Warren Leonard (National Institutes of Health) via Dr. Mark Smyth (Melbourne) at C57BL/6 (B6) N6 and backcrossed to N12 for experimental use. Ly5.1 congenic mice were purchased from the Animal Research Centre in Perth, Australia. Animals were housed under specific pathogen-free conditions and handled in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Age-matched littermate mice used for experimental purposes were between 7 and 14 wk of age.

Immunizations

Mice were immunized i.p. with 100 μg 4-hydroxy-3-nitrophenyl acetyl $(NP)_{13}$–OVA absorbed to aluminum hydroxide (alum) or with 2×10^8 sheep RBCs (Institute of Medical and Veterinary Science, Adelaide, SA, Australia), and spleens were analyzed at time points indicated for primary response and at day 26, 5 d following a boost of 100 μg NP_{13}-OVA given i.v. in PBS at day 21. For adoptive transfer, 3×10^4 OT-II CD4^{+} T cells were given i.v. at the time of immunization. For CFSE labeling, cells were washed twice to remove excess FCS and resuspended at 5×10^7/ml for CFSE staining in buffer containing 5 μM CFSE. Cells were incubated at 37˚C for 10 min andthen washed twice with ice-cold lymphocyte isolation media before being prepared for cell culture or adoptive transfer. For IL-4 neutralization in vivo, 330 μg 11B11 IL-4 Ab was administered i.p. on the day of primary immunization or on day 21 with Ag boost. For recombinant murine (rm)IL-4 treatment, mice received 50 ng/mouse i.p. on day 2 of immunization with 100 μg NP_{13}-OVA absorbed to alum.

Bone marrow chimeras

Cohorts of B6 mice were lethally irradiated by a [^{137}Cs] source (B6, two doses of 0.45 Gy 4 h apart) and reconstituted the following day by i.v. injection with 1×10^7 bone marrow (BM) cells isolated from femurs and tibiae obtained by flushing bone with lymphocyte isolation media in sterile conditions. Mature T cells were depleted from BM by incubation on ice with anti-CD90.2 (Thy1.2, JIJ) ascites (1:200, produced in-house) followed by LowTox guinea pig complement for 40 min at 37˚C (1:20, Cedarlane Laboratories, Burlington, ON, Canada) and two washes in lymphocyte isolation media. A 1:1 ratio of Ly5.1 congenic WT and *Il21r*$^{-/-}$ BM cells were found to result in between 40 and 60% of lymphocytes being derived from each donor. After irradiation, mice were maintained on antibiotic water containing cotrimoxazole (Roche) for 10 d. Mice were immunized at 8–12 wk after reconstitution after checking for chimerism of lymphocytes in blood.

Flow cytometry

RBCs were removed from dispersed splenocytes by hypotonic lysis. A single cell suspension (50 μl) at 2×10^7 cells/ml from spleen and lymph nodes was stained in FACS buffer containing Abs in 96-well V-bottom microtiter plates (Nunc, Roskilde, Denmark). To reduce nonspecific binding, cells were pretreated with anti-CD16 for 20 min (2.4G2 made in-house). Cells were acquired using a FACSCanto cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR). Doublets were excluded by forward scatter height and width, except when analyzing T–B. T–B conjugates were defined as CD4^{+} and B220^{+} double-positive doublet cells. Directly labeled fluorescent Abs and CXCR5 plus biotin were all purchased from eBioscience unless otherwise noted. Bcl-x_L clone 54H6 was from Cell Signaling Technology; fluorescein-conjugated peanut agglutinin was from Vector Laboratories. Detection of NP-specific B cells by 4-hydroxy-3-iodo-5-nitrophenylacetate (NIP)–PE staining was performed as described previously (44). The NIP-OSu ester was purchased from Biosearch Technologies and was haptenated to PE (Molecular Probes).

Immunofluorescence and Immunohistochemistry

Frozen OCT (5 μm; Tissue-Tek) spleen sections were cut using a cryostat (Leica, Wetzlar, Germany) and then fixed in ice-cold acetone for 7 min and dried for 1 h before rehydration in PBS for 5 min. Primary biotin or fluorochrome-conjugated Abs were incubated in 100 μl at room temperature for 2 h followed by amplification with streptavidin-Cy3 (Jackson ImmunoResearch Laboratories) for 1 h. Slides were washed in PBS and then 90% glycerol was used as a mounting agent. Various issues were also embedded in paraffin by the St. Vincent's pathology department following overnight immersion in 10% formalin in PBS. H&E-stained 4-μm sections were cut and stained by the Garvan Institute histology facility. Sections were analyzed using a Leica DM RBE TCS confocal microscope or Leica light microscope (Leica Microsystems, Wetzlar, Germany). The images were processed using the Leica acquisition and analysis software ImageJ (freeware from National Institutes of Health, Bethesda, MD) or Adobe Photoshop, version 7 (Adobe Systems, San Jose, CA).

ELISA

Blood was allowed to clot at room temperature for 1 h before centrifugation at 0.3 relative centrifugal force for 10 min to separate serum for analysis. For anti-NP Ab ELISA, serum Ig was captured by coating plates overnight with anti-mouse Ig (H+L) (2 μg/ml, SouthernBiotech), NP_3-OVA, or NP_{30}-OVA (10 μg/ml, Biosearch Technologies, Petaluma, CA) in ELISA coating buffer. The plate was washed then blocked with 4% milk powder diluted in ELISA buffer for 1 h at 37˚C. Plates were washed and then serum samples were incubated for 2 h at 37˚C at 1:200 in ELISA buffer for Ab detection or neat for cytokine analysis along with 8–15 dilutions at 1:2. After washing, analytes were detected using alkaline phosphatase–conjugated anti-mouse IgG1 (1:2000, BD Biosciences) at 37˚C for 1 h. Standards were purchased from SouthernBiotech and were used at a top dilution of 1 μg/ml. Plates were given five final washes before detection of bound alkaline phosphatase enzyme with 4 nitrophenyl phosphate disodium salt hexahydrate at 1 mg/ml (Sigma-Aldrich). The reaction was stopped using 2 M NaOH. The titer of NP-specific IgG1 was calculated as $\log_2$ of the last dilution factor where the OD was three times that of background. For sIL-4R ELISA, the protocol was a BD Biosciences cytokine ELISA protocol. The capture Ab was purified rat anti-mouse CD124 (catalog no. 552952), and the detection Ab was biotin rat anti-mouse CD124 (catalog no. 552508) (both from BD Pharmingen). The standard used was murine IL-4R/Fc chimera (catalog no. 530-MR) from R&D Systems. For IgE ELISA, plates were coated with 5 μg/ml anti-mouse IgE (R35-72, BD Biosciences, catalog no. 553413). Standard was purified IgE isotype control (MEB-38, BioLegend, catalog no. 401801) starting at 2 μg/ml. Secondary Ab was biotin mouse anti-mouse IgK (187.1, BD Biosciences, catalog no. 59750) at 1:1000 dilution and then streptavidin–alkaline phosphatase at 1:1000, detected with *p*-nitrophenyl phosphate, and absorbance was read at 405 nm. For IL-4 ELISA, IL-4 was detected after 4 h stimulation at 37˚C in cell culture media with PMA (5 ng/ml, Biomol, Plymouth Meeting, PA), ionomycin (1μg/ml, Invitrogen), and GolgiStop (1:1000, BD Biosciences).

Single-cell sorting of GC B cells and somatic hypermutation assay

GL7^{+}Fas^{+}CD19^{+} B cells were collected by flow cytometry activated sorting at day 14 after NP-OVA immunization and OT-II transfer as de-

scribed above. Single cells were collected directly into 10 μl Taq buffer (Invitrogen), 0.5 mg/ml protease K (Promega), 0.1 mM EDTA, and 0.1% Tween 20 in skirted PCR plates (Thermo Fisher Scientific, Scoresby, VIC, Australia) and frozen at −80°C. Plates were spun at 2100rpm for 5 min and DNA was digested by heating to 56°C for 40 min, followed by 98°C for 8 min. The dominant VH186.2 segment was amplified as described earlier (18). In brief, the sequence containing the VH186.2 intron was amplified using nested primers, and 2.5 μl of the first round product was used in the second PCR: first round, 5′-ACACAGGACCTCACCATG-3′ and 5′-TCACAAGAGTCCGATAGACC-3′ (with 35 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1.5 min); second round, 5′-GGGTGA-CAATGACATCCA-3′ and 5′-GAGGAGACTGTGAGAGTGGTGCC-3′ (with 32 cycles of 95°C for 10 s, 66°C for 30 s, and 72°C for 30 s) using Taq DNA polymerase (Invitrogen). Excess primers and nucleotides were removed from the PCR product using ExoSAP-IT (USB, Cleveland, OH) according to the manufacturer's instructions before sequencing at the Garvan Institute in-house facility.

Measurement of IL-4R by quantitative PCR and pyrosequencing

For quantitative RT-PCR, the relative amounts of cDNAs were determined in triplicate by quantitative RT-PCR analysis using the ABI Prism 7700 sequence detection system (Applied Biosystems); positive samples were located in the linear range of a standard curve. Fold modulation was calculated by employing a comparative threshold (Ct) method (relative abundance of genes = $2^{\Delta Ct}$, where ΔCt is the difference between the Ct of target and the arithmetic mean of Cts of GAPDH). B cell stimulation included IgM (5 ug/ml) with or without IL-4 (10 ng/ml). T cell stimulation included CD3 (plate-bound 10 μg/ml) with or without IL-4 (10 ng/ml). Primers for IL-4Ra included the ABI TaqMan assay Mm00439634_m1 (detects total) or the ABI TaqMan assay Mm01275139_m1 (detects membrane only).

Intronic IL-4Rα (pre-mRNA) was measured by an allele-specific pyrosequencing assay as described previously (45). Pyrosequencing was performed on cDNA synthesized from the DNase-treated RNA. Assays were performed according to the manufacturer's instructions on the PSQ96 system (Biotage). Primers for pyrosequencing of IL-4Ra (intron 3) were: forward, 5′-GCATGAAGACACGTGGGAATTG-3′; reverse: biotin-5′-TCAACAGCTACCCTGGGTTTTC-3′; sequence, 5-CTGGGCTACAAG-TTACTG-3.

Statistical analysis

The *p* values between datasets were determined by ANOVA or a two-tailed Student *t* test assuming equal variance.

Results

IL-21–responsive T cells restore productive GC reactions in Il21r$^{-/-}$ *mice*

Mice made genetically deficient in either IL-21 or its receptor (*Il21r*$^{-/-}$) have GC reactions of reduced magnitude (2, 8, 43). To analyze the contribution of IL-21 to the ability of CD4$^+$ T cells to provide effective help to B cells, we established a system in which T cell help could be provided from Ag-specific IL-21R–deficient or –sufficient CD4$^+$ T cells to influence GC reactions in *Il21r*$^{-/-}$ mice. OVA-specific TCR transgenic wild-type (WT) OT-II T cells or *Il21r*$^{-/-}$ OT-II T cells bearing the Thy1.1 marker were transferred into Thy1.2 *Il21r*$^{-/-}$ mice and immunized with NP-OVA adsorbed to alum. In the absence of OT-II T cell help, endogenous *Il21r*$^{-/-}$ B cells generated a low frequency of GC B cells (Fig. 1A, 1B). IL-21–responsive OT-II T cells were able to improve GC reactions by endogenous *Il21r*$^{-/-}$ B cells, increasing the fraction (Fig. 1A, 1B) and absolute numbers (Fig. 1C) of GC B cells in *Il21r*$^{-/-}$ recipients on day 7 of primary immunization. Additionally, WT OT-II cells improved the average size of GC in *Il21r*$^{-/-}$ hosts determined histologically (Supplemental Fig. 1). Interestingly, *Il21r*$^{-/-}$ OT-II T cells also improved the fraction (Fig. 1A, 1B) and absolute numbers (Fig. 1C) of GC B cells on day 7 after primary immunization relative to *Il21r*$^{-/-}$ mice that had not received OT-II cells, which may reflect the increased frequency of Ag-specific T cells.

After secondary immunization, the GC reactions in *Il21r*$^{-/-}$ mice were even more compromised, as shown by a paucity of GC B cells in *Il21r*$^{-/-}$ mice (Fig. 1B, 1C). However, the provision of WT OT-II cells, transferred at primary immunization, ensured that an effective GC reaction occurred in *Il21r*$^{-/-}$ mice after secondary immunization (Fig. 1B, 1C, 1E). The provision of WT OT-II T cells also enabled the differentiation of Ab-forming *Il21r*$^{-/-}$ B cells after secondary immunization, identified as bright IgG1 containing plasmablasts in the bridging channels (Fig. 1E). In contrast, *Il21r*$^{-/-}$ recipients of *Il21r*$^{-/-}$ OT-II T cells responded poorly to secondary immunization with significantly reduced percentages and numbers of GC B cells compared with recipients of WT OT-II cells (Fig. 1B, 1C, 1E).

To test the functional output of these GCs, affinity maturation was assessed by measuring the amount of IgG1 produced that could bind to NP hapten when coupled at very high (NP_{23}) or low (NP_3) levels with BSA. Only very high-affinity Abs are capable of binding robustly to NP_3-BSA, which can be detected using ELISA. In accordance with our observations from histological sections and flow cytometry, only *Il21r*$^{-/-}$ mice that had received WT OT-II T cells were able to restore the production of both low-affinity (Fig. 2A) and high-affinity (Fig. 2B) Abs, showing that IL-21 receptiveness in CD4$^+$ T cells is important for effective T cell help. Although the numbers of *Il21r*$^{-/-}$ GC B cells remained lower than those for WT GC B cells, there seemed to be no absolute requirement in this system for B cells to respond to IL-21 to make high-affinity Abs. Taken together, these data indicate that IL-21–receptive Tfh cells are able to support the differentiation of affinity-matured Ab-producing B cells from B cells with reduced immune function.

Il21r$^{-/-}$ *B cells undergo somatic hypermutation and affinity maturation but fail to thrive*

Immunization with the NP hapten results in the clonal expansion of B cells that are characterized by usage of a particular common variable H chain element, VH186.2 (46). To assess whether both WT and *Il21r*$^{-/-}$ B cells could undergo somatic hypermutation, we quantified the frequency of amino acid mutations in CDRs 1 and 2 and framework regions in the sequence of the VH186.2 H chain fragment of GC B cells. We FACS sorted and sequenced the VH186.2 region from single GL7$^+$Fas$^+$ GC B cells at day 14 of the NP-OVA response in WT *Il21r*$^{-/-}$ mice and *Il21r*$^{-/-}$ mice that had received either WT or *Il21r*$^{-/-}$ OT-II T cells. All groups overwhelmingly used the VH186.2 H chain rather than other related noncanonical clones (Fig. 2C, Supplemental Table I). Despite the differences in the magnitude of the GC response, all groups showed a similar rate of mutation in the CDR regions as opposed to framework (Fig. 2C, Supplemental Table I). These findings demonstrate that although *Il21r*$^{-/-}$ GC B cells were reduced in frequency, IL-21 was not absolutely required for the process of somatic hypermutation.

GC B cells that harbored an L→W mutation at amino acid 33, which confers 10-fold stronger binding to NP (47, 48), were detected in all cases (Fig. 2D, Supplemental Table I). These data suggest that the reduced frequency of Ab-forming *Il21r*$^{-/-}$ B cells was caused by an inability to sustain GC B cells. Improved survival and expansion of somatically mutated B cell clones could therefore be achieved by IL-21 acting on B cells or by IL-21–independent help from WT Tfh cells.

IL-21 increases the numbers of Ag-specific CD4$^+$ T cells and Tfh cells

The model of OT-II transfer and NP-OVA immunization confirmed a role for IL-21 acting on T cells for the differentiation/survival of affinity-matured Ab-producing B cells from growth factor (IL-21)–deprived B cells. However, what IL-21 was doing to CD4$^+$ T cells to impart these effects remained unknown. We therefore examined

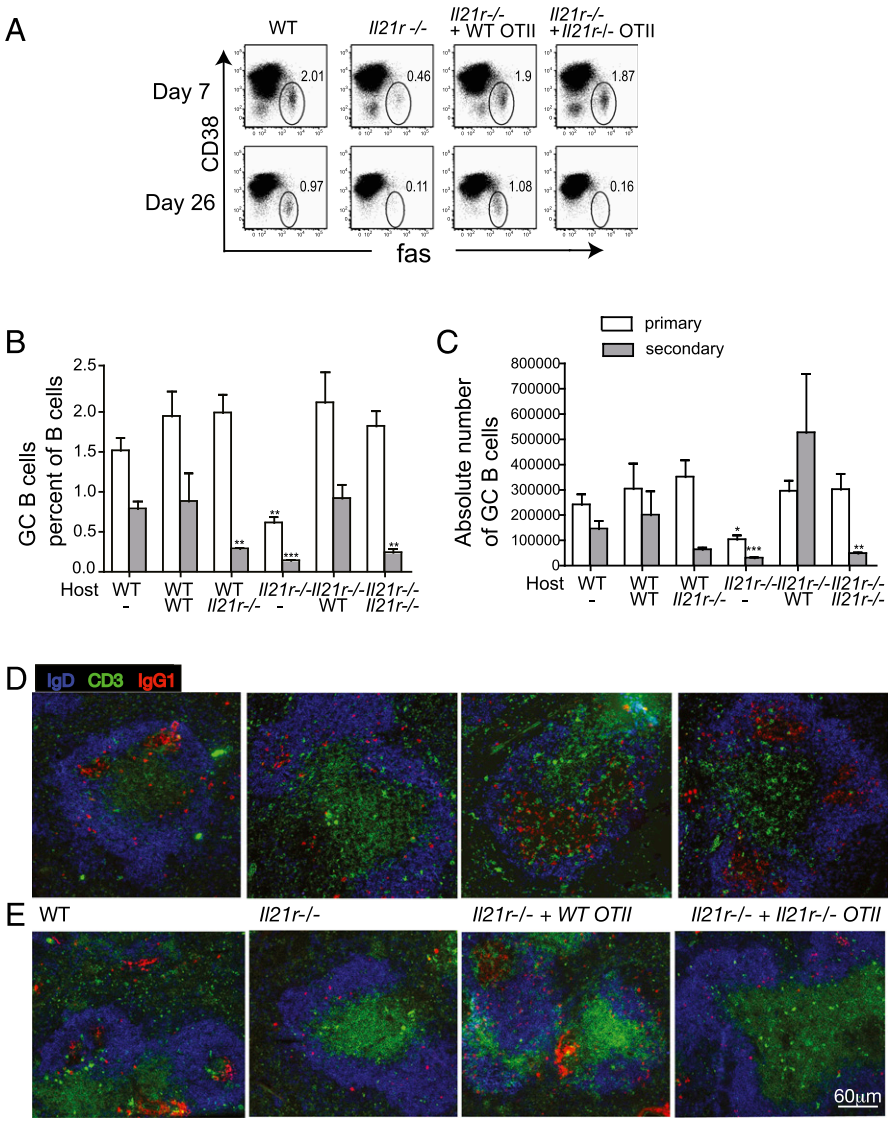

FIGURE 1. IL-21–receptive Ag-specific $CD4^+$ T cells rescue GC responses in $Il21r^{-/-}$ mice. WT or $Il21r^{-/-}$ OT-II cells were delivered i.v to WT and $Il21r^{-/-}$ hosts at the time of i.p immunization with 100 μg NP_{13}-OVA absorbed to alum. Spleens were analyzed at day 7, or at day 26, 5 d following a boost of 100 μg NP_{13}-OVA in PBS at day 21. (**A**) Dot plots showing gating strategy for $CD38^{lo}Fas^+$ GC B cells. (**B**) Percentages and (**C**) absolute numbers of $CD38^{lo}Fas^+$ GC B cells in both the primary and secondary responses described above; data are shown as means ± SEM. The *p* values were calculated by one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Representative confocal images of immunostained histological sections of spleen from the groups in (A) at (**D**) 7 and (**E**) 26 d showing immunofluorescent detection of IgD (blue), CD3 (green), and IgG1 (red).

WT and $Il21r^{-/-}$ donor OT-II T cells during primary and secondary responses to understand how IL-21 might be supporting Tfh cells. WT OT-II cells were recovered at greater numbers relative to $Il21r^{-/-}$ OT-II cells from both WT and $Il21r^{-/-}$ hosts (Fig. 3A). WT OT-II T cells expanded to 10-fold greater levels in $Il21r^{-/-}$ hosts than in WT hosts, perhaps reflecting higher levels of IL-21 available to WT OT-II cells (as IL-21 is not used by endogenous $Il21r^{-/-}$ cells) (Fig. 3A). In accordance with their decreased numbers, $Il21r^{-/-}$ donor T cells exhibited a slightly reduced modulation of molecules expressed on the surface of Tfh cells, including lower expression of the chemokine receptor CXCR5 and reduced ICOS expression after secondary immunization (Fig. 3B). By contrast, levels of the coinhibitory molecule PD-1 were markedly higher on donor $Il21r^{-/-}$ Tfh cells than on WT Tfh cells in both the primary and secondary responses (Fig. 3B).

In an effort to replicate our analyses of $Il21r^{-/-}$ Tfh cells in a nontransgenic system, we examined polyclonal Tfh cells and GC reactions that form following immunization in response to T-dependent Ag in mixed BM chimeras consisting of WT BM expressing the congenic marker Ly5.1 and $Il21r^{-/-}$ BM (Th1.1, Ly5.2). Twelve weeks later, and after checking chimerism following reconstitution, mice were immunized with NP-OVA in alum and the relative contribution of both WT and $Il21r^{-/-}$ lymphocytes to the immune response was determined. Reconstitution with $Il21r^{-/-}$ BM alone showed a reduced fraction of Tfh cells in response to immunization with NP-OVA in alum compared with recipients of WT BM cells (Fig. 3C, 3D). $Il21r^{-/-}$ Tfh cells were represented at similar or slightly lower percentages compared with WT Tfh cells on day 7 after primary immunization (Fig. 3D), but the percentages of WT and $Il21r^{-/-}$ Tfh cells were equivalent on day 21 of immunization (Fig. 3D). In contrast, 5 d after secondary immunization, significantly fewer $Il21r^{-/-}$ Tfh cells were observed relative to WT Tfh cells in the same mice (Fig. 3D). IL-21–receptive Tfh cells made a markedly greater contribution than did $Il21r^{-/-}$ Tfh cells to the total Tfh cell population following recall immunization (Fig. 3E). IL-21 offered a marked competitive advantage to GC B cells, memory B cells, and plasma cells in a B cell–intrinsic fashion (Supplemental Fig. 1). Although polyclonal $Il21r^{-/-}$ and WT Tfh cells appeared in similar numbers after the primary response to NP-OVA, the poor numbers of $Il21r^{-/-}$ Tfh cells observed 5 d after secondary exposure to NP-OVA may have been due to undetected quantitative differences in Tfh cells at day 21 or due to qualitative differences between $Il21r^{-/-}$ and WT cells.

IL-21 has an early effect on positioning and survival of Ag-specific T cells

We next focused our attention on an early time point following immunization (day 3), when $CD4^+$ T cells have been reported to

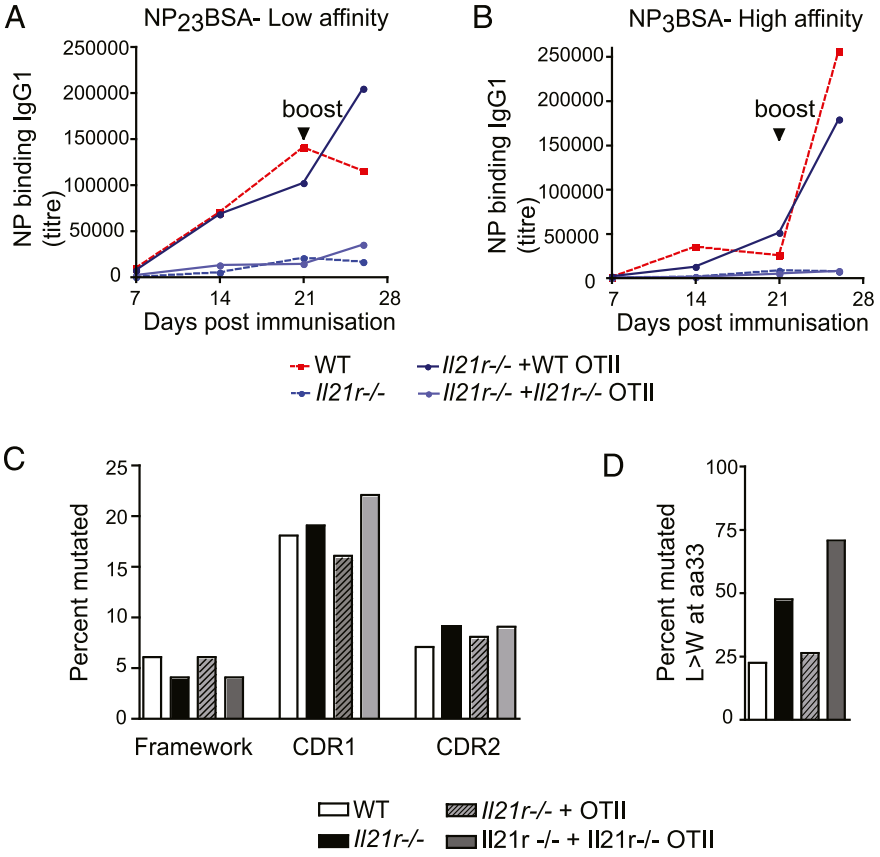

FIGURE 2. IL-21–receptive Ag-specific $CD4^+$ T cells help the differentiation of affinity-matured Ab-producing $Il21r^{-/-}$ B cells. Quantification of (**A**) low- and (**B**) high-affinity NP-specific Ab titers in the serum of WT and $Il21r^{-/-}$ mice and $Il21r^{-/-}$ recipients of 3×10^4 WT or $Il21r^{-/-}$ OT-II cells delivered i.v. at the time of i.p. immunization with 100 μg NP_{13}-OVA absorbed to alum. NP-specific Ab was measured in serum on days 7 and 14 following primary immunization, on the day of secondary boost (day 21), and 5 d following a boost of 100 μg NP_{13}-OVA in PBS by ELISA capture using NP_{23}-BSA or NP_3-BSA, respectively. (**C**) The mean percentages of $GL7^+$ Fas^+CD19^+ GC B cell clones, FACS purified on day 14 of immunization, bearing amino acid changes from the germline VH186.2 sequence in indicated regions. (**D**) The mean percentages of clones that bore an L→W mutation at position amino acid 33. Data represent the mean of all individual GC B cell sequences analyzed (see also Supplemental Table I).

begin expressing surface molecules associated with the Tfh cell phenotype (49–51). To study the importance of IL-21 for early expansion and positioning of Ag-specific T cells, we transferred CFSE-labeled WT and $Il21r^{-/-}$ OT-II T cells into WT and $Il21r^{-/-}$ hosts immunized with NP-OVA. We quantified positioning of CFSE-labeled WT and $Il21r^{-/-}$ OTII T cells by examining consecutive transverse sections from each group and scoring each for whether $CFSE^+$ T cells were in the red pulp, T cell zone, or B cell follicle (Fig. 3F). Both WT and $Il21r^{-/-}$ OT-II cells were represented equally in the red pulp (Fig. 3G). In contrast, IL-21–responsive OT-II cells were more frequent in the T cell zones (Fig. 3H) and B cell follicles (Fig. 3I) compared with $Il21r^{-/-}$ OT-II T cells. IL-21 clearly played a role in clonal expansion or survival of Ag-specific OT-II cells because even by day 3 there was a marked reduction in the numbers of $Il21r^{-/-}$ OT-II T cells recovered, regardless of whether the host mouse was IL-21R deficient or sufficient (Fig. 3J).

Flow cytometry can be used to detect stable interactions of T and B cells, termed T–B conjugates (17). These highly purified conjugates contained increased class-switched, somatically mutated, B cells at later stages of the immune response (17). Interestingly, despite the strong competitive advantage observed for IL-21–responsive B cells, the number of conjugates correlated with the genotype of the T cell donor, rather than the genotype of the host B cells (Fig. 4A, 4B). A significantly greater number of CFSE-labeled WT OT-II T cells were bound to B cells in both hosts than in their $Il21r^{-/-}$ counterparts (Fig. 4B), suggesting that IL-21 receptiveness by $CD4^+$ T cells favored the formation of strong synapses with B cells. Although $Il21r^{-/-}$ OT-II T cells formed a significantly reduced number of conjugates with B cells than did WT OT-II T cells, a relatively higher proportion of the residual $Il21r^{-/-}$ OT-II T cells was bound to B cells (Fig. 4A). PD-1 expression was, again, higher on $Il21r^{-/-}$ than on WT OT-II T cells, and expression of PD-1 was higher on B cell–bound, rather than free, OT-II T cells (Fig. 4C, 4D). In contrast, there was little difference observed in the expression of CXCR5, ICOS, CCR7, ICAM1, or BTLA between $Il21r^{-/-}$ OT-II cells and WT OT-II cells at this time point (Supplemental Fig. 2).

To determine whether receptiveness to IL-21 was influencing proliferation of OT-II cells in vivo, we analyzed the peaks of CFSE dilution by flow cytometry, but we observed no differences in the number of peaks as defined by halving of CFSE expression during division (Supplemental Fig. 2C). We next speculated that $Il21r^{-/-}$ Ag-specific $CD4^+$ T cells might be more susceptible to cell death. This may explain why a higher ratio of $Il21r^{-/-}$ OT-II T cells could be detected in conjugates. Indeed, we found that on day 3 of the response to NP-OVA, OT-II T cells that could not respond to IL-21 had decreased expression of the prosurvival molecule Bcl-x_L (Fig. 4E, 4F), whereas no difference in BCL2 could be detected (Fig. 4E). To determine whether IL-21 was also capable of sustaining Bcl-x_L expression in polyclonal $CD4^+$ T cells, we immunized WT and $Il21r^{-/-}$ mice with NP-OVA on alum and analyzed Bcl-x_L expression on day 3. There was an IL-21–dependent increase in the expression of Bcl-x_L in $ICOS^+CD4^+$ T cells and to a greater extent in $CXCR5^+ICOS^+$ Tfh cells (Fig. 4G, 4H). Taken together, these findings indicate that IL-21 delivers an important survival signal to Ag-specific Tfh cells.

IL-21–independent T cell help is mediated by IL-4

IL-21–responsive OT-II T cells could "rescue" $Il21r^{-/-}$ B cells for the generation of affinity-matured Ab, but the identity of the IL-21–independent help remained unknown. Because in addition to IL-21, the cytokine IL-4 is important for the generation for IgG1 Ab responses, we neutralized IL-4 with the Ab 11b11 after WT OT-II rescue of $Il21r^{-/-}$ B cells. Anti–IL-4, administered with the primary immunization, significantly reduced the fraction of

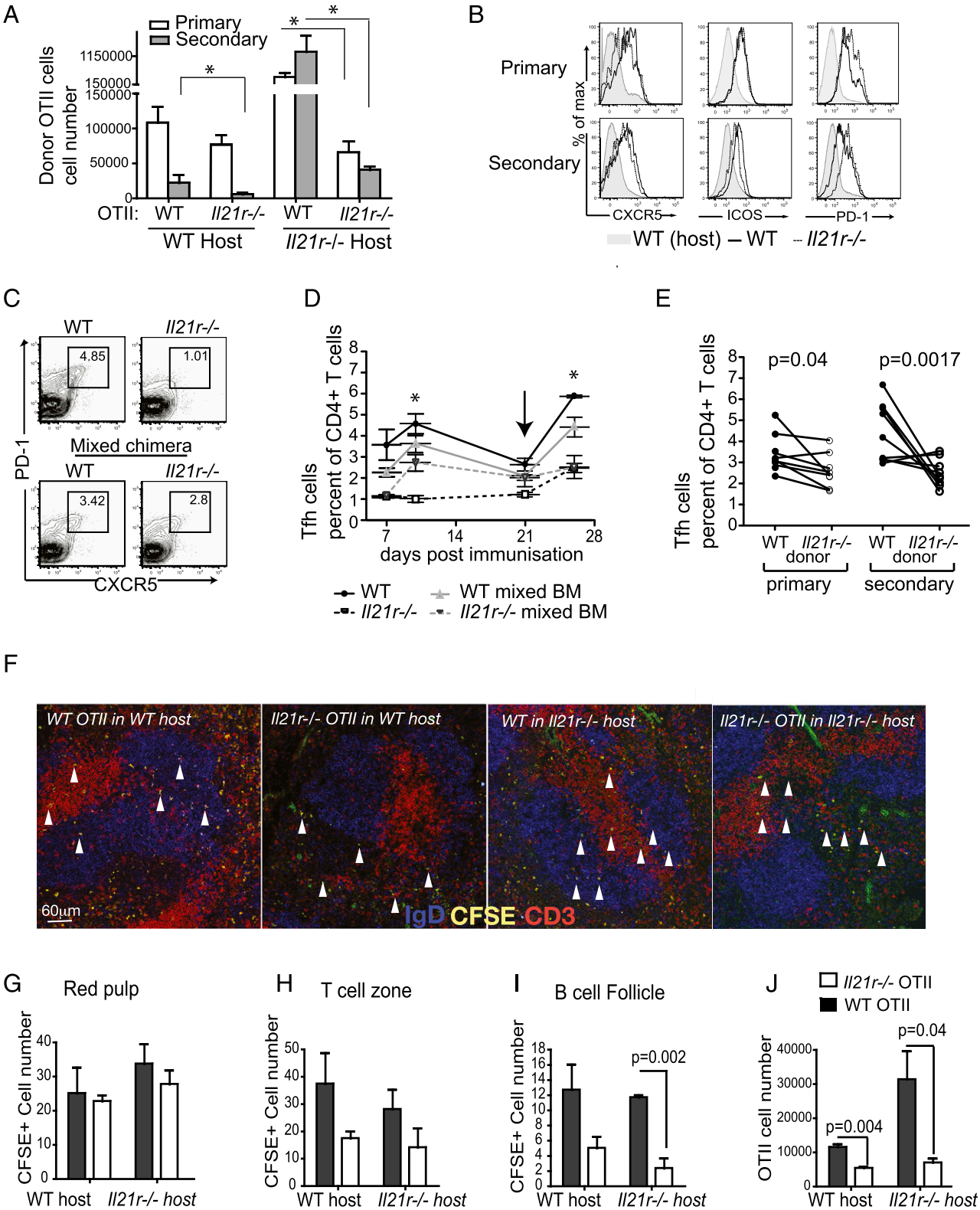

FIGURE 3. *Il21r*$^{-/-}$ CD4^{+} T cells are reduced in frequency and exhibit differential expression of Tfh cell surface markers. (**A**) Quantification of the number of donor WT and *Il21r*$^{-/-}$ OT-II T cells in the spleen on day 7 and day 26 of the primary and secondary response as shown in Fig. 2. The *p* values from one-way ANOVA of <0.01 are represented as asterisks where *Il21r*$^{-/-}$ values are significantly different from WT values. Data are presented as the mean of three experiments where $n = 9$ per group. (**B**) Splenic Tfh markers CXCR5, ICOS, and PD-1 on host polyclonal WT (filled), WT OT-II (solid line), or *Il21r*$^{-/-}$ OT-II (dashed line) donor CD4 T cells after primary (*top*) and secondary (*bottom*) immunization shown as histograms from three independent experiments with equivalent results. BM chimeras are shown from WT, *Il21r*$^{-/-}$, and 50:50% mixed *Il21r*$^{-/-}$ and WT donors immunized with 100 µg NP_{13}-OVA absorbed to alum 12 wk after reconstitution and checking for chimerism; spleens were analyzed on day 10 of immunization. (**C**) FACS gating for CD4^{+} T cells showing CXCR5^{+}PD-1^{+} Tfh cells (**D**) PD-1^{+}CXCR5^{+} Tfh cells as a percentage of Ly5.1^{+} WT or Ly5.2^{+} *Il21r* CD4^{+} T cells from BM chimera; asterisks indicate when values for *Il21r*$^{-/-}$ and WT from mixed BM chimeras are significantly different ($*p < 0.05$) to *Il21r*$^{-/-}$ alone and as (**E**) percentages of Ly5.1^{+} WT or Ly5.2+*Il21r*$^{-/-}$ CXCR5^{+}PD-1^{+} Tfh cells that contributed to the total Tfh cell population. (**F**) (*Figure legend continues*)

NP-specific GC B cells and NP-specific memory phenotype B cells that were detected after re-exposure to Ag (Fig. 5A and 5B, respectively). By contrast, when IL-4 was neutralized at the time of the secondary immunization, there was no effect on NP-specific B cells (Fig. 5A, 5B). Moreover, anti–IL-4 delivered at primary immunization prevented the WT OT-II–induced increase in both low affinity (Fig. 5C) and high affinity (Fig. 5D). To test whether IL-4 could influence the ability of GC B cells to form in *Il21r*$^{-/-}$ recipients of *Il21r*$^{-/-}$ OT-II cells, we administered rmIL-4 on day 2 of immunization with NP-OVA adsorbed to alum. The provision of rmIL-4 increased the percentage of GC (CD38^{lo}Fas^{+}) B cells in the spleens of *Il21r*$^{-/-}$ mice (Fig. 5E, 5F). rmIL-4 slightly boosted the fraction of GC *Il21r*$^{-/-}$ B cells (Fig. 5E), but not IgG1^{+} GC B cells in the primary response (Fig. 5G). More notable was the influence of rmIL-4 on GC *Il21r*$^{-/-}$ B cells (Fig. 5F) and IgG1^{+} GC B cells (Fig. 5H) that emerged after secondary immunization, increasing them to equal the percentage of WT GC B cells. Taken together, these findings indicated that IL-4 acting in the primary response aided the generation of Ab-forming *Il21r*$^{-/-}$ B cells that could respond to secondary immunization.

These findings demonstrated that IL-21–independent help was mediated by IL-4.

IL-21 modulates IL-4 production and IL-4Ra expression

To determine whether IL-21 receptiveness influenced the ability of Tfh cells to secrete IL-4, we FACS purified polyclonal CXCR5^{+} ICOS^{+} *Il21r*$^{-/-}$ and WT Tfh cells 7 d after immunization with NP-OVA in alum. IL-4 was then measured by ELISA in the supernatants of FACS-purified Tfh cells stimulated with PMA and ionomycin for 4 h. IL-21–receptive Tfh cells secreted a greater amount of IL-4 than did *Il21r*$^{-/-}$ Tfh cells (Fig. 6A), indicating an effect of IL-21 on IL-4 production or IL-4–producing Th cells.

As a result of the proximity of *Il21r* and *Il4r* on chromosome 7 in mice, *Il21r*$^{-/-}$ B6 mice (N12) mice carry the *Il4ra* allele from the original embryonic stem cell strain background 129/SvJ (129). To determine whether the expression of the IL-21R influences the IL-4R, we analyzed the expression of IL-4Ra on both *Il21r*$^{-/-}$ and WT (129 and B6) CD4^{+} T cells and B cells. IL-4Ra was detected on the surface of anti-IgM–stimulated B cells from WT and *Il21r*$^{-/-}$ and on CD4^{+} T cells stimulated with plate-bound anti-CD3 mice in the presence and absence of rmIL-4 by flow cytometry. FACS analyses showed a small, but significant, decrease in the expression of IL-4Ra on *Il21r*$^{-/-}$ cells compared with WT cells (Fig. 6B, 6C).

Utilizing primers that enabled selective amplification of total *Il4ra* or membrane-bound *Il4ra*, we observed decreased levels of *Il4ra* in both CD4^{+} T cells and B cells. Unstimulated CD4^{+} T cells and CD4^{+} T cells simulated with anti-CD3 mAb plus rmIL-4 from *Il21r*$^{-/-}$ T cells exhibited levels of total (Fig. 6D) and membrane-bound (Fig. 6E) *Il4ra* mRNA that were lower than levels of *Il4ra* in CD4^{+} T cells from either B6 or 129 mice. Similarly, unstimulated *Il21r*$^{-/-}$ B cells and *Il21r*$^{-/-}$ B cells stimulated with anti-IgM had decreased levels of total (Fig. 6F) and membrane-bound (Fig. 6G) *Il4ra* mRNA compared with levels of *Il4ra* mRNA from WT 129 and B6 mice. By contrast, the expression of *Il4ra* in CD4^{+} T cells and B cells from B6 and 129 mice was equivalent under all conditions (Fig. 6D–G).

During analyses of the locus, we also observed decreased expression of another *Il4ra* neighboring gene named Gleeshar in *Il21r*$^{-/-}$ mice relative to WT mice (Fig. 6H). The sequence of Gleeshar encoded on mouse chromosome 7 is defined by just one GenBank accession (no. BG144240) (http://www.ncbi.nlm.nih.gov/genbank) from GC B cells.

Reduced expression of Il4ra *is specific to the* Il21r$^{-/-}$ *allele*

Il21r$^{-/-}$ mice have defects in Th2 responses (30, 31) and in costimulation of lymphocyte Ag receptors that influence growth and survival. Therefore, the differences observed on IL-4Ra expression may simply reflect qualitative or quantitative differences in the baseline IL-4Ra–expressing T and B cell populations in *Il21r*$^{-/-}$ mice relative to their WT counterparts.

To remove some variability from differences in IL-4Ra–expressing T and B cell subsets in *Il21r*$^{-/-}$ and WT mice, we next determined whether the low expression of *Il4ra* was a feature of the Il21R knockout allele (129) in the same mice. To do this, we employed pyrosequencing, which measures the relative abundance of alleles in an F_1 cross of *Il21r*$^{-/-}$ and B6 mice. As discussed earlier, *Il21ra* and *Il4ra* sit 27 Kb apart on chromosome 7 in the mouse. As a result of this proximity, heterozygous *Il21r*$^{+/-}$ mice carry *Il4ra* from B6 mice on the WT allele and *Il4ra* from 129 mice on the *Il21r*$^{-/-}$ allele, and we could therefore measure the relative abundance of the *Il4ra* alleles by sequencing across one single nucleotide polymorphism that distinguished B6 *Il4ra* from 129 *Il4ra* (Fig. 6I).

Pyrosequencing demonstrated an increased abundance of the WT (B6) *Il4ra* allele in unstimulated T cells and after 2.5 h of stimulation in the presence or absence of rmIL-4. In contrast, after 5 h of stimulation, the abundance of *Il4ra* contributed by the WT (B6) and *Il21*$^{-/-}$ (129) alleles in CD4^{+} T cells was indistinguishable (Fig. 6J). In WT × *Il21r*$^{-/-}$ (F_1) B cells, both before and during 2.5 and 5 h of stimulation with anti-IgM in the presence or absence of rmIL-4, the abundance of *Il4ra* mRNA was underrepresented by the *Il21r*$^{-/-}$ allele (Fig. 6K).

Taken together, these findings indicate that the low levels of *Il4ra* were a feature of the *Il21r*$^{-/-}$ allele. To distinguish whether the reduced *Il4ra* in *Il21r*$^{-/-}$ mice reflected either the deletion of *Il21ra* or was simply due to an underlying difference in the expression of the 129 and B6 *Il4ra* alleles, we compared *Il4ra* expression in CD4^{+} T cells from 129 × B6 F_1 mice. These findings demonstrate that there was no significant difference between the abundance of B6 and 129 *Il4ra* alleles (Fig. 6L), indicating that the reduced expression of *Il4ra* in *Il21r*$^{-/-}$ mice reflected the absence of *Il21ra*.

Decreased soluble IL-4Ra correlates with increased IgE in sera

There are two forms of the IL-4R; one is membrane bound and the other is a soluble form (sIL-4R). Proteolytic cleavage releases sIL-4R, containing the extracellular portion of IL-4R, from the transmembrane and intracellular domains that remain cell-bound. sIL-4R inhibits the biologic actions of IL-4 in vitro (28) and in vivo (29, 52). sIL-4R detected in serum by ELISA on day 10 following immunization with NP-OVA in alum showed

Representative histological sections of spleen showing positioning of donor CFSE-labeled WT OT-II cells and *Il21r*$^{-/-}$ OT-II T cells in WT and *Il21r*$^{-/-}$ hosts. IgD immunostained cells are in blue, CD3 immunostained cells are in red, and CFSE-stained cells in yellow. Quantification is shown of the average number of T cells in more than three fields from 20 transverse histological sections scored for position within the (**G**) red pulp, (**H**) T cell zone, and (**I**) B cell follicle (n = 5 mice/group). (**J**) Total numbers of donor WT and *Il21r*$^{-/-}$ OT-II T cells in the spleen of WT and *Il21r*$^{-/-}$ hosts on day 3 of immunization. Data are shown as means ± SEM from two experiments where n = 5 per group. Only p values for significant differences are shown. The p values <0.001 were calculated using one-way ANOVA and are represented by asterisks.

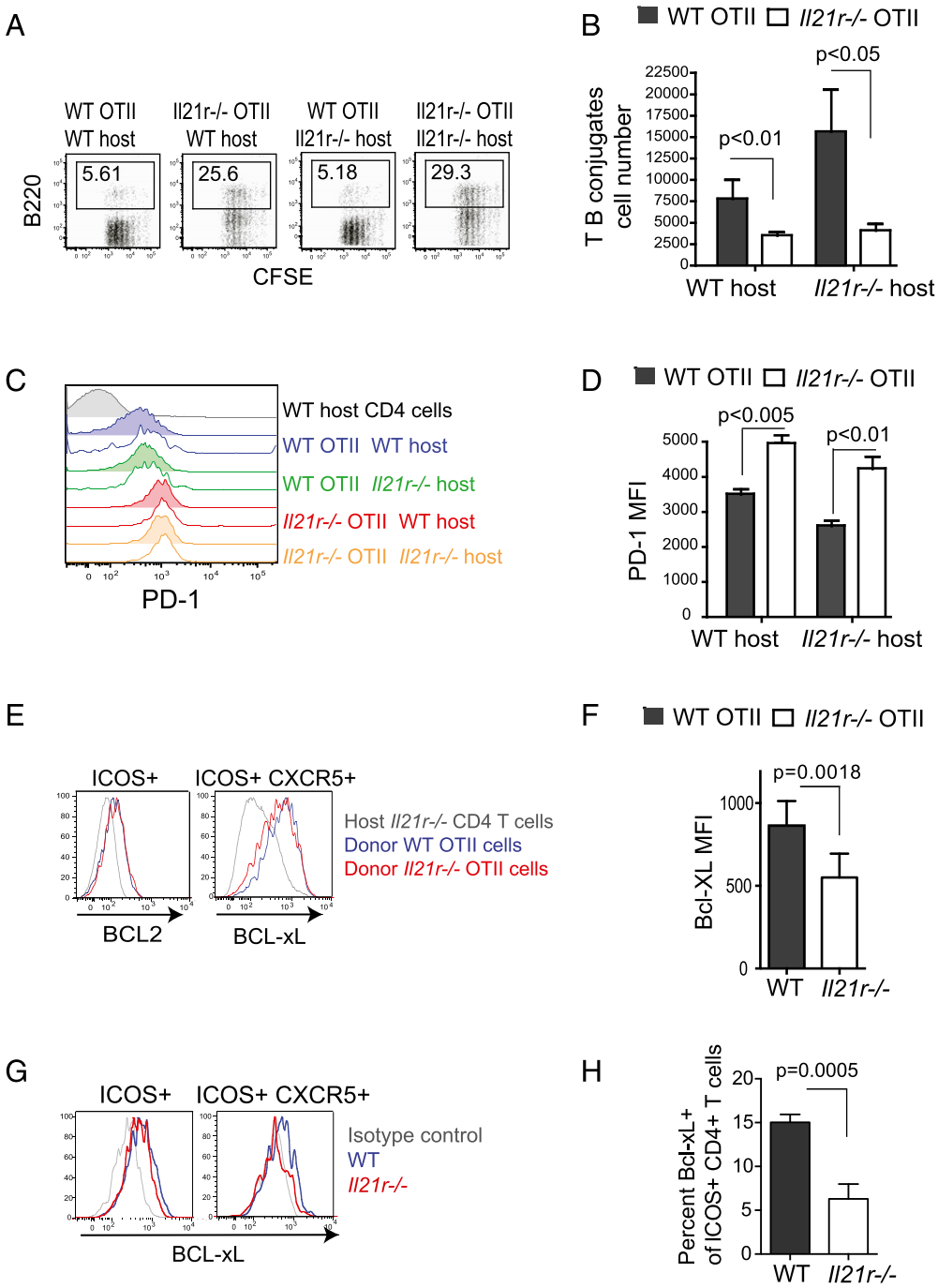

FIGURE 4. Quantitative and qualitative differences between *Il21r*$^{-/-}$ and WT Tfh cells. CFSE-labeled WT or *Il21r*$^{-/-}$ OT-II CD4 T cells (5×10^5) were adoptively transferred (i.v.) at the time of immunization (i.p.) with NP-OVA and analyzed on day 3. (**A**) Representative flow cytometry dot plots gated on donor Ly5.1$^+$ WT or Ly5.2$^+$ *Il21r* OT-II T cells showing OT-II T cells bound to B220$^+$ cells as doublets, and (**B**) quantification of the total number of T–B conjugates in the spleen of WT and *Il21r*$^{-/-}$ hosts. (**C**) Histograms showing PD-1 expression, where filled histograms are from unbound CD4$^+$ T cells and unfilled lines are from conjugate CD4$^+$ T cells. WT host CD4$^+$ T cells (gray line), WT OT-II cells transferred into WT hosts (blue line), WT OT-II cells transferred into *Il21r*$^{-/-}$ hosts (green line), *Il21r*$^{-/-}$ OT-II cells transferred into WT host (red line), and *Il21r*$^{-/-}$ OT-II cells transferred into *Il21r*$^{-/-}$ hosts (orange line) are shown. (**D**) Quantification of mean fluorescence intensity (MFI) of PD-1 immunofluorescence determined by FACS on gated WT and *Il21r*$^{-/-}$ OT-II T cells (±SEM). (**E**) Representative histograms showing BCL2 and Bcl-x_L expression in host *Il21r*$^{-/-}$ CD4$^+$ T cells, WT and *Il21r*$^{-/-}$ ICOS$^+$ OT-II T cells, and CXCR5$^+$ICOS$^+$ OT-II Tfh cells on day 3 postimmunization. Data are representative of two experiments in which four mice were pooled. (**F**) Quantification of the MFI of Bcl-x_L in CXCR5$^+$ICOS$^+$ Tfh cells from individual mice in (E). (**G**) Representative histograms showing Bcl-x_L in WT or *Il21r*$^{-/-}$ ICOS$^+$ and CXCR5$^+$ICOS$^+$ Tfh cells 3 d after i.p. immunization with 100 μg NP_{13}-OVA absorbed to alum. (**H**) Quantification of the percentage of *Il21r*$^{-/-}$ and WT Bcl-x_L^+CXCR5$^+$ICOS$^+$ Tfh cells from individual mice in (G). Data are shown as means ± SD from two experiments where n = 4–8.

a significant decrease in sIL-4R in *Il21r*$^{-/-}$ mice when compared with WT mice (Fig. 7A).

This effect was not specific to the immunogen NP-OVA because we also observed a significant reduction in the concentration of sIL-4R in sera from *Il21r*$^{-/-}$ mice relative to WT mice following immunization with the polyvalent Ag SRBC (Fig. 7B). The reduction in sIL-4R in *Il21r*$^{-/-}$ mice was observed on days 4, 7, 10, and 14 after SRBC immunization with the most sig-

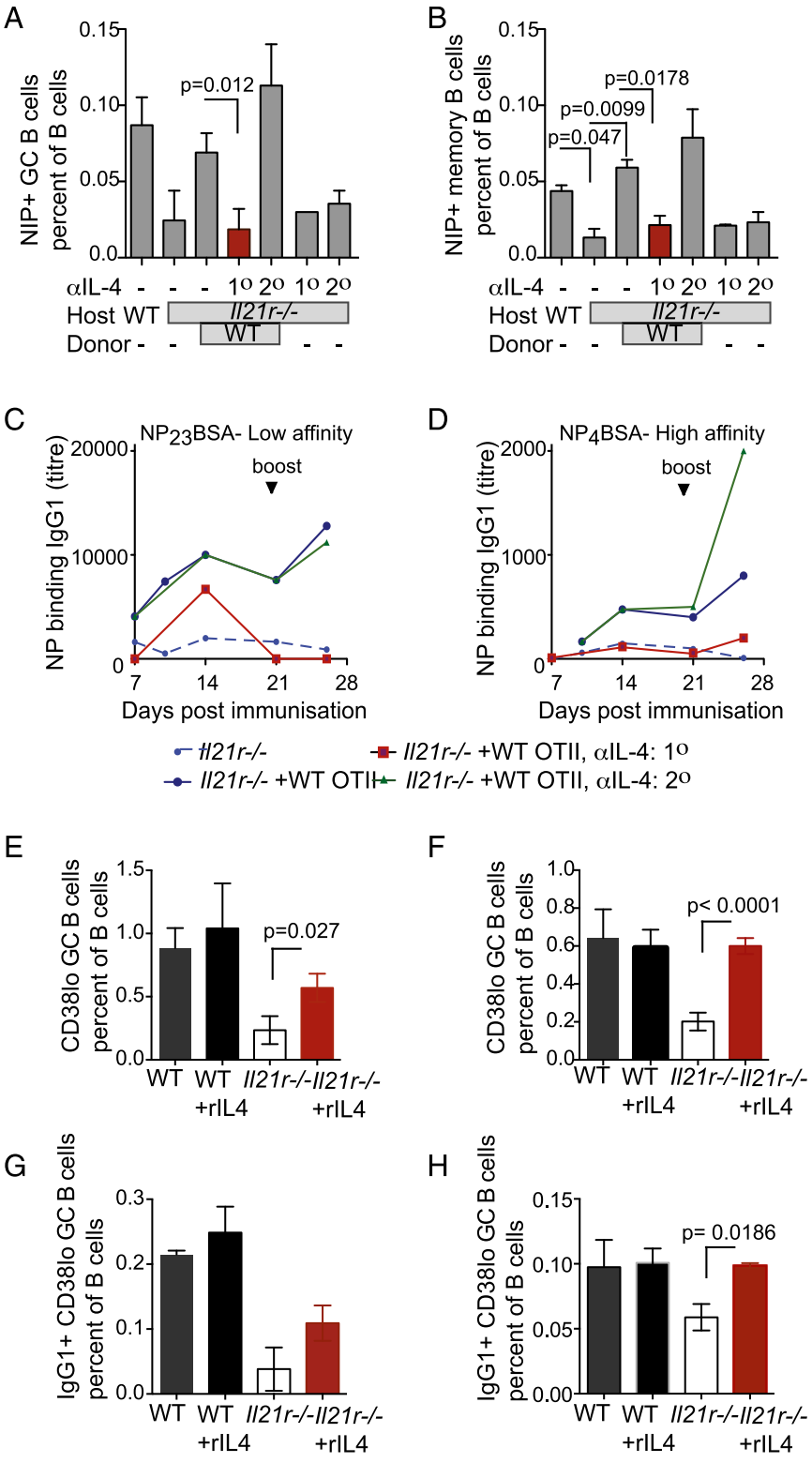

FIGURE 5. IL-21–independent T cell help is mediated by IL-4. WT *Il21r*$^{-/-}$ mice and *Il21r*$^{-/-}$ recipients of 3×10^5 WT OT-II T cells were treated with IL-4–blocking Ab (11B11) either on the day of primary immunization (1°) with 100 μg NP_{13}-OVA absorbed to alum or on the day of secondary boost (day 21) (2°). The GC reaction in the spleen was compared between groups. Quantification is shown of the percentage of (**A**) NP-specific (NIP$^+$) GC (CD38^{lo}Fas$^+$) B cells and (**B**) NP-specific (NIP$^+$) memory (CD38^{hi}GL7$^+$) B cells as a percentage of total B cells. Serum was collected weekly and at 5 d after the secondary boost was assessed by ELISA to detect (**C**) low affinity or (**D**) high affinity to either NP_{23}-BSA or NP_3-BSA, respectively. Data are presented as the mean of two combined experiments where n = 2–5 per group. Quantification is shown of the percentage of GC (CD38^{lo}Fas$^+$) B cells in the spleens of WT and *Il21r*$^{-/-}$ recipients of 3×10^4 *Il21r*$^{-/-}$ OT-II cells immunized with NP-OVA adsorbed to alum and (**E**) treated with rIL-4 on day 2, and analyzed on day 7, or (**F**) treated with rIL-4 on day 2 and analyzed on day 5 after boost on day 21. Quantification is shown of the percentage of IgG1$^+$ GC (CD38^{lo}Fas$^+$) B cells in the spleens of rmIL-4–treated WT and *Il21r*$^{-/-}$ mice after primary (**G**) and secondary (**H**) immunization with NP-OVA adsorbed to alum. Data are from two similar experiments, showing the means ± SD; n = 5 per group.

nificant difference between WT B6 and *Il21r*$^{-/-}$ mice observed on day 10 (Fig. 7B).

As discussed earlier, sIL-4R has been shown to block B cell binding of IL-4 and IgE production. We therefore measured the concentration of IgE in sera of WT and *Il21r*$^{-/-}$ mice and found it correlated inversely with the concentration of sIL-4R in sera (Fig. 7C). These findings suggest that the high levels of IgE that we, and others previously (16), have observed in *Il21r*$^{-/-}$ mice are associated with low production of sIL-4R, which may be a contributing factor to the dysregulation of IgE in *Il21r*$^{-/-}$ mice.

Discussion

The interaction between CD4$^+$ T cells and B cells is important for the differentiation of both Tfh cells and affinity-matured Ab-producing B cells (49–51). Tfh cell–produced cytokines, including IL-21, play an important role in the GC reaction following T-dependent immunization (2, 8, 43, 53). Both T and B cells express

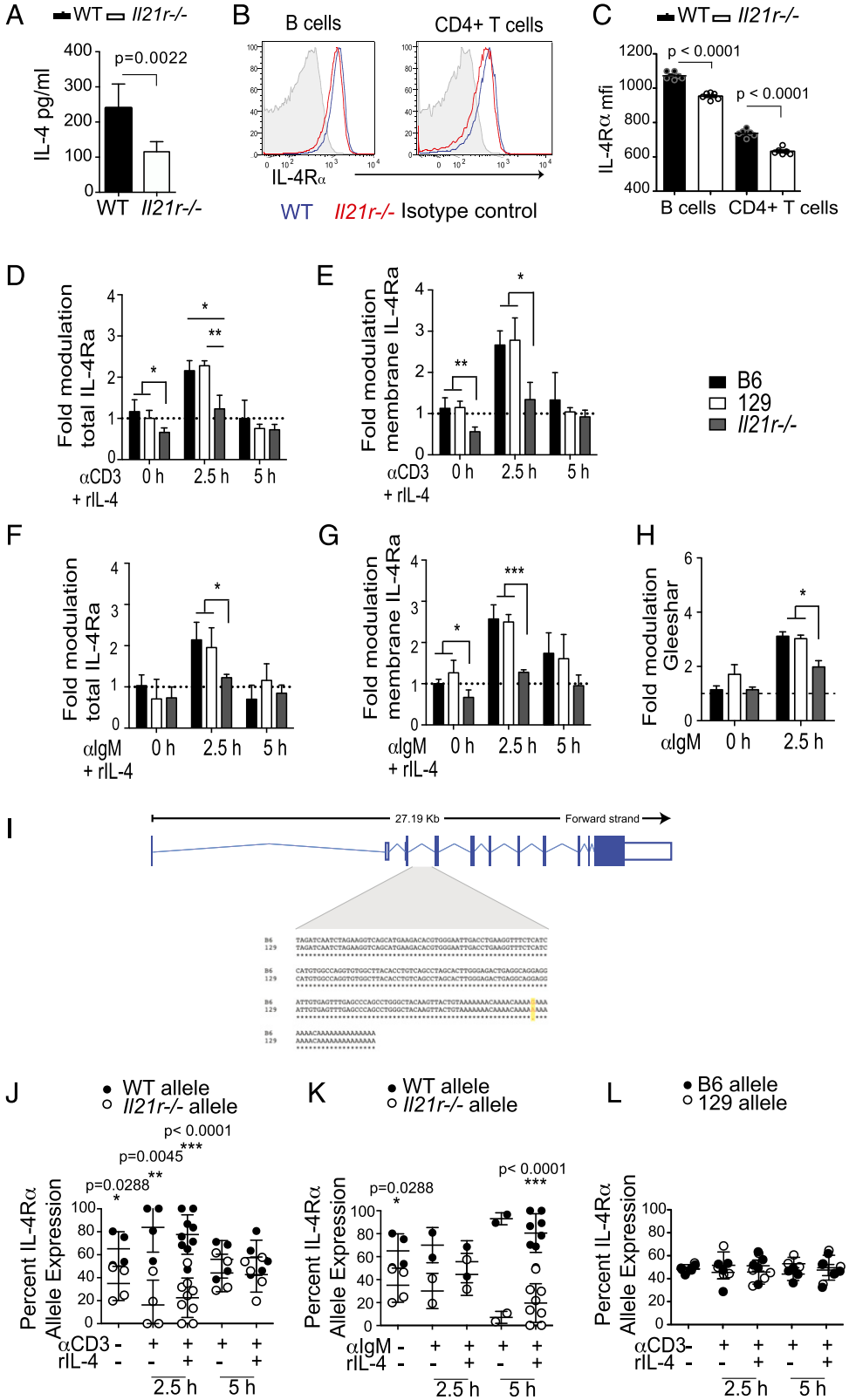

FIGURE 6. IL-21R influences T cell expression of IL-4 and the IL-4R on both T cells and B cells. IL-4 production (**A**) by *Il21r*$^{-/-}$ and WT CD4^{+} T cells purified from the spleen of mice on day 7 of immunization with 100 μg NP_{13}-OVA absorbed to alum. Two × 10^{6} cells/ml were restimulated with PMA and ionomycin for 4 h ex vivo and IL-4 was measured by ELISA. (A) Mean fluorescence intensity (MFI) of IL-4Ra measured on WT B6 and *Il21r*$^{-/-}$ B cells and CD4^{+} T cells shown as in histograms from FACS analyses (**B**) and means and MFI from individual mice ± SEM (**C**) from two experiments (n = 6–9). Total (**D**) and membrane specific (**E**) IL-4Rα mRNA levels in CD4^{+} T cells from splenocytes of B6, 129, and *Il21r*$^{-/-}$ mice stimulated with plate-bound anti-CD3 Ab and rIL-4 are shown. Total (**F**) and membrane-specific (**G**) B cells from splenocytes of B6, 129, and *Il21r*$^{-/-}$ mice stimulated with anti-IgM Ab and rIL-4 measured by quantitative PCR. Data are shown as means ± SD from n = 5 mice of two separate experiments with equivalent results. (**H**) Single nucleotide polymorphism identified to distinguish B6 and 129 Il4ra alleles (**I**). Intronic IL-4Rα (pre-mRNA) measured by allele-specific pyrosequencing assay for (**J**) *Il4ra* in anti-CD3 Ab with or without rIL-4, (**K**) anti-IgM Ab with or without rIL-4–stimulated (*Figure legend continues*)

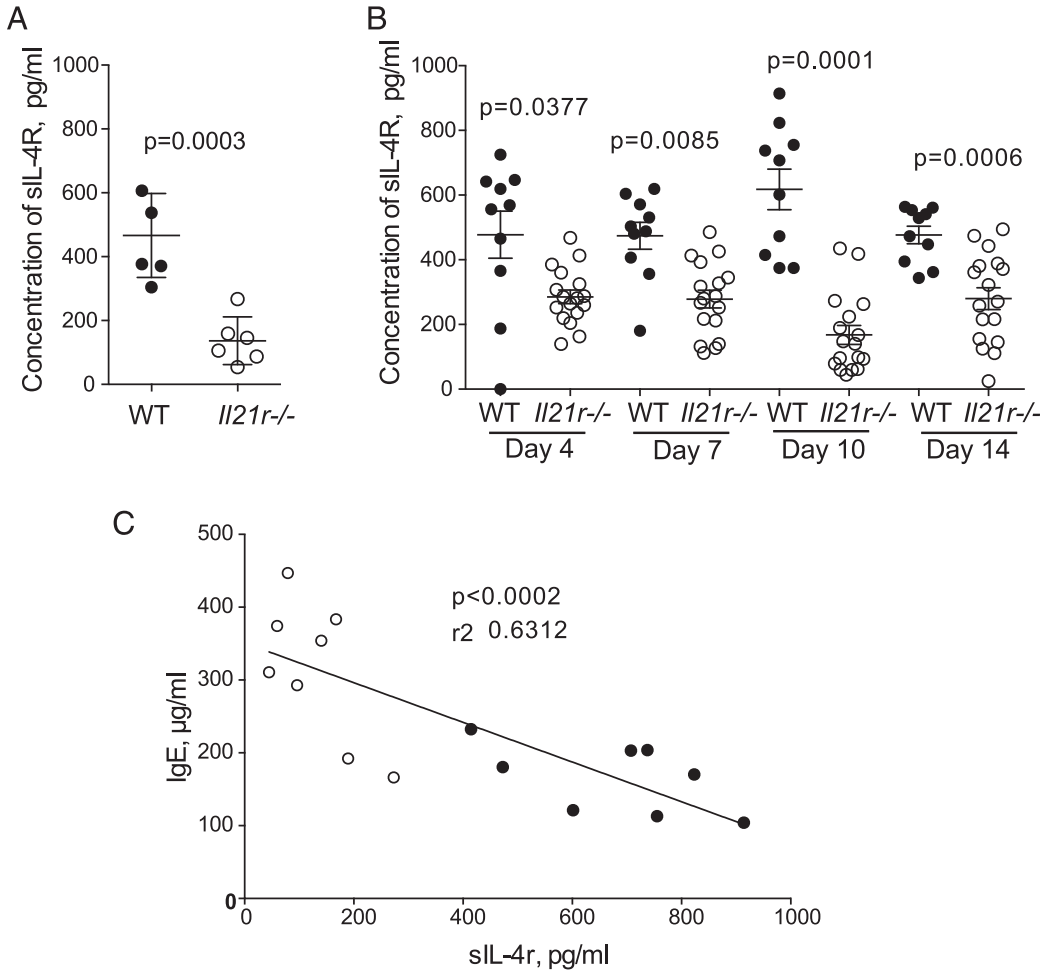

FIGURE 7. Increased production of IgE in *Il21r*$^{-/-}$ mice correlates with reduced production of soluble IL-4R. (**A**) sIL-4R measured in serum of B6 and *Il21r*$^{-/-}$ mice on day 10 following i.p. immunization with 100 μg NP_{13}-OVA absorbed to alum. The experiment was repeated twice with equivalent results (n = 6 mice/group). (**B**) sIL-4R measured in serum of WT (n = 10) and *Il21r*$^{-/-}$ (n = 17) mice on days 4, 7, 10, and 14 following i.p. immunization with SRBCs; results are shown for individual mice with means ± SEM. The p values are shown for WT versus *Il21r*$^{-/-}$ determined by a Student t test. (**C**) IgE and sIL-4R were measured on day 10 of SRBC immunization by ELISA; results are from individual mice, where *Il21r*$^{-/-}$ (○) and B6 (●), as tested for correlation as determined by linear regression analyses, are shown; r^2 and p values are indicated.

the receptor for IL-21 (10, 43), and the ligation of IL-21R by IL-21 initiates signaling pathways and transcription of genes, such as Bcl6, that have important roles in the differentiation and survival of Tfh cells and Ab-forming B cells (4, 5, 43). IL-21 has been shown to influence the differentiation of Ab-forming B cells through its actions on both B cells and CD4$^+$ T cells (2, 8, 9). In contrast, several studies have questioned whether IL-21 has a CD4$^+$ T cell–intrinsic role (10, 11).

IL-21 has exhibited varying degrees of influence on Tfh cells and humoral immune responses, depending on the form of Ag and adjuvant (2, 8, 11, 43). In addition to a context-dependent role for IL-21, IL-6 can act redundantly with IL-21 to support Tfh cell differentiation, and a deficiency of both cytokines severely limits the generation of Tfh cells (12, 13). The nature of this redundancy and the manner by which cytokines influence Tfh cells remain incompletely understood. However, IL-6 has been shown to activate STAT1, which is important for Tfh cell generation (54) and to stimulate production of IL-4 and IL-21 through c-Maf induction (55). IL-21 similarly activates c-Maf, but whether IL-21 influences IL-4 through c-Maf remains unknown.

In this study, we provide evidence that IL-21 supports the differentiation and survival of Ag-specific CD4$^+$ T cells. Reduced numbers of *Il21r*$^{-/-}$ OT-II Tfh cells were observed in both the primary and secondary response to immunization, and a reduced frequency of *Il21r*$^{-/-}$ OT-II Tfh cells was found bound to B cells in conjugates early in the primary response. The reduced frequency of *Il21r*$^{-/-}$ Tfh cells is likely to have contributed to the weaker GC reaction observed following recall immunization. In addition to the quantitative differences observed, *Il21r*$^{-/-}$ OT-II Tfh cells exhibited further qualitative differences to WT OT-II Tfh cells, including increased expression of the coinhibitory molecule PD-1. By contrast, endogenous *Il21r*$^{-/-}$ Tfh cells appeared in similar numbers to WT Tfh cells after primary immunization, but they were significantly reduced after secondary immunization in 50:50% *Il21r*$^{-/-}$/WT mixed BM chimeras, indicating a cell-intrinsic effect of IL-21–IL-21R signaling. The reason for the more marked effect of IL-21 on the survival/expansion of OT-II

splenocytes from *Il21r*$^{+/-}$ heterozygous mice, and (**L**) anti-CD3 Ab with or without rIL-4–stimulated splenocytes from 129 × B6 (F_1) mice is shown; values are from individual mice (including means ± SD) from three experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells may reflect the observation that OT-II cells have low affinity for OVA and to thrive may be particularly dependent on the costimulatory signal delivered by IL-21. This study did not determine the source of the CXCR5$^+$PD-1$^+$ Tfh cell population (or a component of this population) that expanded after secondary exposure to Ag. This population was unlikely to have expanded de novo, because the secondary boost was delivered i.v. without adjuvant. However, whether the cells expanded from a resident Tfh cell population generated during the primary response (56) or from memory T cells, as has been recently suggested (57), remains unknown. The early effect of IL-21 on Tfh cells was more suggestive of increased survival than increased proliferation, which was supported by lower expression of the prosurvival protein Bcl-x_L in *Il21r*$^{-/-}$ Tfh cells relative to their IL-21R–sufficient counterparts and is consistent with a previous report showing that IL-21 improves T cell survival through activation of PI3K (58).

IL-21–receptive B cells, in turn, had a marked competitive advantage over *Il21r*$^{-/-}$ B cells in mixed BM chimeras, which was evident in the GC B cell, memory B cell, and plasma cell populations. *Il21r*$^{-/-}$ B cells exhibited a poor affinity-matured Ab response despite undergoing somatic hypermutation and affinity-based selection in the GC. Our findings indicate that IL-21 aided the survival and/or expansion of GC B cells available for selection or during the process of selection rather than having a critical effect on the processes of class switch or somatic hypermutation. This finding contrasts with previous studies that have observed a critical role for IL-21 acting on B cells for affinity maturation (10, 43). The difference in our findings with that of these previous studies may be due to differences in the time point of analyses, which may be influenced by the reduced life-span of *Il21r*$^{-/-}$ GC B cells and the model systems used rather than an inability of *Il21r*$^{-/-}$ B cells to undergo these processes per se.

Il21r$^{-/-}$ Tfh cells were modest producers of IL-4 and (in part, as a consequence) inefficient B cell helpers. Only IL-21–responsive Ag-specific T cells were able to provide help to *Il21r*$^{-/-}$ B cells during the primary response that ensured a successful secondary GC reaction to NP-OVA. These findings suggested that *Il21r*$^{-/-}$ OT-II Tfh cells had not supported the differentiation of an isotype-switched Ag-specific memory B cell population during the primary response. The success of the secondary GC reaction was exemplified by affinity maturation of the Ab response from *Il21r*$^{-/-}$ B cells. IL-4, known to influence both isotype switch and B cell survival, was critical for IL-21–independent B cell help and the differentiation of *Il21r*$^{-/-}$ isotype-switched memory phenotype B cells. Our findings are consistent with a previous study that demonstrated the important role of IL-21 in the development of memory B cell responses (53). The superior ability of WT OT-II T cells to rescue *Il21r*$^{-/-}$ B cells in this study contrasted with the inefficiency of endogenous WT T cells in this and previous studies (10, 43, 53). The reason why OT-II cells, but not endogenous CD4$^+$ T cells, were effective at rescuing *Il21r*$^{-/-}$ B cells may reflect the relatively large precursor frequency of IL-4–producing Ag-specific OT-II Tfh cells.

Despite reduced Th2 responses, mice deficient in IL-21R or its ligand harbor increased amounts of the IL-4–dependent Ab isotype IgE (16), and increased IgE has been detected in sera of patients with loss-of-function mutations in the IL-21R gene (40). High IgE levels in *Il21r*$^{-/-}$ mice that also have poor IgG1 Ab responses is a paradoxical observation because an IgG1$^+$ B cell intermediate population may be the precursor of IgE-producing B cells (41). Through activation of Tfh cell–associated molecules such as Bcl6, IL-21 reinforces the GC response, supporting the differentiation and survival of B cells that produce affinity-matured IgG1 Ab while inhibiting IgE production (42, 43). The genes encoding the receptors for IL-4 and IL-21 are adjacent to each other, but it is unlikely that our observations reflect a unique effect in the *Il21r*$^{-/-}$ mouse line because mice made genetically deficient in the IL-21 ligand and humans with loss-of-function mutations in IL-21R also exhibit raised IgE levels (40, 59). In this regard, it remains possible that transcription of both IL-4R and IL-21R are modulated by shared regulatory elements.

Although the membrane-bound IL-4R has a known role in ligand binding and subsequent signaling, the role of the sIL-4R has been more difficult to define (52). Several in vitro and in vivo studies have demonstrated that the sIL-4R binds IL-4 with high affinity and can function as an antagonist of IL-4 activity, competing with the mIL-4R on target cells for the binding of IL-4 (20, 26, 27, 29, 52). The production of sIL-4Ra is reduced in *Il21r*$^{-/-}$ mice, in accordance with reduced membrane-bound IL-4Ra and reduced IL-4 production. The reduced amount of IL-4 produced by immune cells from *Il21r*$^{-/-}$ mice was matched by reduced sIL-4Ra, which may enable IL-4 to act even at very low doses to support IgE production in the absence of IL-21–IL-21R signaling and may contribute to the high IgE levels observed in the absence of IL-21–IL-21R signaling.

Our findings demonstrate that IL-21 acts on both CD4$^+$ T cells and B cells to generate an affinity-matured Ab response of normal magnitude. The evidence that IL-21 made better Tfh cells that could more effectively contribute to humoral immunity, even in the presence of immunodeficient B cells, indicates that Tfh cells could be useful therapeutic targets for improved vaccine strategies. Additionally, the findings in this study are consistent with the notion that both IL-21 and IL-4 are required for optimal IgG1 Ab responses (17, 18, 60). The observation that responsiveness to IL-21 influences responsiveness to IL-4 highlights the important collaboration of these two cytokines in Ab production.

Disclosures

The authors have no financial conflicts of interest.

References

1. Ansel, K. M., L. J. McHeyzer-Williams, V. N. Ngo, M. G. McHeyzer-Williams, and J. G. Cyster. 1999. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J. Exp. Med.* 190: 1123–1134.
2. Vogelzang, A., H. M. McGuire, D. Yu, J. Sprent, C. R. Mackay, and C. King. 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29: 127–137.
3. King, C. 2009. New insights into the differentiation and function of T follicular helper cells. *Nat. Rev. Immunol.* 9: 757–766.
4. Yu, D., S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, et al. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31: 457–468.
5. Nurieva, R. I., Y. Chung, G. J. Martinez, X. O. Yang, S. Tanaka, T. D. Matskevitch, Y. H. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325: 1001–1005.
6. Ettinger, R., S. Kuchen, and P. E. Lipsky. 2008. The role of IL-21 in regulating B-cell function in health and disease. *Immunol. Rev.* 223: 60–86.
7. Leonard, W. J., R. Zeng, and R. Spolski. 2008. Interleukin 21: a cytokine/cytokine receptor system that has come of age. *J. Leukoc. Biol.* 84: 348–356.
8. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29: 138–149.
9. Rasheed, M. A., D. R. Latner, R. D. Aubert, T. Gourley, R. Spolski, C. W. Davis, W. A. Langley, S. J. Ha, L. Ye, S. Sarkar, et al. 2013. Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. *J. Virol.* 87: 7737–7746.
10. Zotos, D., J. M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L. M. Corcoran, D. I. Godfrey, K. M. Toellner, M. J. Smyth, et al. 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J. Exp. Med.* 207: 365–378.

11. Bessa, J., M. Kopf, and M. F. Bachmann. 2010. Cutting edge: IL-21 and TLR signaling regulate germinal center responses in a B cell-intrinsic manner. *J. Immunol.* 184: 4615–4619.
12. Eto, D., C. Lao, D. DiToro, B. Barnett, T. C. Escobar, R. Kageyama, I. Yusuf, and S. Crotty. 2011. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One* 6: e17739.
13. Karnowski, A., S. Chevrier, G. T. Belz, A. Mount, D. Emslie, K. D'Costa, D. M. Tarlinton, A. Kallies, and L. M. Corcoran. 2012. B and T cells collaborate in antiviral responses via IL-6, IL-21, and transcriptional activator and coactivator, Oct2 and OBF-1. *J. Exp. Med.* 209: 2049–2064.
14. Kühn, R., K. Rajewsky, and W. Müller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science* 254: 707–710.
15. Toellner, K. M., S. A. Luther, D. M. Sze, R. K. Choy, D. R. Taylor, I. C. MacLennan, and H. Acha-Orbea. 1998. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *J. Exp. Med.* 187: 1193–1204.
16. Ozaki, K., R. Spolski, C. G. Feng, C. F. Qi, J. Cheng, A. Sher, H. C. Morse, III, C. Liu, P. L. Schwartzberg, and W. J. Leonard. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science* 298: 1630–1634.
17. Reinhardt, R. L., H. E. Liang, and R. M. Locksley. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat. Immunol.* 10: 385–393.
18. King, I. L., and M. Mohrs. 2009. IL-4-producing $CD4^+$ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J. Exp. Med.* 206: 1001–1007.
19. Rochman, Y., R. Spolski, and W. J. Leonard. 2009. New insights into the regulation of T cells by γ_c family cytokines. *Nat. Rev. Immunol.* 9: 480–490.
20. Renz, H., K. Bradley, K. Enssle, J. E. Loader, G. L. Larsen, and E. W. Gelfand. 1996. Prevention of the development of immediate hypersensitivity and airway hyperresponsiveness following in vivo treatment with soluble IL-4 receptor. *Int. Arch. Allergy Immunol.* 109: 167–176.
21. Henderson, W. R., Jr., D. B. Lewis, R. K. Albert, Y. Zhang, W. J. Lamm, G. K. Chiang, F. Jones, P. Eriksen, Y. T. Tien, M. Jonas, and E. Y. Chi. 1996. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J. Exp. Med.* 184: 1483–1494.
22. Ozaki, K., K. Kikly, D. Michalovich, P. R. Young, and W. J. Leonard. 2000. Cloning of a type I cytokine receptor most related to the IL-2 receptor β chain. *Proc. Natl. Acad. Sci. USA* 97: 11439–11444.
23. Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408: 57–63.
24. Leonard, W. J., and R. Spolski. 2005. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat. Rev. Immunol.* 5: 688–698.
25. Vogelzang, A., and C. King. 2008. The modulatory capacity of interleukin-21 in the pathogenesis of autoimmune disease. *Front. Biosci.* 13: 5304–5315.
26. Chilton, P. M., and R. Fernandez-Botran. 1993. Production of soluble IL-4 receptors by murine spleen cells is regulated by T cell activation and IL-4. *J. Immunol.* 151: 5907–5917.
27. Chilton, P. M., and R. Fernandez-Botran. 1997. Regulation of the expression of the soluble and membrane forms of the murine IL-4 receptor. *Cell. Immunol.* 180: 104–115.
28. Maliszewski, C. R., T. A. Sato, T. Vanden Bos, S. Waugh, S. K. Dower, J. Slack, M. P. Beckmann, and K. H. Grabstein. 1990. Cytokine receptors and B cell functions. I. Recombinant soluble receptors specifically inhibit IL-1- and IL-4-induced B cell activities in vitro. *J. Immunol.* 144: 3028–3033.
29. Henderson, W. R., Jr., E. Y. Chi, and C. R. Maliszewski. 2000. Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J. Immunol.* 164: 1086–1095.
30. Fröhlich, A., B. J. Marsland, I. Sonderegger, M. Kurrer, M. R. Hodge, N. L. Harris, and M. Kopf. 2007. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. *Blood* 109: 2023–2031.
31. Pesce, J., M. Kaviratne, T. R. Ramalingam, R. W. Thompson, J. F. Urban, Jr., A. W. Cheever, D. A. Young, M. Collins, M. J. Grusby, and T. A. Wynn. 2006. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J. Clin. Invest.* 116: 2044–2055.
32. Suto, A., H. Nakajima, K. Hirose, K. Suzuki, S. Kagami, Y. Seto, A. Hoshimoto, Y. Saito, D. C. Foster, and I. Iwamoto. 2002. Interleukin 21 prevents antigen-induced IgE production by inhibiting germ line Cε transcription of IL-4-stimulated B cells. *Blood* 100: 4565–4573.
33. Wurster, A. L., V. L. Rodgers, A. R. Satoskar, M. J. Whitters, D. A. Young, M. Collins, and M. J. Grusby. 2002. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon γ-producing Th1 cells. *J. Exp. Med.* 196: 969–977.
34. Suto, A., A. L. Wurster, S. L. Reiner, and M. J. Grusby. 2006. IL-21 inhibits IFN-γ production in developing Th1 cells through the repression of Eomesodermin expression. *J. Immunol.* 177: 3721–3727.
35. Suto, A., D. Kashiwakuma, S. Kagami, K. Hirose, N. Watanabe, K. Yokote, Y. Saito, T. Nakayama, M. J. Grusby, I. Iwamoto, and H. Nakajima. 2008. Development and characterization of IL-21-producing $CD4^+$ T cells. *J. Exp. Med.* 205: 1369–1379.
36. Lajoie, S., I. Lewkowich, N. S. Herman, A. Sproles, J. T. Pesce, T. A. Wynn, M. J. Grusby, Q. Hamid, and M. Wills-Karp. 2014. IL-21 receptor signalling partially mediates Th2-mediated allergic airway responses. *Clin. Exp. Allergy* 44: 976–985.
37. Avery, D. T., C. S. Ma, V. L. Bryant, B. Santner-Nanan, R. Nanan, M. Wong, D. A. Fulcher, M. C. Cook, and S. G. Tangye. 2008. STAT3 is required for IL-21-induced secretion of IgE from human naive B cells. *Blood* 112: 1784–1793.
38. Pène, J., L. Guglielmi, J. F. Gauchat, N. Harrer, M. Woisetschläger, V. Boulay, J. M. Fabre, P. Demoly, and H. Yssel. 2006. IFN-γ-mediated inhibition of human IgE synthesis by IL-21 is associated with a polymorphism in the IL-21R gene. *J. Immunol.* 177: 5006–5013.
39. Wood, N., K. Bourque, D. D. Donaldson, M. Collins, D. Vercelli, S. J. Goldman, and M. T. Kasaian. 2004. IL-21 effects on human IgE production in response to IL-4 or IL-13. *Cell. Immunol.* 231: 133–145.
40. Kotlarz, D., N. Ziętara, G. Uzel, T. Weidemann, C. J. Braun, J. Diestelhorst, P. M. Krawitz, P. N. Robinson, J. Hecht, J. Puchałka, et al. 2013. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. *J. Exp. Med.* 210: 433–443.
41. Erazo, A., N. Kutchukhidze, M. Leung, A. P. Christ, J. F. Urban, Jr., M. A. Curotto de Lafaille, and J. J. Lafaille. 2007. Unique maturation program of the IgE response in vivo. *Immunity* 26: 191–203.
42. Kitayama, D., A. Sakamoto, M. Arima, M. Hatano, M. Miyazaki, and T. Tokuhisa. 2008. A role for Bcl6 in sequential class switch recombination to IgE in B cells stimulated with IL-4 and IL-21. *Mol. Immunol.* 45: 1337–1345.
43. Linterman, M. A., L. Beaton, D. Yu, R. R. Ramiscal, M. Srivastava, J. J. Hogan, N. K. Verma, M. J. Smyth, R. J. Rigby, and C. G. Vinuesa. 2010. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J. Exp. Med.* 207: 353–363.
44. Wolniak, K. L., R. J. Noelle, and T. J. Waldschmidt. 2006. Characterization of (4-hydroxy-3-nitrophenyl)acetyl (NP)-specific germinal center B cells and antigen-binding $B220^-$ cells after primary NP challenge in mice. *J. Immunol.* 177: 2072–2079.
45. McGuire, H. M., A. Vogelzang, N. Hill, M. Flodström-Tullberg, J. Sprent, and C. King. 2009. Loss of parity between IL-2 and IL-21 in the NOD Idd3 locus. *Proc. Natl. Acad. Sci. USA* 106: 19438–19443.
46. McHeyzer-Williams, M. G., M. J. McLean, P. A. Lalor, and G. J. Nossal. 1993. Antigen-driven B cell differentiation in vivo. *J. Exp. Med.* 178: 295–307.
47. Bothwell, A. L., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1981. Heavy chain variable region contribution to the NPb family of antibodies: somatic mutation evident in a γ2a variable region. *Cell* 24: 625–637.
48. Cumano, A., and K. Rajewsky. 1985. Structure of primary anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies in normal and idiotypically suppressed C57BL/6 mice. *Eur. J. Immunol.* 15: 512–520.
49. Choi, Y. S., R. Kageyama, D. Eto, T. C. Escobar, R. J. Johnston, L. Monticelli, C. Lao, and S. Crotty. 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 34: 932–946.
50. Kerfoot, S. M., G. Yaari, J. R. Patel, K. L. Johnson, D. G. Gonzalez, S. H. Kleinstein, and A. M. Haberman. 2011. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* 34: 947–960.
51. Kitano, M., S. Moriyama, Y. Ando, M. Hikida, Y. Mori, T. Kurosaki, and T. Okada. 2011. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* 34: 961–972. doi: 10.1016/j.immuni.2011.03.025
52. Sato, T. A., M. B. Widmer, F. D. Finkelman, H. Madani, C. A. Jacobs, K. H. Grabstein, and C. R. Maliszewski. 1993. Recombinant soluble murine IL-4 receptor can inhibit or enhance IgE responses in vivo. *J. Immunol.* 150: 2717–2723.
53. Rankin, A. L., H. MacLeod, S. Keegan, T. Andreyeva, L. Lowe, L. Bloom, M. Collins, C. Nickerson-Nutter, D. Young, and H. Guay. 2011. IL-21 receptor is critical for the development of memory B cell responses. *J. Immunol.* 186: 667–674.
54. Choi, Y. S., D. Eto, J. A. Yang, C. Lao, and S. Crotty. 2013. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J. Immunol.* 190: 3049–3053.
55. Tsukamoto, H., S. Senju, K. Matsumura, S. L. Swain, and Y. Nishimura. 2015. IL-6-mediated environmental conditioning of defective Th1 differentiation dampens antitumour immune responses in old age. *Nat. Commun.* 6: 6702.
56. Fazilleau, N., M. D. Eisenbraun, L. Malherbe, J. N. Ebright, R. R. Pogue-Caley, L. J. McHeyzer-Williams, and M. G. McHeyzer-Williams. 2007. Lymphoid reservoirs of antigen-specific memory T helper cells. *Nat. Immunol.* 8: 753–761.
57. Fairfax, K. C., B. Everts, E. Amiel, A. M. Smith, G. Schramm, H. Haas, G. J. Randolph, J. J. Taylor, and E. J. Pearce. 2015. IL-4-secreting secondary T follicular helper (Tfh) cells arise from memory T cells, not persisting Tfh cells, through a B cell-dependent mechanism. *J. Immunol.* 194: 2999–3010.
58. Ostiguy, V., E. L. Allard, M. Marquis, J. Leignadier, and N. Labrecque. 2007. IL-21 promotes T lymphocyte survival by activating the phosphatidylinositol-3 kinase signaling cascade. *J. Leukoc. Biol.* 82: 645–656.
59. Shang, X. Z., K. Y. Ma, J. Radewonuk, J. Li, X. Y. Song, D. E. Griswold, E. Emmell, and L. Li. 2006. IgE isotype switch and IgE production are enhanced in IL-21-deficient but not IFN-γ-deficient mice in a Th2-biased response. *Cell. Immunol.* 241: 66–74.
60. Yusuf, I., R. Kageyama, L. Monticelli, R. J. Johnston, D. Ditoro, K. Hansen, B. Barnett, and S. Crotty. 2010. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J. Immunol.* 185: 190–202.