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In Vivo Regulation of Bcl6 and T Follicular Helper Cell Development

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Follicular helper T (T_{FH}) cells, defined by expression of the surface markers CXCR5 and programmed death receptor-1 (PD-1) and synthesis of IL-21, require upregulation of the transcriptional repressor Bcl6 for their development and function in B cell maturation in germinal centers. We have explored the role of B cells and the cytokines IL-6 and IL-21 in the in vivo regulation of Bcl6 expression and T_{FH} cell development. We found that T_{FH} cells are characterized by a Bcl6-dependent downregulation of P-selectin glycoprotein ligand 1 (PSGL1, a CCL19- and CCL21-binding protein), indicating that, like CXCR5 and PD-1 upregulation, modulation of PSGL1 expression is part of the T_{FH} cell program of differentiation. B cells were neither required for initial upregulation of Bcl6 nor PSGL1 downregulation, suggesting these events preceded T–B cell interactions, although they were required for full development of the T_{FH} cell phenotype, including CXCR5 and PD-1 upregulation, and IL-21 synthesis. Bcl6 upregulation and T_{FH} cell differentiation were independent of IL-6 and IL-21, revealing that either cytokine is not absolutely required for development of Bcl6⁺ T_{FH} cells in vivo. These data increase our understanding of Bcl6 regulation in T_{FH} cells and their differentiation in vivo and identifies a new surface marker that may be functionally relevant in this subset. *The Journal of Immunology*, 2010, 185: 313–326.

Antibody production is critical for pathogen clearance and prevention of reinfection, with Ab responses to most protein Ags strictly T cell dependent (TD) (1). Follicular helper T (T_{FH}) cells play a critical role in providing B cell help to TD Ags given their movement to the B cell follicle (2) and the germinal center (GC) (3, 4), the primary site of B cell somatic mutation with affinity maturation (5, 6) and Ig class switching (7). This help is mediated in part by IL-21, a cytokine produced by T_{FH} cells that, in cooperation with B cell AgR and TD signals, promotes GC B cell proliferation and maturation (8–12) and, in conjunction with IL-4, Ig class switching (13). Consistent with their location in the B cell follicle, T_{FH} cells upregulate the chemokine receptor

CXCR5, allowing for migration toward the ligand CXCL13 (B lymphocyte chemoattractant [BLC]) expressed in the follicle (3, 4). ICOS is also upregulated on T_{FH} cells and is required for their development and expansion (14–16), as are B cells (14, 17, 18) via their expression of the ligand for ICOS (B7RP.1) (9, 14). Similarly, IL-6 and IL-21 are important for the in vitro development of IL-21-producing CD4 T cells (19, 20) and T_{FH} cell development in vitro and in vivo following immunization with protein Ags (8, 9). IL-6 is also important for Ab responses in several systems (20–22). Yet, the role that these cytokines play in T cell maturation is not restricted to the T_{FH} cell subset, given their requirement for Th17 differentiation and maintenance (23–25).

Bcl6 is a transcriptional repressor that was originally identified in GC B cells, with its expression in these cells necessary for GC formation (26). It is also selectively expressed by T_{FH} cells compared with other CD4 T cell subsets (9, 12). We and others have recently shown that it is required for T_{FH} cell development and the subsequent formation of TD GC responses (18, 27, 28). Bcl6 represses a program of gene activation, including that of other transcription factors (18, 27, 28) and microRNAs (28) that promotes expression of proteins needed for T_{FH} cell trafficking and function. These observations further established T_{FH} cells as a subset independent from the Th1, Th2, and Th17 lineages; however, other studies have demonstrated that IFN- γ , IL-4, and IL-17 can be secreted by T_{FH} cells, with subsequent shaping of the Ab and autoantibody responses (18, 29–33) indicating plasticity in differentiation (34). IL-21 and IL-6 can induce Bcl6 expression in vitro in mouse T cells (9, 27), with IL-12 playing a similar role in human cells (35, 36), although the role that these cytokines play in Bcl6 regulation in vivo is less clear.

We recently described a population of CD4 T cells in lupus-prone MRL mice that is marked by downregulation of P-selectin glycoprotein ligand 1 (PSGL1). These cells migrate to the extrafollicular sites of Ab production in the spleen (37). Extrafollicular PSGL1^{lo} cells are similar to T_{FH} cells in that they express IL-21,

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Abbreviations used in this paper: alum, aluminum hydroxide; BLC, B lymphocyte chemoattractant; GC, germinal center; KO, knockout; LCMV, lymphocytic choriomeningitis virus; NP-CGG, nitrophenyl-chicken gammaglobulin; PD-1, programmed death receptor-1; PNA, peanut lectin (agglutinin); PSGL1, P-selectin glycoprotein ligand 1; TD, T cell-dependent; T_{FH}, follicular helper T; WT, wild-type.

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require ICOS and B cells for development, and are necessary for generation of class-switched Ab and autoantibody production; however, unlike T_{FH} cells, they lack expression of CXCR5. This absence, combined with expression of CXCR4, presumably enables their movement to extrafollicular locations (38). Modification of PSGL1 by glycosyltransferases permits T cell migration to inflammatory sites via binding to P- or E-selectin expressed on endothelial cells (39, 40); however, unmodified PSGL1 can bind CCL19 and CCL21 (41), suggesting that PSGL1 may act as a retention signal for T cells in the T zone.

These findings indicated that T cells with reduced surface expression of PSGL1 are capable of migration out of the T cell zone to sites of B cell help. Logically then, T_{FH} cells would likely be characterized by downregulation of PSGL1, because they too migrate to sites of B cell responses. We have sought in this study to address this question, in parallel with further dissection of the developmental requirements for T_{FH} cells. We specifically asked how the expression of Bcl6 is integrated with that of PSGL1, the inflammatory cytokines IL-6 and IL-21, and the presence of B cells, using in vivo models of Ag-specific CD4 T cell activation. We found that T_{FH} cells are characterized by a Bcl6-dependent downregulation of PSGL1, indicating that this is part of the T_{FH} cell program of differentiation. B cells were not required for initial upregulation of Bcl6 or PSGL1 downregulation, suggesting these events preceded T–B cell interactions, although they were required for full development of the T_{FH} cell phenotype, including CXCR5 and programmed death receptor-1 (PD-1) upregulation, and IL-21 synthesis. Interestingly, Bcl6 upregulation was independent of both IL-6 and IL-21, revealing that neither is absolutely required for development of Bcl6⁺ T_{FH} cells in vivo. These data increase our understanding of Bcl6 regulation in T_{FH} cells and their differentiation in vivo.

Materials and Methods

Mice

Mice were housed in specific pathogen-free conditions at the Yale School of Medicine (New Haven, CT) or the La Jolla Institute for Allergy and Immunology (La Jolla, CA). B6 mice were purchased from the National Cancer Institute (Bethesda, MD) (Figs. 1–6) or The Jackson Laboratory (Bar Harbor, ME) (Figs. 7–9). IL-6-deficient (B6.129S2-*Il6*^{tm1Kopf/J}), B cell-deficient (μ MT; B10.129S2(B6)-*Igh-6*^{tm1Cgn/J}), OT-II TCR transgenic (C57BL/6-Tg [TeraTerb]425Cbn/J), CD19-deficient (B6.129P2[C]-Cd19^{tm1CreCgn/J}), and MD4 (HEL Tg) (C57BL/6-Tg [IghelMD4]4Ccg/J) were purchased from The Jackson Laboratory, as were MRL/*Fas*^{pr} (MRL/MpJ-*Fas*^{pr}/2J) and BALB/c animals. IL-21-deficient (B6.129S5-*Il21*^{tm1Lex/Mmcd}) mice were purchased from MMRRC. DO11.10 mice (42) were provided by K. Bottomly (Yale School of Medicine). Bcl6-deficient B6 animals were generated as described previously (43). To produce Fas-deficient Bcl6-deficient animals, we backcrossed Bcl6 heterozygotes to the MRL/*Fas*^{pr} strain to the N2 generation, producing Bcl6 heterozygous Fas-deficient mice, followed by intercrosses to generate three groups of homozygous *Fas*^{pr} animals: Bcl6 intact, Bcl6 heterozygous, and Bcl6 deficient. These mice were used at ages 7–8 wk (given the shortened lifespan of Bcl6-deficient mice) in the experiments depicted in Fig. 4E–I, always with appropriate littermate controls. All other mice were used at 6–8 wk of age, save for wild-type (WT) MRL/*Fas*^{pr} animals sacrificed at age 16–24 wk. The Institutional Animal Care and Use Committee of Yale University or La Jolla Institute for Allergy and Immunology approved all procedures involving mice.

Immunizations and infections

A total of 0.5×10^6 OT-II CD4 T cells were transferred via retro-orbital injection into recipient mice. Twelve to 24 h later, 50 μ g OVA precipitated in 200 μ l aluminum hydroxide (alum) were administered by i.p. injection. Six days postimmunization, spleens of recipient mice were harvested and crushed through 40- μ m cell strainers. Red cell lysis was performed by hypotonic disruption as described previously (37). For nitrophenyl-chicken gammaglobulin (NP-CGG) immunizations, 100 μ g NP-CGG (Biosearch Technologies, Novato, CA) was precipitated in alum and 200 μ l administered i.p. Lymphocytic choriomeningitis virus (LCMV) stocks were

prepared and quantified as described previously (44). All infections were done by i.p. injection of $1-2 \times 10^5$ PFU of LCMV Armstrong per mouse.

Flow cytometry and cell sorting

Abs used for staining included CD4, CD44, TCR β , B220 (all from eBioscience, San Diego, CA), PD-1, ICOS, PSGL1, Thy1.1, CXCR5, CD62L, streptavidin, BTLA, Fas, IFN- γ , KJ1–26, IgM, IgG, CD138, CXCR4 (all from BD Biosciences, San Jose, CA), and peanut lectin (agglutinin) (PNA)-biotin (Vector Laboratories, Burlingame, CA). In general, 10×10^6 cells were surface stained in ice-cold $1 \times$ PBS plus 0.5% BSA for 30 min at 4°C. For Figs. 1–6, CXCR5 detection was performed for 30 min at 25°C to enhance detection of surface expression. For Figs. 7–9, CXCR5 labeling was a three-step stain as described previously (18). Cells were washed and immediately analyzed unfixed on an LSRII Multilaser Cytometer (BD Biosciences). Dead cells were excluded using Hoechst (Sigma-Aldrich, St. Louis, MO). Intracellular IgM and IgG staining was performed by standard fixation and permeabilization methods directly ex vivo. Staining for surface IgM was done prior to fixation. For intracellular cytokine staining, splenocytes were stimulated ex vivo for 4–5 h with LCMV gp61–80 peptide (2.0 μ g/ml) or PMA (20 ng/ml) and ionomycin (1 μ M). Intracellular IL-21 staining was done using an IL-21R-Fc chimeric protein (R&D Systems, Minneapolis, MN), followed by PE- or allophycocyanin-labeled anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) (18). For cell sorting, magnetic separation was performed using a biotin-based magnetic separation kit (EasySep; StemCell Technologies, Vancouver, British Columbia, Canada) to enrich for CD4 T cells, followed by surface staining as described previously. Sorting was performed on a FACSAria (BD Biosciences), with postsorting purity ~95%. A nondepleting CD4 Ab (BD Biosciences) was used for experiments with sorted cell transfers.

B cell transfers

A total of 0.5×10^6 OT-II CD4 T cells were transferred via retro-orbital injection into WT or B cell-deficient (μ MT) mice. Twelve to 24 h later, 50 μ g OVA precipitated in 200 μ l alum was administered by i.p. injection. Four days postimmunization, spleens were harvested and total CD4 T cells sorted using a nondepleting Ab. The percentage of OT-II CD4 Thy1.1⁺ cells was assessed by flow cytometry, and an equivalent number of OT-II CD4 T cells was then transferred to new recipients, either WT or B cell deficient, that were immunized 4 d previously i.p. with 50 μ g OVA in alum (at the same time as the first set of recipient mice) so that transferred cells would return to the same antigenic and inflammatory environment. Three days posttransfer of sorted CD4 T cells (7 d after the first immunization), spleens of recipient mice were harvested and processed for flow cytometry.

Quantitative PCR

For Figs. 1, 2, and 5, RNA from sorted cell populations was isolated using the mini or microRNasy Kit (Qiagen, Valencia, CA), followed by conversion to cDNA using iScript (Bio-Rad, Hercules, CA). Quantitative PCR was performed on a Stratagene MX4005P Thermal Cycler using Stratagene Brilliant SYBR Master Mix (Stratagene, La Jolla, CA) and the following primers: *bcl6*, 5'-CACACTCGAATTCATCTCG-3' (forward) and 5'-TAT-TGCACCTTGGTGTGG-3' (reverse); *il21*, 5'-TGAAAGCCTGTGGAA-GTTGCAACC-3' (forward) and 5'-AGCAGATTCACAGGACCA-CA-3' (reverse); *rorc*, 5'-CCGCTGAGAGGGCTTAC-3' (forward) and 5'-TGCAGGAGTAGGCCACATTACA-3' (reverse); *prdm1*, 5'-GCCAAC-CAGGAACCTTCTGTGT-3' (forward) and 5'-AGGATAAACACCCGAG-GGT-3' (reverse); and *rpl9*, 5'-TGAAGAAATCTGTGGTGC-3' (forward) and 5'-GCACTACGGACATAGGAAC-3' (reverse). L9 (*rpl9*) is a ribosomal protein gene that is expressed at a constant level between naive and effector T cells. For Fig. 8, RNA isolation and quantitative PCR analysis was performed as described previously (18). Briefly, SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) was used during cDNA synthesis. iTaq Sybr Green with Rox (Bio-Rad) was used on a Roche Lightcycler 480 for RT-PCR with the following primers: *bcl6*, 5'-CCTGTGAAATCTGTGGCACTCG-3' (forward) and 5'-CGCAGTTGGCTTTTGTGACG-3' (reverse); *il21*, 5'-GCT-CCACAAGATGTAAAGGGGC-3' (forward) and 5'-CCACGAGGTCAATG-ATGAATGTC-3' (reverse); and *prdm1* 5'-ACATAGTGAACGACCACCC-TG-3' (forward) and 5'-CTTACCACGCAATAACCTCTTTG-3' (reverse).

Western blots

Cells were sorted by flow cytometry from spleens of 16- to 24-wk-old WT MRL/*Fas*^{pr} mice, followed by lysis in radioimmunoprecipitation assay buffer. The supernatants from spun cell lysates were loaded onto a 12% SDS-PAGE gel in loading buffer. Roughly 13–15 μ g total protein was loaded per lane for each of the sorted populations, and 30 μ g protein

was loaded for positive controls. Blots were incubated with an anti-Bcl6 Ab (Cell Signaling Technology, Beverly, MA) overnight and, after exposure, stripped and reprobed overnight with anti-Blimp-1 (Santa Cruz Biotechnology, Santa Cruz, CA). After exposure, blots were restripped and probed for actin (Santa Cruz Biotechnology).

Retroviral transduction of primary T cells

OT-II Thy1.1 splenocytes were isolated and activated with Con A for 24 h in vitro. A control retrovirus (MigR1, an empty construct) or one expressing Bcl6 was transduced into activated OT-II splenocytes by centrifugation (2500 rpm) at 37°C for 90 min, and 0.5×10^6 OT-II CD4 T cells were immediately transferred i.v. into recipient mice. Twelve to 24 h after transfer, mice were immunized i.p. with 50 µg OVA precipitated in alum. Six days later, spleens were harvested and stained as above.

ELISA

Anti-LCMV Ab was measured by ELISA using sonicated cell lysate from LCMV-infected BHK-21 cells as capture Ag. Ninety-six-well Polysorp microtiter plates (Nunc, Naperville, IL) were coated overnight with lysate in PBS and then UV inactivated (300 mJ in Stratalinker 1800; Stratagene). HRP-conjugated goat anti-mouse IgG secondary Ab was used for detection (Caltag Laboratories, Burlingame, CA) (44). For NP-specific ELISA, plates were coated with NP₁₉-conjugated OVA (Biosearch Technologies). Anti-NP IgM and IgG Abs were detected using HRP-conjugated goat anti-mouse IgM, IgG, or IgG1 Abs (Southern Biotechnology Associates, Birmingham, AL). ODs were converted to units based on standard curves with sera from B6 mice immunized with NP-CGG using a four-parameter logistic equation (Softmax Pro 3.1 software; Molecular Devices, Sunnyvale, CA).

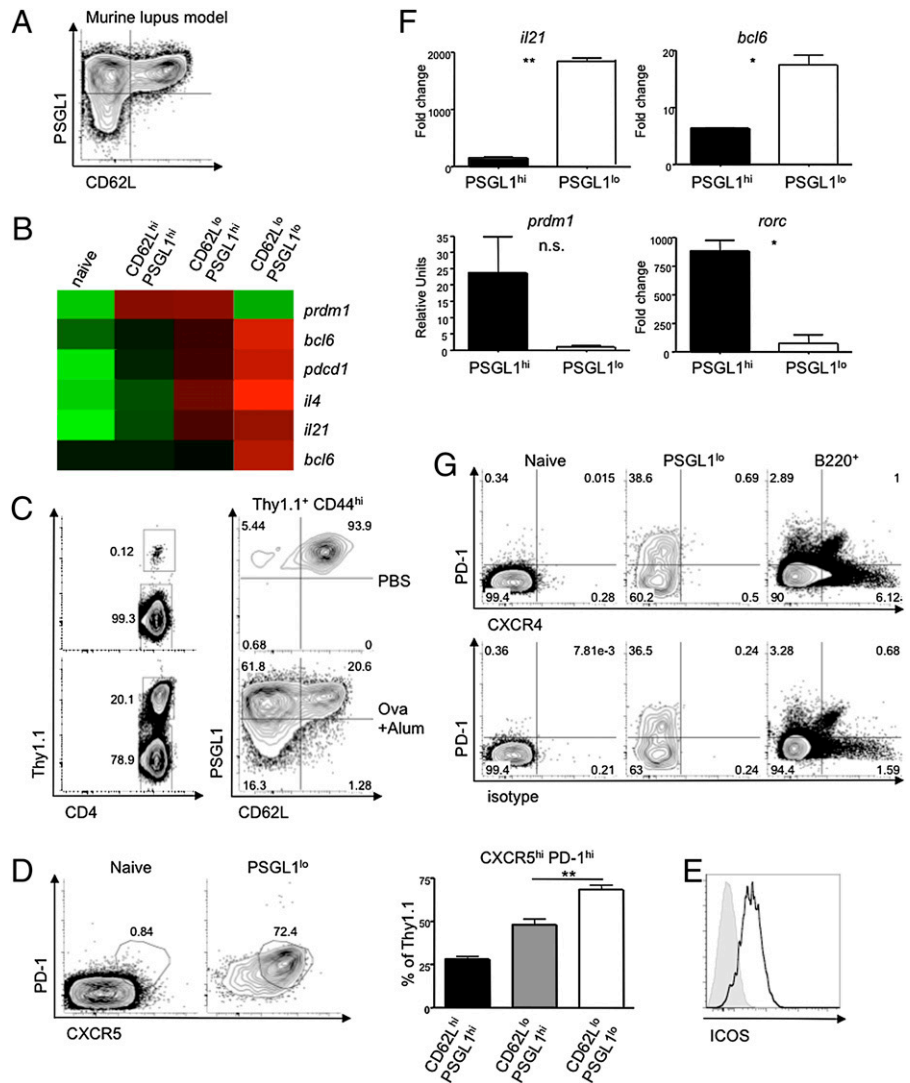
Microscopy

Spleens were harvested and immediately frozen in OCT tissue-freezing medium. Sections were cut to 6-µm thickness on a cryostat and stained for immunofluorescence using CD4 Pacific Blue (eBioscience), PNA biotin (Vector Laboratories), IgD FITC (BD Biosciences), and streptavidin Alexa-555 (Invitrogen). Unconjugated PSGL1 (BD Biosciences) was directly coupled to Alexa-647 using a mAb labeling kit (Invitrogen). All images were acquired on a Zeiss LSM 510 NLO confocal microscope (×25) equipped with a MaiTai titanium:sapphire 2-photon laser (Spectra-Physics) for Pacific Blue detection.

Gene expression microarrays

CD62L^{hi}PSGL1^{hi}, CD62L^{lo}PSGL1^{hi}, and CD62L^{lo}PSGL1^{lo} subsets (all of which were CD4⁺TCRβ⁺B220⁻CD44^{hi}) were sorted from spleens of aged (16–24 wk old) female lupus-prone MRL/Fas^{lpr} mice, with control naive (CD4⁺CD44^{lo}CD62L^{hi}) cells sorted from young 6- to 7-wk-old female animals. RNA from three independent sorts was submitted to the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University for amplification and hybridization to Affymetrix Mouse Genome 430 2.0 GeneChips. Data were analyzed using a GeneSpring GX Analysis Platform (Agilent, Palo Alto, CA). Genes that were differentially expressed ($p \leq 0.05$) among CD62L^{lo}PSGL1^{lo}, CD62L^{lo}PSGL1^{hi}, CD62L^{hi}PSGL1^{hi}, and naive subsets ($n = 3$) were identified using two-way ANOVA. The gene expression data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE20735.

FIGURE 1. PSGL1 is downregulated on T_{FH} cells. **A**, Expression of PSGL1 and CD62L on CD4⁺CD44^{hi}B220^{lo} splenocytes in aged lupus-prone MRL/Fas^{lpr} mice. **B**, Selected genes are differentially expressed in CD4 PSGL1^{lo} T cells from lupus-prone MRL/Fas^{lpr} mice. **C**, Expression of PSGL1 and CD62L on transferred OT-II Thy1.1 CD4 T cells in unimmunized or immunized mice. *Left panels* are gated on CD4⁺B220^{lo} cells. **D**, Expression of CXCR5 and PD-1 on gated OT-II Thy1.1 CD4 T cells 6 d after transfer to B6 mice and immunization. Bar graph represents the percentage of cells that are CXCR5^{hi}PD-1^{hi} of indicated group. Results are representative of three independent experiments using four mice per group; ** $p = 0.0075$. **E**, Expression of ICOS on OT-II Thy1.1 CD4 T cells 6 d after transfer to B6 mice and immunization. Naive CD4 T cells are shown in gray shade, and PSGL1^{lo} T cells by the black line. **F**, Expression of *il21*, *bcl6*, *prdm1*, and *rorc* transcripts in sorted PSGL1^{hi} and PSGL1^{lo} OT-II Thy1.1 CD4 T cells 6 d after transfer to B6 mice and immunization with OVA in alum. Results are representative of three independent experiments. Fold change is in comparison with sorted naive CD4 T cells; *il21*, ** $p = 0.0012$; *bcl6*, * $p = 0.0223$; and *rorc*, * $p = 0.0221$. **G**, Expression of CXCR4 on naive, PSGL1^{lo} T_{FH}, and B220⁺ cells 7 d after transfer to B6 mice and immunization. The PSGL1^{lo} cells are in the gated OT-II Thy1.1 CD4 T cell population. Results are representative of two experiments using four mice per experiment.



Statistics

Data were analyzed with Student *t* test with Prism4 or 5 (GraphPad Software; GraphPad, San Diego, CA). Number of asterisks represents the degree of significance with respect to *p* value. Bar graphs for experiments involving cell percentages and RT-PCR show mean \pm SEM.

Results

T_{FH} cells are marked by downregulation of PSGL1

We recently demonstrated that lupus-prone MRL/*Fas*^{lpr} mice have a subset of activated CD4 T cells with reduced surface expression of PSGL1 (Fig. 1A) (37). In MRL mice, PSGL1^{lo} cells were capable of promoting isotype-switched autoantibody formation, a process dependent upon their secretion of IL-21, and one associated with upregulation of activation-induced cytidine deaminase in plasma cells. To further characterize these cells, we compared their gene expression profile to that of naive and activated PSGL1^{hi} CD4 T cells, also from MRL mice. Several gene products were upregulated in the PSGL1^{lo} population compared with naive and activated PSGL1^{hi} cells, including those that encode Bcl6, PD-1, IL-4, and IL-21 (Fig. 1B). Blimp-1 (*prdm1*) was downregulated in PSGL1^{lo} cells compared with activated PSGL1^{hi} cells, consistent with the finding that Bcl6 can repress Blimp-1 and vice versa (18, 45–47).

We hypothesized that T_{FH} cells are characterized by reduced surface expression of PSGL1, because IL-21 secretion and PD-1 expression are known markers of this population, as is Bcl6 (12, 17). To address this hypothesis, we tracked development of Ag-specific PSGL1^{lo} cells following immunization of B6 mice. We adoptively transferred H-2^b-restricted OVA-specific OT-II TCR-transgenic CD4 T cells to Thy1-mismatched recipients, followed by i.p. immunization with OVA mixed in the adjuvant alum and analysis of splenocytes 6 d later. Following immunization, PSGL1^{lo} cells made up ~15–20% of activated OT-II Thy1.1 CD4 T cells, whereas unimmunized controls retained a small number of naive transferred cells (Fig. 1C). PSGL1^{lo} cells in immunized mice were enriched for cells expressing PD-1, consistent with our array data, as well as upregulation of CXCR5 and ICOS, additional markers of T_{FH} cells (Fig. 1D, 1E). Similar results were obtained when H-2^d-restricted DO11.10 TCR-transgenic cells were transferred to BALB/c recipients followed by immunization of OVA in alum (Supplemental Fig. 1A).

To rule out the possibility that development of PSGL1^{lo} cells was an artifact of adoptive transfers of TCR transgenic T cells, we immunized B6 mice with NP-CGG in alum, followed by analysis of spleens 8 d later. In this study, as in mice with adoptively transferred TCR transgenic cells, there was expansion of PSGL1^{lo} as well as CXCR5^{hi} and PD-1^{hi} cells (Supplemental Fig. 1B).

CXCR5, PD-1, and ICOS are upregulated following Ag encounter, as well as on T_{FH} cells (48). To help determine whether PSGL1 downregulation is a specific marker of the latter, we asked whether PSGL1^{lo} cells expressed IL-21 and Bcl6, other constituents of T_{FH} cells. Activated OT-II Thy1.1 CD4 T cells following transfer and immunization were sorted into PSGL1^{hi} and PSGL1^{lo} subsets, with mRNA expression analyzed by RT-PCR. OT-II PSGL1^{lo} T cells had upregulation of transcripts for *il21* and *bcl6* in comparison with PSGL1^{hi} cells (Fig. 1F), as did PSGL1^{lo} cells from B6 mice immunized with NP-CGG in alum (Supplemental Fig. 1C). PSGL1^{hi} cells expressed Blimp-1 (*prdm1*) transcripts, whereas PSGL1^{lo} cells expressed relatively little Blimp-1 (Fig. 1F), consistent with the findings that Bcl6 represses Blimp-1 (45–47) and that Bcl6 and Blimp-1 distinguish separate populations of effector CD4 T cells (18, 49). Because Th17 cells produce IL-21, we also determined the mRNA expression of *rorc*, the gene encoding ROR γ t, the transcription factor for their development (50). We found that *rorc*

expression was downregulated in PSGL1^{lo} cells in comparison with PSGL1^{hi} cells (Fig. 1F), indicating that the presence of IL-21 in PSGL1^{lo} cells was not due to a Th17 phenotype.

As we have previously demonstrated that extrafollicular PSGL1^{lo} cells from MRL/*Fas*^{lpr} mice expressed high levels of CXCR4, but not CXCR5 (37), we assessed the former on PSGL1^{lo} T_{FH} cells in nonautoimmune mice (37). Seven days following transfer of OT-II Thy1.1 cells into WT mice and immunization, we found that CXCR4 was not upregulated on PSGL1^{lo} T_{FH} cells; however, the presence of CXCR4 was detected on a subset of B cells, presumably extrafollicular plasma cells, as expected after immunization (Fig. 1G) (38).

To explore concomitant expression of the markers CXCR5, PD-1, and PSGL1, we analyzed activated CD4 T cells isolated from mice 8 d following immunization with NP-CGG in alum. As expected from previous reports, we found that activated CD4 T cells with the highest expression of CXCR5 also had high expression of

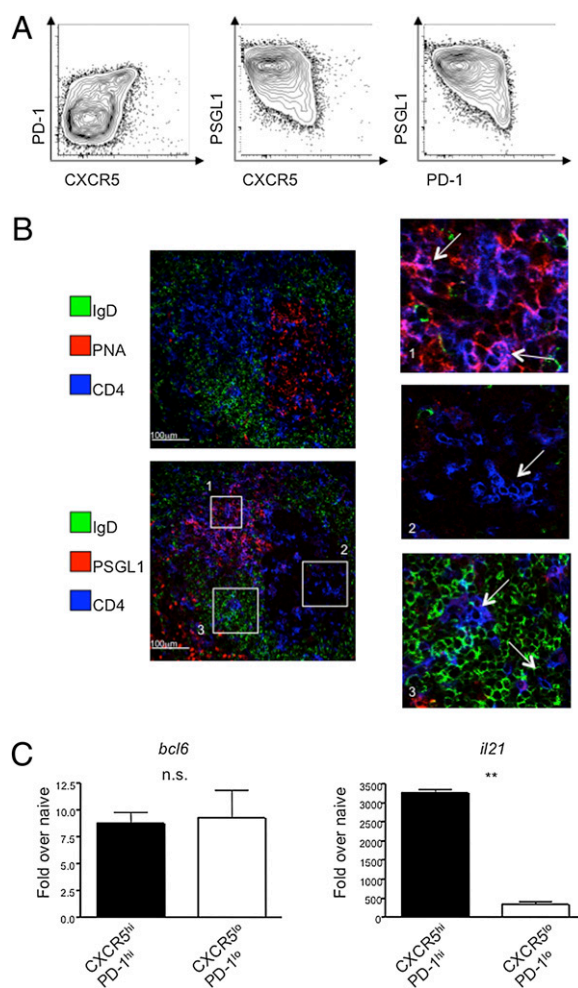


FIGURE 2. PSGL1^{lo} T_{FH} cells are located in GCs and express Bcl6. *A*, Expression of CXCR5, PD-1, and PSGL1 on CD44^{hi} CD4 T cells taken from spleens of B6 mice 8 d after immunization with NP-CGG in alum. *B*, Microscopy of spleens taken from B6 mice 8 d after immunization with NP-CGG in alum. Sections were stained with four Abs. For simplicity, the top panel shows staining to detect IgD, PNA, and CD4; the bottom panel shows that for IgD, PSGL1, and CD4. Inset panels (1–3) show increased magnification of labeled areas. Arrows indicate PSGL1^{hi} (panel 1) or PSGL1^{lo} (panels 2, 3) CD4 T cells. *C*, Expression of *bcl6* and *il21* mRNA levels in sorted CD44^{hi}CD4⁺PSGL1^{lo}CXCR5^{lo}PD-1^{lo} or CD44^{hi}CD4⁺PSGL1^{hi}CXCR5^{hi}PD-1^{hi} cell subsets. Results are representative of two independent experiments. Fold change is in comparison with sorted naive CD4 T cells. *p* = 0.8765 (*bcl6*); ***p* = 0.0016 (*il21*).

PD-1. Similarly, CD4 T cells with the highest expression of CXCR5, or PD-1, had the lowest levels of PSGL1 (Fig. 2A). This finding indicated that downregulation of PSGL1 is part of a T_{FH} cell program of differentiation that includes upregulation of PD-1 and CXCR5.

A principal criterion for identifying T_{FH} cells is their location in GCs (3, 4). Thus, we asked whether GC T cells lacked expression of PSGL1. B6 mice were immunized with NP-CGG in alum, and 8 d later, their spleens were harvested and examined by immunofluorescence confocal microscopy (Fig. 2B). Splenic GCs, identified by staining with PNA, contained CD4 T cells that lacked PSGL1. CD4 T cells in the B cell follicle also lacked PSGL1, whereas the majority of cells in the T zone expressed high levels of PSGL1 (Fig. 2B, insets; compare PSGL1 staining of T cells in T zone [inset 1], GC [inset 2], and follicle [inset 3]). The location in the GC indicates that downregulation of PSGL1 is an additional marker of the T_{FH} cell lineage. Although GC T cells are all PSGL1^{lo}, follicular CD4 T cells found outside the GC also are characterized by downregulation of this protein.

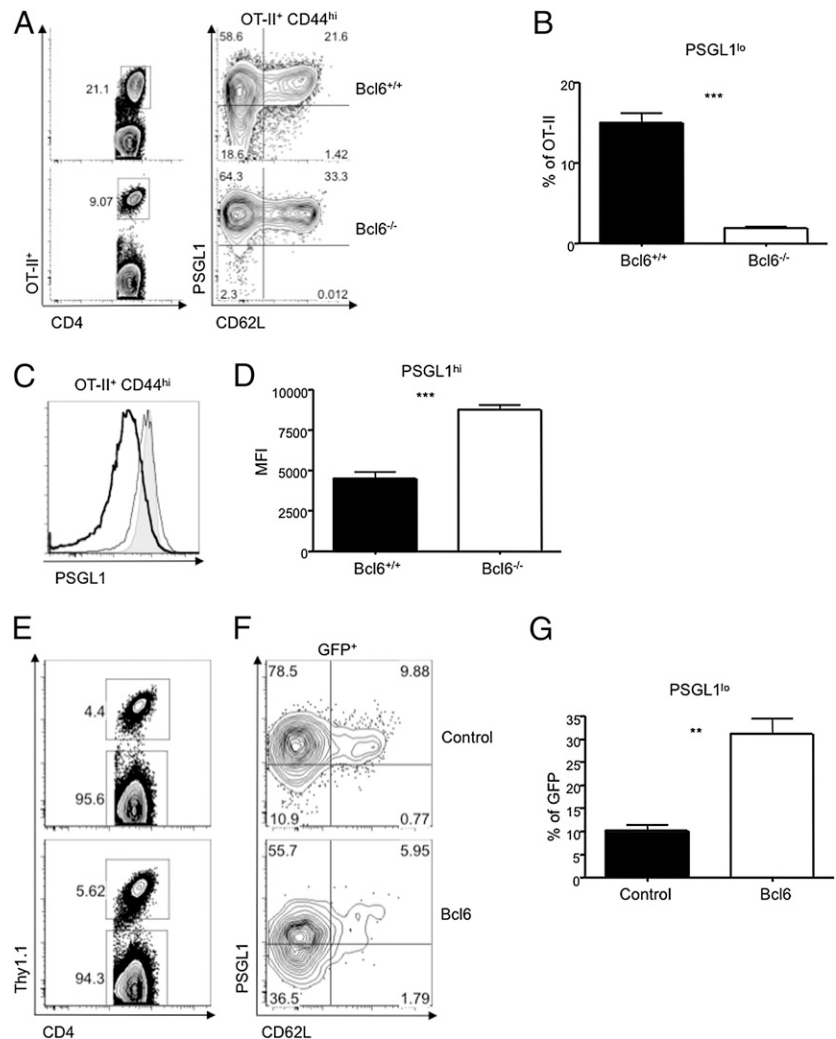
Although the majority of PSGL1^{lo} cells were positive for CXCR5 and PD-1, ~20–30% expressed lower amounts of both (Fig. 1D). This indicated that downregulation of PSGL1 and upregulation of CXCR5 and PD-1 were not necessarily coordinated. To further investigate the relationship among CD4 T cells expressing these markers, we sorted splenic PSGL1^{lo} cells into CXCR5^{lo}PD-1^{lo} and CXCR5^{hi}PD-1^{hi} populations 8 d after immunization of B6 mice

with NP-CGG in alum, followed by analysis of *bcl6* and *il21* transcript expression. The CXCR5^{hi}PD-1^{hi}PSGL1^{lo} population expressed significantly more *il21* than the CXCR5^{lo}PD-1^{lo}PSGL1^{lo} cells (Fig. 2C, right panel); however, both populations had equal amounts of *bcl6* (Fig. 2C, left panel). These data suggested that Bcl6 expression and PSGL1 downregulation can occur independently of upregulation of CXCR5, PD-1, and IL-21.

Bcl6 drives PSGL1 downregulation

Because T_{FH} cells require Bcl6 for their development (18, 27, 28), we next asked whether PSGL1^{lo} cells would have similar requirements. To accomplish this goal, we crossed Bcl6-deficient mice to OT-II TCR-transgenic animals to use in adoptive transfer and immunization experiments. To avoid the spontaneous activation of Bcl6-deficient T cells (43), we used as transfer donors lethally irradiated B6 mice reconstituted with bone marrow from Bcl6-deficient animals as described previously (18). We transferred small numbers of naive OT-II Bcl6-intact or OT-II Bcl6-deficient CD4 T cells into congenically mismatched B6 recipients, followed by immunization with OVA in alum. Six days after immunization, spleens of recipient mice were harvested and analyzed for the percentages of PSGL1^{lo} cells. Roughly 15% of activated Bcl6-intact OT-II Thy1.1 T cells became PSGL1^{lo} (Fig. 3A, 3B), similar to our previous result (Fig. 1C). By contrast, OT-II Thy1.2⁺ Bcl6-deficient T cells failed to give rise to a significant number of PSGL1^{lo} cells

FIGURE 3. Bcl6 is required for downregulation of PSGL1. *A*, Expansion of adoptively transferred OT-II CD4 T cells (left panels) and expression of CD62L and PSGL1 on transferred CD44^{hi}Bcl6^{+/+} or Bcl6^{-/-} CD4 T cells (right panels) following immunization of recipient mice. *B*, Percentage of PSGL1^{lo} CD4 T cells among total transferred T cells, representative of the data shown in *A*, compiled from three independent experiments using four mice per group; ****p* < 0.0001. *C*, Mean fluorescence intensity of PSGL1 on transferred OT-II⁺ Bcl6^{+/+} or Bcl6^{-/-} CD4 T cells following immunization of recipient mice with OVA in alum. Naive CD4 T cells represented by shaded gray, OT-II CD44^{hi}Bcl6^{+/+} CD4 T cells by the black line, and OT-II CD44^{hi}Bcl6^{-/-} CD4 T cells by the gray line. *D*, Mean fluorescence intensity of PSGL1 gated on OT-II CD44^{hi}PSGL1^{hi} CD4 T cells. Bar graph is representative of three independent experiments, using four mice for each group; ****p* < 0.0001. *E*, Expansion of OT-II Thy1.1 CD4 T cells transduced with a GFP-expressing retrovirus 6 d after transfer to B6 mice and immunization with OVA in alum. *F*, Expression of CD62L and PSGL1 on OT-II Thy1.1 GFP⁺CD44^{hi}CD4 T cells 6 d after transfer to B6 mice and immunization. *G*, Percentages of PSGL1^{lo} cells of total transferred OT-II GFP⁺ CD4 T cells, with results representative of the data in *E* and of three independent experiments using three mice for each group; ***p* = 0.0061.



(Fig. 3A, 3B). This observation indicated that Bcl6 is required for development of PSGL1^{lo} cells.

In these adoptive transfer experiments, we noted that the mean fluorescence intensity of PSGL1 on all activated CD4 T cells and on gated PSGL1^{hi} cells (Fig. 3C, 3D, respectively) was significantly increased in the absence of Bcl6, suggesting a direct role for Bcl6 in repression of PSGL1. To test this possibility, we overexpressed Bcl6 in OT-II Thy1.1 T cells using a GFP-bearing retrovirus,

followed by transfer into B6 hosts and immunization with OVA in alum (Fig. 3E). When Bcl6 was overexpressed, the percentage of PSGL1^{lo} cells among Thy1.1 CD44^{hi}GFP⁺ cells increased ~3-fold compared with cells expressing a control retrovirus (Fig. 3F, 3G). These data indicate that Bcl6 expression drives PSGL1 downregulation, further strengthening the concept that PSGL1 is part of the program for T_{FH} cell differentiation controlled by this transcriptional repressor.

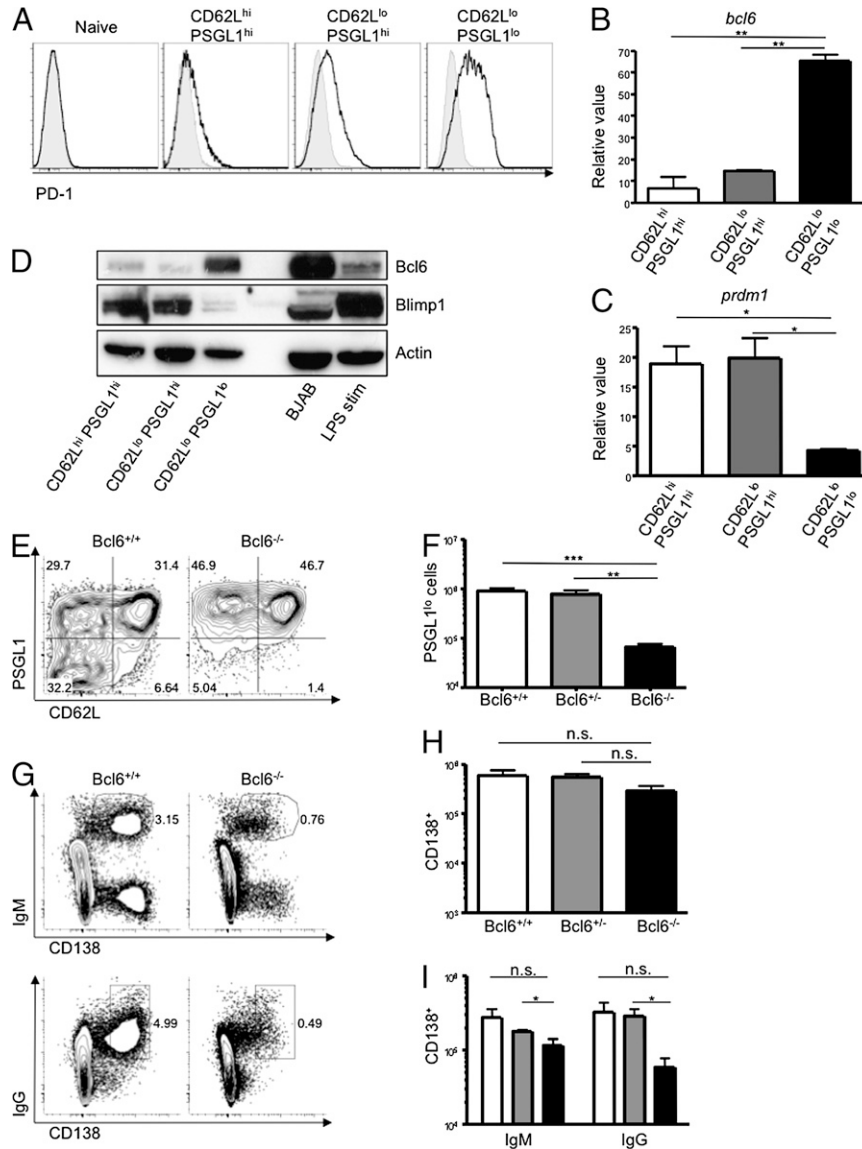


FIGURE 4. Bcl6 is required for development of extrafollicular helper T cells and formation of IgG-producing plasma cells in MRL/Fas^{lpr} mice. **A**, Expression of PD-1 on naive, CD62L^{hi}PSGL1^{hi}, CD62L^{lo}PSGL1^{hi}, and CD62L^{lo}PSGL1^{lo} cells from MRL/Fas^{lpr} mice. Gray shaded is isotype control, black line is PD-1. **B** and **C**, Expression of *bcl6* and *prdm1* (Blimp-1) transcripts in sorted CD62L^{hi}PSGL1^{hi}, CD62L^{lo}PSGL1^{hi}, and CD62L^{lo}PSGL1^{lo} cells isolated from spleens of 12- to 16-wk-old MRL/Fas^{lpr} mice. Results are representative of three experiments. In **B**, ***p* = 0.0029 (comparing CD62L^{lo}PSGL1^{hi} and CD62L^{lo}PSGL1^{lo} cells), ***p* = 0.0093 (CD62L^{hi}PSGL1^{hi} versus CD62L^{lo}PSGL1^{lo}), and in **C**, **p* = 0.0427 (CD62L^{lo}PSGL1^{hi} versus CD62L^{lo}PSGL1^{lo}), **p* = 0.0391 (CD62L^{hi}PSGL1^{hi} versus CD62L^{lo}PSGL1^{lo}). **D**, Protein expression of Bcl6 and Blimp-1 in sorted CD62L^{hi}PSGL1^{hi}, CD62L^{lo}PSGL1^{hi}, and CD62L^{lo}PSGL1^{lo} cells isolated from spleens of 12- to 16-wk-old MRL/Fas^{lpr} mice. The human B cell line BJAB was used as a positive control for Bcl6 and purified B cells activated in vitro with LPS for 48 h served as a positive control for Blimp-1. Actin was used as a loading control. Results are representative of two experiments. **E** and **F**, Spleens from 7- to 8-wk-old Bcl6-intact or -deficient MRL/Fas^{lpr} mice were analyzed for the presence of PSGL1^{lo} cells. Results are representative of three independent experiments from three cohorts of mice, with four to five mice per group; ****p* < 0.0001 (Bcl6^{+/+} versus Bcl6^{-/-}); ***p* = 0.0038 (Bcl6^{+/-} versus Bcl6^{-/-}). **G-I**, Percentage and number of CD138⁺ plasma cells analyzed for intracellular expression of IgM and IgG isolated from spleens of 7- to 8-wk-old Bcl6-intact or -deficient MRL/Fas^{lpr} mice. In **I**, open bars represent Bcl6^{+/+}, gray bars represent Bcl6^{+/-}, and black bars represent Bcl6^{-/-} MRL/Fas^{lpr} mice. Results are representative of three independent experiments from three cohorts of mice, with four to five mice per group. In **H**, *p* = 0.1497 (Bcl6^{+/+} versus Bcl6^{-/-}); *p* = 0.0817 (Bcl6^{+/-} versus Bcl6^{-/-}). In **I**, IgM *p* = 0.1591 (Bcl6^{+/+} versus Bcl6^{-/-}), **p* = 0.0491 (Bcl6^{+/-} versus Bcl6^{-/-}); IgG, *p* = 0.1209 (Bcl6^{+/+} versus Bcl6^{-/-}); and **p* = 0.0285 (Bcl6^{+/-} versus Bcl6^{-/-}).

Bcl6 is required for development of extrafollicular helper T cells

We previously described the presence of CXCR4^{hi}, CXCR5^{lo}, and IL-21-producing B helper T cells that migrate to extrafollicular sites in lupus-prone murine strains (37). Microarray analysis of these extrafollicular helper cells from MRL/*Fas*^{lpr} mice suggested additional similarities to T_{FH} cells, such as upregulation of PD-1 and Bcl6 (Fig. 1A, 1B). Indeed, we found increased expression of

surface PD-1 (Fig. 4A) and Bcl6 at the transcript and protein level in PSGL1^{lo} extrafollicular Th cells isolated from the spleens of aged MRL/*Fas*^{lpr} mice (Fig. 4B, 4D). Conversely, Blimp-1 transcript levels and protein were decreased, compared with PSGL1^{hi} CD4 effector T cells, similar to our findings in T_{FH} cells (Fig. 4C, 4D) (18).

To test whether Bcl6 played a role in the development of extrafollicular Th cells, we generated Bcl6-intact and -deficient *Fas*^{lpr} mice. Although these animals develop robust extrafollicular foci

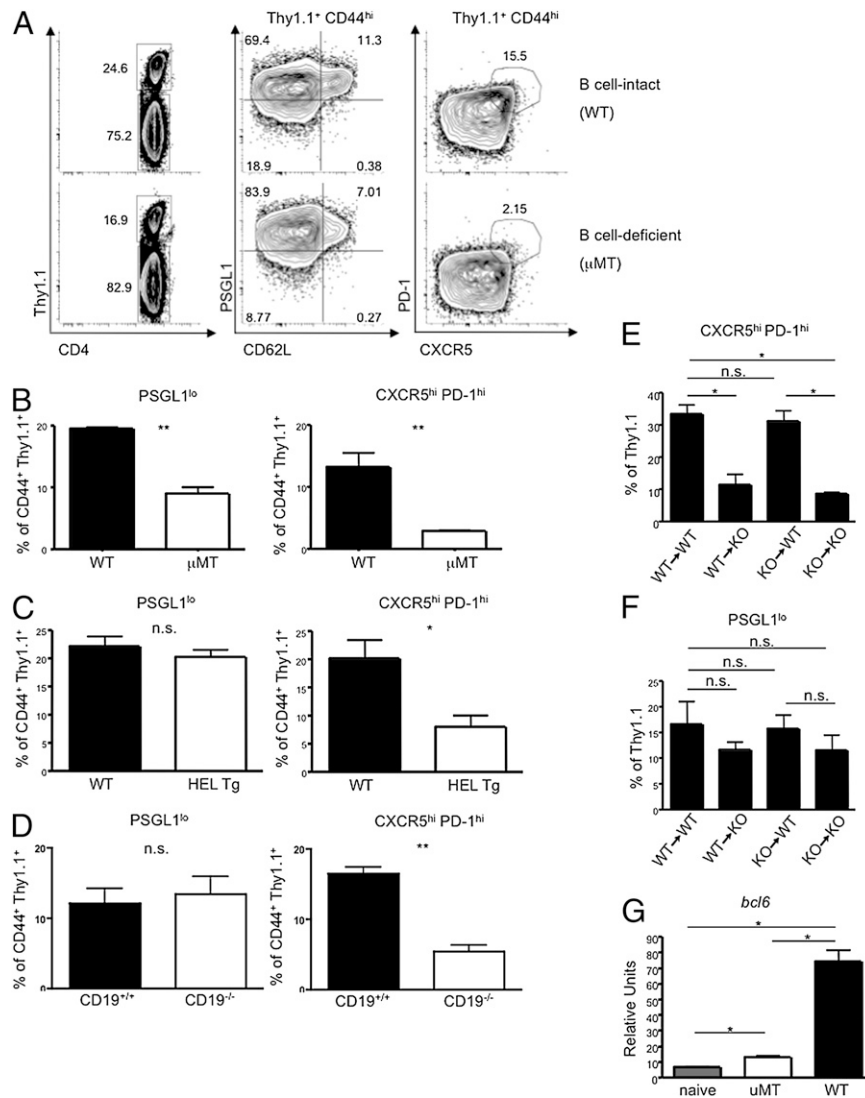


FIGURE 5. B cells are required for development of CXCR5^{hi} PD-1^{hi} T_{FH} cells, but not downregulation of PSGL1. *A*, Expansion of OT-II Thy1.1 CD4 T cells 6 d after adoptive transfer and immunization of recipient B6 mice with OVA in alum in the presence or absence of B cells (*left panels*). Expression of CD62L and PSGL1 (*middle panels*) and PD-1 and CXCR5 (*right panels*) on the expanded OT-II Thy1.1 CD44^{hi} CD4 T cells in B cell-intact or -deficient recipients. *B*, Percentages of T_{FH} cells of OT-II Thy1.1 CD44^{hi} CD4 T cells taken from B cell-intact and -deficient mice are shown, with ***p* = 0.0049 (PSGL1^{lo} T cells) and ****p* = 0.0093 (CXCR5^{hi} PD-1^{hi} T cells). Data shown are representative of that in *A*, and of four independent experiments, using four mice per group. *C*, Percentages of T_{FH} cells of OT-II Thy1.1 CD44^{hi} CD4 T cells 7 d after immunization of B6 (WT) or HEL Ig transgenic (MD4) mice with OVA in alum; **p* = 0.4702 (PSGL1^{lo} T cells) and **p* = 0.0370 (CXCR5^{hi} PD-1^{hi} T cells). Data are representative of two independent experiments using four mice per group. *D*, Percentages of T_{FH} cells of OT-II Thy1.1 CD44^{hi} CD4 T cells 7 d after immunization of CD19-intact or -deficient recipients with OVA in alum. Bar graph shows data representative of two independent experiments using four mice per group; *p* = 0.7029 (PSGL1^{lo} cells); ***p* = 0.0036 (CXCR5^{hi} PD-1^{hi} cells). *E*, Percentages of CXCR5^{hi} PD-1^{hi} cells of OT-II Thy1.1 CD44^{hi} cells 3 d after transfer (7 d after immunization) from B cell-intact or B cell-deficient mice. Bar graph shows data representative of three independent experiments using two to three mice per group; **p* = 0.0129 (WT→WT versus KO→KO), *p* = 0.6576 (WT→WT versus KO→WT); **p* = 0.0354 (WT→WT versus WT→KO); and **p* = 0.0218 (KO→WT versus KO→KO). *F*, Percentages of PSGL1^{lo} cells of OT-II Thy1.1 CD44^{hi} cells 3 d after transfer (7 d after immunization) to B cell-intact or B cell-deficient mice. Bar graph shows data representative of three independent experiments, using two to three mice per group; *p* = 0.4327 (WT→WT versus KO→KO); *p* = 0.8822 (WT→WT versus KO→WT); *p* = 0.3921 (WT→WT versus WT→KO); and *p* = 0.3920 (KO→WT versus KO→KO). *G*, Expression of *bcl6* mRNA in naive CD4 T cells and OT-II Thy1.1 CD44^{hi} T cells sorted 7 d after transfer to B6 WT or B cell-deficient (μMT) hosts, followed by immunization with OVA in alum; **p* = 0.0112, transfer to naive versus WT recipients; **p* = 0.0138, transfer to WT versus μMT recipients; and **p* = 0.0163, transfer to naive versus μMT recipients. Data are representative of three independent experiments.

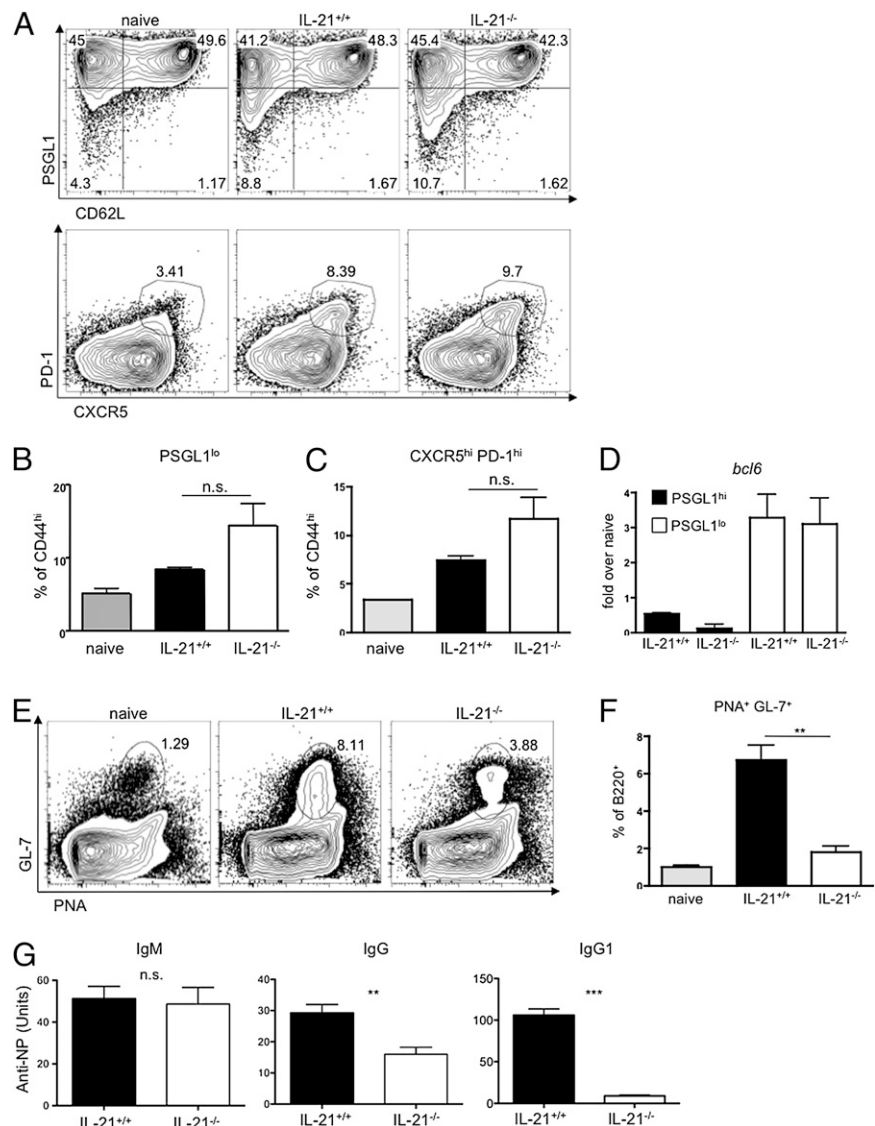
in conjunction with the accelerated autoimmunity seen in the Fas-deficient background (data not shown), we did not attempt to assess development of lupus phenotypes, given their shortened lifespan (Bcl6-deficient mice succumb to lymphoproliferation by 10–12 wk of age [43]) and the lack of the full complement of lupus-potentiating MRL genes. Nonetheless, there was expansion of PSGL1^{lo} extrafollicular Th cells in Bcl6-intact or Bcl6-heterozygous Fas-deficient mice by 7–8 wk of age, whereas in the absence of Bcl6, the percentage and number of PSGL1^{lo} cells were dramatically reduced (Fig. 4E, 4F). To determine whether such reduced development affected plasma cell formation and Ab responses, we assessed the percentage and number of syndecan (CD138)-positive IgM and IgG plasma cells. Similar to our findings in ICOS-deficient MRL/Fas^{lpr} mice (37), the reduced number of extrafollicular Th cells had no effect on the total number of syndecan⁺ plasma cells (Fig. 4H), with only a slight decrease in the number of such cells that were IgM⁺ (Fig. 4G, 4I). However, IgG-producing plasma cells were reduced ~10-fold in the absence of Bcl6 (Fig. 4G, 4I), suggesting that extrafollicular Th cells, likely through their ability to produce IL-21 and express CD40L (37), are required for isotype switch to occur. Although not excluded in this study, it is unlikely that deficiency in Bcl6 could directly affect the formation of Ab-producing GC B cells or T_{FH} cells in these animals, as CXCR5^{hi} T_{FH} cells have not been observed

in our hands in these animals (37), and Blimp-1-dependent extrafollicular foci are the primary site of isotype-switched somatically mutated autoantibody production in the MRL/Fas^{lpr} model, whereas GC formation is largely absent (51). This supports a model in which deficiency of Bcl6 leads to the absence of IL-21-producing extrafollicular Th cells, resulting in reduced isotype switch autoantibody production. Collectively, these data suggest that T_{FH} and extrafollicular Th cells share similar function in terms of B cell maturation and reliance upon Bcl6 for development.

B cells are not required for downregulation of PSGL1

The primary function of T_{FH} cells is to promote GC B cell survival and maturation, including IL-21 secretion and CD40 engagement (48). B cells are also required for T_{FH} cell development (14, 17, 18) indicating that T–B interactions are bidirectional. To determine whether development of PSGL1^{lo} T cells also requires B cells, we transferred OT-II Thy1.1 splenocytes into B cell-intact or -deficient hosts, followed by immunization of OVA in alum, with splenic T cells analyzed 6 d later. Following immunization of B cell-deficient recipients, we observed a 6- to 8-fold reduction in CXCR5^{hi}PD-1^{hi} cells as a percentage of transferred Thy1.1 cells, compared with B cell-intact control mice; however, by comparison, PSGL1^{lo} cells exhibited only a 2-fold decrease

FIGURE 6. IL-21 is not required for development of T_{FH} cells or Bcl6 upregulation in vivo. **A**, Development of T_{FH} cells in IL-21-intact or -deficient mice 7 d after immunization with NP-CGG in alum compared with unimmunized (naive) mice, using PSGL1 (*top panels*) or CXCR5 and PD-1 (*bottom panels*) as markers. **B**, Percentages of PSGL1^{lo} T cells of CD44^{hi} CD4 cells 7 d after immunization with NP-CGG in alum or in unimmunized (naive) mice. Bar graph shows data representative of that revealed in the *top panels* of **A**, compiled from two independent experiments, using five mice per group; *p* = 0.0959. **C**, Percentages of CXCR5^{hi}PD-1^{hi} T cells of CD44^{hi} CD4 cells 7 d after immunization with NP-CGG in alum or in unimmunized (naive) mice. Data represented in the *bottom panels* of **A** are compiled from two independent experiments using five mice per group; *p* = 0.1044. **D**, Bcl6 expression in CD4 CD44^{hi} cells sorted into PSGL1^{hi} and PSGL1^{lo} subsets 7 d after immunization of IL-21-intact and -deficient mice with NP-CGG in alum. Expression is shown as fold over sorted naive CD4 T cells. Data are representative of two independent experiments. **E**, Analysis of GC cell formation (GL-7⁺PNA⁺) in IL-21-intact, -deficient, or unimmunized (naive) mice. TCRβ⁺IgD⁻ cells were gated. **F**, Percentage of GC B cells (GL-7⁺PNA⁺) among B220⁺ cells. Data shown are representative of that shown in **E** and of two independent experiments using four mice per group; ***p* = 0.0011. **G**, Levels of anti-NP Abs in the serum of IL-21-intact or -deficient mice bled 10 d after immunization with NP-GCC in alum. IgM, *p* = 0.8110; total IgG, ***p* = 0.0043; and IgG1, ****p* < 0.0001. Data compiled from one experiment, five mice per group.



(Fig. 5A, 5B). To further investigate the possible disparity between the role of B cells in development of PSGL1^{lo} and CXCR5^{hi} PD-1^{hi} cells, we transferred OT-II Thy1.1 CD4 T cells into B cell-transgenic animals expressing a BCR specific for HEL (consequently unable to respond to OVA immunization) (52) or into CD19-deficient mice (in which B cells are not properly activated) (53). Seven days after immunization, PSGL1^{lo} cells as a percentage among transferred cells were equivalent among HEL Ig transgenics, CD19-deficient mice, and controls (Fig. 5C, 5D). However, there was a statistically significant decrease in CXCR5^{hi}PD-1^{hi} cells, a finding in concert with previous analysis using these markers to identify T_{FH} cells (9, 17, 18). These data indicated that activated Ag-specific B cells are required for development of CXCR5^{hi}PD-1^{hi} cells but not necessarily PSGL1^{lo} cells.

The formation of PSGL1^{lo} cells in the absence of robust development of CXCR5^{hi}PD-1^{hi} cells (Fig. 5A–D) indicated that downregulation of PSGL1 and upregulation of CXCR5 and PD-1 were independent events, with expansion of cells expressing the latter molecules dependent on the presence of B cells and PSGL1 downregulation possible in the absence of Ag-specific B cell signals. To further explore this idea, we used a model wherein T_{FH} cells initially developed in the presence or absence of B cells, followed by transfer to a new set of previously immunized B cell-deficient or B cell-intact hosts for a short maintenance phase. In these experiments, OT-II Thy1.1 CD4 T cells were initially transferred into either WT or B cell-deficient (μ MT) B6 hosts, followed by priming with OVA in alum. Four days later, CD4 T cells were sorted and transferred into immunized B cell-intact or -deficient recipients. Four conditions were tested: 1) primed in and then transferred to WT hosts (WT→WT); 2) primed in and transferred to μ MT mice (knockout [KO]→KO); 3) primed in B cell-sufficient, and transferred to, B cell-deficient hosts (WT→KO); and 4) primed in B cell-deficient, and transferred to, B cell-sufficient hosts (KO→WT). The percentages of PSGL1^{lo} cells and CXCR5^{hi}PD-1^{hi} cells were analyzed 3 d after the second T cell transfer, a time point that was 7 d after initial immunization. In comparison with mice that were B cell sufficient throughout, there was significantly reduced development of CXCR5^{hi}PD-1^{hi} T_{FH} cells in animals in which B cells were absent during both the priming and maintenance phases (compare WT→WT to KO→KO; Fig. 5E), similar to our results following immunization of μ MT, HEL Ig transgenic, or CD19-deficient mice (Fig. 5B–D, respectively). By contrast, PSGL1^{lo} cells were slightly reduced in conditions where B cells were absent throughout, but this decrease was not statistically significant (Fig. 5F). CXCR5^{hi}PD-1^{hi} and PSGL1^{lo} T_{FH} cells that developed in mice initially in the absence of B cells, followed by transfer to mice that were B cell intact, expanded to percentages similar to those observed in WT mice (compare WT→WT to KO→WT; Fig. 5E, 5F). When T cells were transferred from mice that were B cell intact to mice that were B cell deficient, the percentage of CXCR5^{hi}PD-1^{hi} cells dropped significantly, with percentages in the latter comparable to those that were in a B cell-deficient environment for the duration of the experiment; by contrast, there was again less effect on the recovery of PSGL1^{lo} cells (compare WT→WT to WT→KO; Fig. 5E, 5F). In all groups, the reduction in CXCR5^{hi}PD-1^{hi} cells was more dramatic than PSGL1^{lo} cells, suggesting that expression of these surface molecules is not coordinated and that B cells play an important role in regulating these markers, particularly CXCR5 and PD-1. These data also suggest that B cells are dispensable for early development of CXCR5^{hi}PD-1^{hi} T_{FH} cells following priming, yet they are critical later in the immune response.

These findings, in concert with our observation that PSGL1^{lo} cells expressed Bcl6, regardless of the expression of CXCR5 and

PD-1 (Fig. 2C), and that Bcl6 drives PSGL1 downregulation (Fig. 3C–F) suggested that Bcl6 upregulation and subsequent PSGL-1 downregulation occurred prior to interaction with B cells. To directly test the role of B cells in induction of Bcl6, we transferred OT-II Thy1.1 CD4 T cells to WT and B cell-deficient B6 mice, followed by immunization with OVA in alum, and 7 d later, we sorted OT-II CD4 Thy1.1⁺ cells from recipients for assessment of *bcl6* transcripts. Expression of *bcl6* was dramatically reduced in the absence of B cells (Fig. 5G), consistent with our published data (18); however, transcript levels were increased in comparison with naive CD4 T cells, indicating some *bcl6* expression can occur

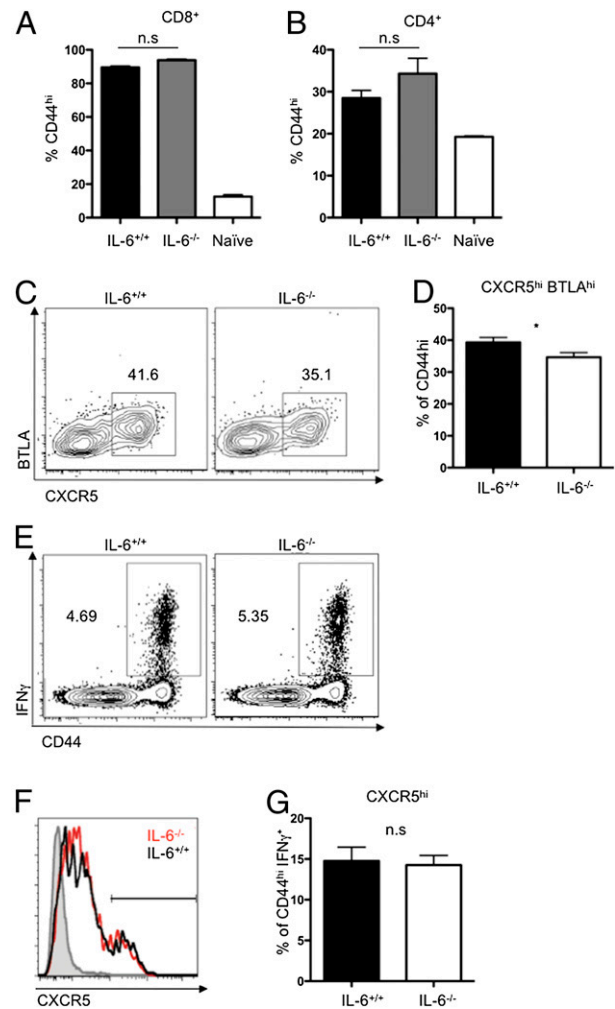


FIGURE 7. IL-6 is not required for development of T_{FH} cells following infection with LCMV. *A* and *B*, Expansion of activated (CD44^{hi}CD62L[−]) CD8 (*A*) and CD4 (*B*) T cells from spleens of IL-6-intact and -deficient mice isolated 8 d after LCMV infection. *C*, Expression of BTLA and CXCR5 on CD44^{hi} CD4 T cells 8 d after LCMV challenge in IL-6-intact or -deficient mice. *D*, Percentages of T_{FH} cells among CD44^{hi} CD4 T cells 8 d after LCMV challenge in IL-6-intact or -deficient mice. Data are compiled from three independent experiments, using nine mice per group; **p* = 0.0441. *E*, Intracellular staining for IFN- γ production in CD4 T cells from spleens isolated 8 d after LCMV infection in IL-6-intact or -deficient mice. Cells were restimulated with LCMV I-A^b binding peptide gp61–80 for 5 h in vitro. *F*, Expression of CXCR5 on LCMV-specific (CD44^{hi}IFN- γ ⁺) CD4 T cells. Gray shaded line represents naive CD4 T cells; black line represents CD44^{hi}IFN- γ ⁺ CD4 T cells from IL-6-intact mice; red line represents CD44^{hi}IFN- γ ⁺ CD4 T cells from IL-6-deficient mice. *G*, Percentages of T_{FH} cells of LCMV-specific (IFN- γ ⁺) CD4 T cells 8 d after LCMV infection in IL-6-intact or -deficient mice. Data are compiled from two independent experiments, using nine mice per group.

in the absence of B cell interactions. This result was surprising, given that we only saw a 2-fold reduction in PSGL1^{lo} cells in B cell-deficient mice (Fig. 5B), whereas we expected to see levels of Bcl6 more similar to WT levels. Taken together, these data suggest that once PSGL1 is downregulated, B cells play a role in robust upregulation of Bcl6 that is necessary for expression of certain T_{FH} cell features, such as CXCR5 and PD-1.

IL-21 is not required for T_{FH} cell development

IL-21 produced by T_{FH} cells promotes GC B cell maturation and production of isotype-switched Abs. It is also produced by Th17 cells, acting in an autocrine manner to maintain Th17 phenotypes (23–25). It has been proposed that IL-21 can similarly act in an autocrine manner to promote development of the T_{FH} cell subset (8, 9), a notion based in part upon the observation that in vitro, IL-21 is able to upregulate levels of Bcl6 (9, 27). To explore the roles of IL-21 in PSGL1 downregulation and T_{FH} cell differentiation in vivo, we immunized IL-21-intact or –deficient mice with NP-CGG in alum and analyzed T_{FH} cell formation, GC development, and Bcl6 levels. Surprisingly, we did not observe a difference in the percentage of T_{FH} cells in the presence or absence of IL-21 when gating on the activated CD4 T cell population using either PSGL1 or CXCR5 and PD-1 as markers (Fig. 6A–C). In accordance with this finding, we also did not observe a difference in the levels of Bcl6 in PSGL1^{lo} cells taken from either

IL-21-intact or –deficient animals (Fig. 6D). There was a significant decrease in GC B cells in the IL-21-deficient animals (Fig. 6E, 6F), as well as a decrease in total IgG- and IgG1-specific Abs, whereas IgM levels remained intact (Fig. 6G). In aggregate, these data indicate that IL-21 plays an important functional role in GC formation or maintenance in vivo (at least up to 7 d following immunization) but is not absolutely required for the upregulation of Bcl6 or the initial development of T_{FH} cells following immunization of mice with protein in alum.

IL-6 is not required for the development or function of T_{FH} cells

We hypothesized that IL-6 might be responsible for Bcl6 upregulation and PSGL1 downregulation in vivo in T_{FH} cells, given its in vitro role in Bcl6 induction (27), and in development of T_{FH} cells in vivo following protein immunization (9). To investigate this idea, we transferred sorted naive OT-II Thy1.1 CD4 T cells into IL-6-intact or –deficient recipients, followed by immunization with OVA in alum, with splenic PSGL1^{lo} T_{FH} cell development and Bcl6 expression in the sorted Thy1.1 population analyzed 6 d later. Contrary to a previous report, we did not see differences between IL-6^{–/–} and IL-6^{+/+} hosts in the percentages of Thy1.1⁺ PSGL1^{lo} or CXCR5^{hi}PD-1^{hi} OT-II cells (Supplemental Fig. 2A, 2B) or in the amounts of *bcl6* and *il21* mRNA expression (Supplemental Fig. 2C). One explanation for our results that contrast with data

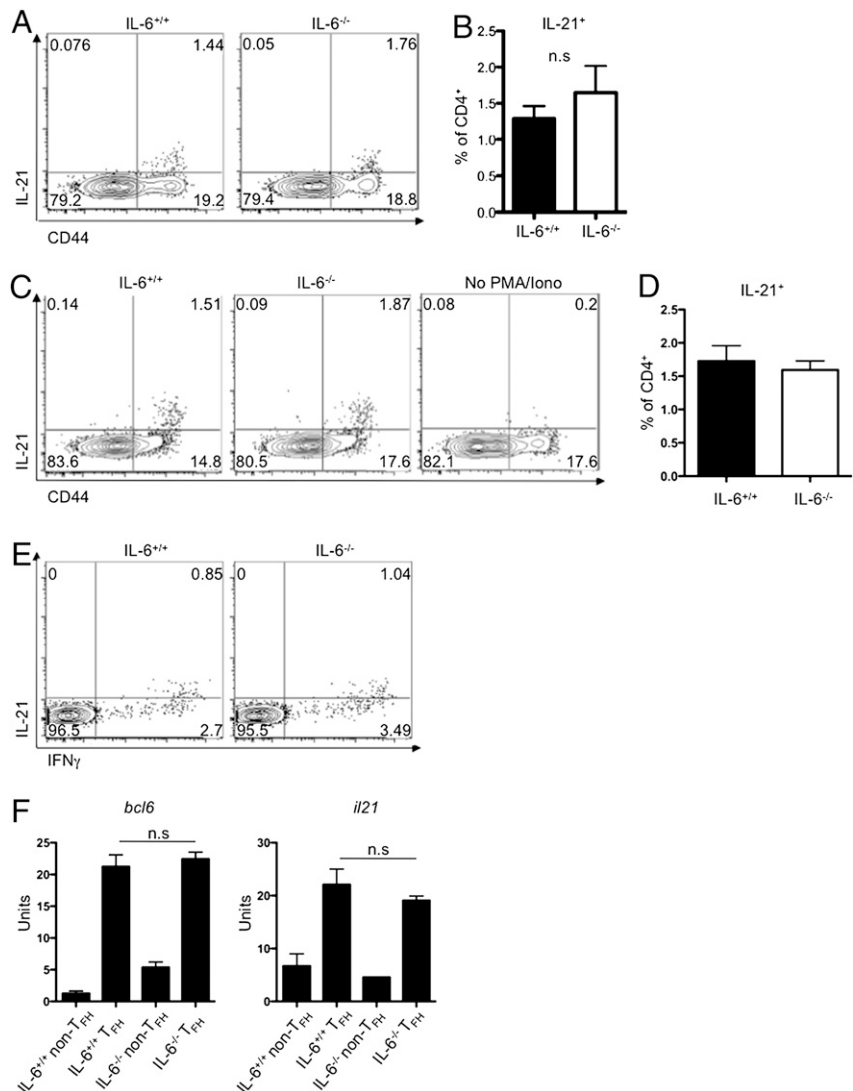


FIGURE 8. IL-6 is not required for Bcl6 expression or IL-21 production in T_{FH} cells following infection with LCMV. *A*, Intracellular staining of IL-21 in LCMV-specific CD4 T cells from spleens isolated 8 d postinfection in IL-6-intact or –deficient mice. Cells were restimulated with LCMV I-A^b binding peptide gp61–80 for 5 h in vitro. *B*, Percentages of IL-21⁺ cells of LCMV-specific CD4 T cells. Data are compiled from two independent experiments, using nine mice per group. *C–E*, IL-21 and IFN-γ production by LCMV-specific CD44^{hi} CD4 T cells assessed using intracellular staining after restimulation with PMA and ionomycin. Data are compiled from two independent experiments, using nine mice per group. *F*, Expression of *bcl6* and *il21* in T_{FH} cells (CD4⁺CD44^{hi}CXCR5^{hi}BTLA^{hi}) and non-T_{FH} (CD4⁺CD44^{lo}CXCR5^{lo}BTLA^{lo}) cells sorted 8 d postinfection of IL-6-intact or –deficient mice.

indicating a role for this cytokine in T_{FH} cell development may be our use of alum as an adjuvant, compared with CFA in published experiments (9).

We next used a second strategy to analyze the requirement for IL-6 in T_{FH} cell development and function. IL-6-deficient mice were infected with LCMV, a virus that elicits a robust T cell and GC response (54–56). Eight days following viral challenge, activated $CD44^{hi}$ splenic CD4 and CD8 T cells expanded comparably in both IL-6-intact and -deficient mice (Fig. 7A, 7B). Using CXCR5 and BTLA as markers of T_{FH} cell (9, 18), a small decrease in the percentage of $CD44^{hi}$ T_{FH} cells was observed in the absence of IL-6 (Fig. 7C, 7D); however, the decrease was diminutive, indicating IL-6 did not play a major role in T_{FH} cell development. To directly quantify T_{FH} cell differentiation of virus-specific CD4 T cells, splenocytes 8 d after viral infection were restimulated with the immunodominant LCMV I-A^b binding peptide gp61–80, followed by staining for IFN- γ to reveal the Ag-specific population. The percentages of IFN- γ ⁺ cells (Fig. 7E) and Ag-specific CXCR5^{hi} cells (Fig. 7F, 7G) were unchanged between IL-6-deficient and -intact controls. These data suggested that IL-6 is not required for the development of T_{FH} cells during LCMV infection.

To determine whether IL-6 had a role in IL-21 secretion, splenocytes from infected mice were stimulated with either gp61 or PMA plus ionomycin, followed by intracellular staining for IL-21. $CD44^{hi}$ CD4 T cells produced similar levels of IL-21 in the absence or presence of IL-6 (Fig. 8A, 8B [gp61] and Fig. 8C–E [PMA/iono]). In addition, there was no difference in *bcl6*, *il21*, or *prdm1* transcripts in the T_{FH} cell subsets sorted from IL-6-intact or -deficient recipients (Fig. 8F and data not shown).

To further address the effects of IL-6 on T_{FH} cell function, we analyzed the formation of GCs in IL-6-intact or IL-6-deficient mice. After LCMV infection, large GC B cell responses ($GL7^{+}$ Fas⁺ cells) were observed in both IL-6-intact and -deficient mice at 8 d following infection (Fig. 9A, 9B). Interestingly, IL-6-deficient mice had lower titers of LCMV-specific IgG at day 8 postinfection compared with IL-6-intact controls; however, this deficiency was absent at day 15 postinfection (Fig. 9C, 9D). The day 8 deficiency may be due to the direct effects of IL-6 on Ab secretion by B cells (57–59), because GC formation was intact at this time point. Regardless, these data in aggregate suggest that IL-6 is not required for the development or function of T_{FH} cells or in the upregulation of Bcl6 or IL-21 following viral infection and indicate that there is an IL-6-independent pathway for the development of T_{FH} cells.

Discussion

The discovery of the cytokines produced by Th1, Th2, and Th17 effector cells, and the transcription factors that control their development, has provided an understanding of how these subsets function and differ from one other (60). However, knowledge of the requirements for development of T_{FH} cells has lagged behind. In this study, we have concentrated on more clearly delineating these requirements, focusing upon the roles that B cells, IL-6, and IL-21 play in Bcl6 regulation and T_{FH} cell development while at the same time identifying PSGL1 downregulation as a new marker for this lineage and linking its expression to that of Bcl6.

We initially observed that Bcl6-driven PSGL1 downregulation was a characteristic of follicular T cells. Unmodified PSGL1 binds CCL19 and CCL21 (41), chemokines responsible for entry of T cells into lymph nodes (61), providing a rationale for its downregulation in T_{FH} cells that leave the T cell zone and migrate toward B cell follicles. Consistent with this observation, the PSGL1^{lo} population also has significantly diminished expression of *psgl1* transcripts (data not shown), suggesting loss of this mol-

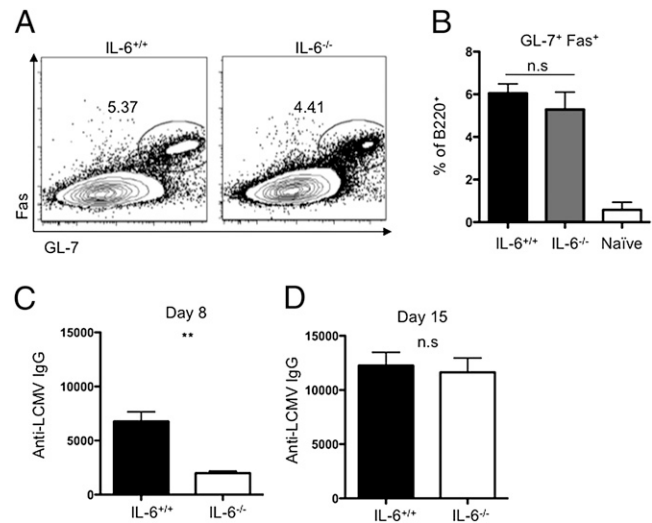


FIGURE 9. IL-6 is not required for function of T_{FH} cells during LCMV infection. *A*, Analysis of GC formation (Fas⁺GL7⁺) in spleens isolated 8 d after LCMV infection from IL-6-intact or -deficient mice. Cells are gated on B220⁺ B cells. *B*, GC B cells as a percentage of total B220⁺ B cells from infected IL-6-intact or -deficient mice 8 d after LCMV infection. Data are compiled from three independent experiments, using nine mice per group. *C* and *D*, Titers of anti-LCMV IgG Ab in the serum of IL-6-intact or -deficient mice bled 8 and 15 d after LCMV infection. Day 8, ***p* < 0.001. Data are representative of four independent experiments.

ecule is part of the T_{FH} cell differentiation program driven by Bcl6 (18, 27, 28). Roughly 200 bp upstream of the transcriptional start site for PSGL1, we identified a region that was homologous to the published consensus site for Bcl6 binding (62). Known targets of Bcl6 in T cells include IL-5, GATA-3, and granzyme B (63–65); however, both transcriptional and posttranslational controls have been reported for some of these genes (64), suggesting Bcl6 may be able to regulate expression of PSGL1 by several mechanisms.

B cells play a critical role in T_{FH} cell development and perhaps in Bcl6 expression, although the timing of T–B cell interaction in this process is not well understood (17, 18, 33). Our results suggest that B cells play a more critical role in the maintenance of the expression of Bcl6, rather than initiation of its upregulation. We base these conclusions upon the findings that PSGL1^{lo} CD4 T cells that are CXCR5^{lo}PD-1^{lo} express Bcl6 and develop in the absence of B cells. Bcl6 expression likely precedes downregulation of PSGL1, because Bcl6 is necessary and sufficient for reduced surface expression of this molecule (Fig. 3). Because a significant percentage of PSGL1^{lo} cells develop in B cell-deficient mice, or in animals that lack Ag-specific B cell activation, it follows that Bcl6 upregulation should precede contact with B cells. We were surprised then when we found low levels of Bcl6 in activated OT-II CD4 T cells that had developed in B cell-deficient hosts. On the basis of the lower percentage of PSGL1^{lo} cells that developed in these animals, we expected Bcl6 to be somewhat reduced; yet, we found that the reduction was quite striking. Despite this finding, we did consistently observe an increase in its expression compared with naive CD4 T cells, supporting the conclusion that Bcl6 upregulation can occur in the absence of B cells and that this is sufficient to induce downregulation of PSGL1.

These results indicate that B cells may play a role in stabilizing or increasing Bcl6 levels, an explanation in line with our findings that B cells are more critical at later stages of T_{FH} cell differentiation. This is a time of more robust development of CXCR5^{hi}PD-1^{hi} cells that synthesize IL-21, a result demonstrated here using a transfer model. A day 4 time point for the initial analysis was chosen; however, we do not presume that this is the point at which

T cells begin their interaction with B cells. It is likely that some T–B interaction occurs even earlier (2, 66). Regardless, ongoing interaction with B cells appears necessary for full commitment to T_{FH} cell development. More work is needed to further explore the exact time and location at which this critical T–B interaction takes place and when and where Bcl6 is upregulated.

These data do not contradict our recently published work (18), in which we demonstrated that overexpression of Bcl6 in the absence of Ag-specific B cell interaction can drive T_{FH} cell development. Data presented in this paper suggest that B cells are necessary to stabilize or increase levels of Bcl6, a step that is required for development of fully functional CXCR5^{hi}PD-1^{hi} IL-21-secreting T_{FH} cells. By overexpressing Bcl6 using a retrovirus, the levels of Bcl6 protein are experimentally enhanced, and the requirement for signals from B cells is negated, allowing T_{FH} cell development to occur in the absence of this critical interaction. However, as noted above, our findings indicate that B cells are not the signal that drives initial Bcl6 upregulation, as we find Bcl6 expression in PSGL1^{lo}CXCR5^{lo}PD-1^{lo} cells, and these cells can develop in the absence of interaction with Ag-specific activated B cells.

Previous studies have indicated that IL-21 is necessary for T_{FH} cell development in vivo (8, 9). Our data contrast with these results and are in line with more recent data (67, 68), indicating that T_{FH} cells can develop independently of this cytokine. Our finding that Bcl6 upregulation in T cells was also independent of IL-21 supports this conclusion, given the former's requirement in T_{FH} cell genesis, although these findings too are at odds with in vitro studies (9, 27). Nonetheless, our data indicate the type and route of immunization may determine the dependency of T_{FH} cell development on an inflammatory cytokine, a notion supported by the recent finding that T_{FH} cells arise independently of IL-21 following viral challenge (69) and unchanged frequencies of T_{FH} cell in sanroque mice in the absence of IL-21 (70). We do note, though, that GC B cell maturation does require this cytokine, as shown by others (8, 9, 67, 68) (also Fig. 6E, 6F).

We next focused our efforts upon the role of IL-6 in Bcl6 induction and T_{FH} cell formation, as it too has been shown to play a role in T_{FH} cell and GC development in vivo following protein immunization (9) and to drive Bcl6 upregulation in vitro (27). We therefore were surprised to find that it was not required for the development or function of T_{FH} cells, or in their upregulation of Bcl6, following protein immunization of IL-6-deficient and -sufficient mice. Variations in experimental systems may account for the differences between our results and those previously published (9), such as differences in adjuvant use and routes of immunization. We also used transfer of TCR-transgenic CD4 T cells, creating a system where Ag-specific cells were specifically tracked, rather than analysis of global CD4 T cell responses.

As the TCR-transgenic cells we transferred in these experiments were IL-6 sufficient, it was also formally possible that autocrine production of cytokine was sufficient to drive Bcl6 upregulation and T_{FH} cell development. Therefore, we turned to a second in vivo system, acute LCMV infection. Upon viral infection, T_{FH} cell formation and function (the latter measured by IL-21 mRNA levels and protein secretion) and Bcl6 upregulation were normal in the absence of IL-6. Although we observed a difference in LCMV-specific IgG Ab responses 8 d following viral challenge, this presumably was due to the direct effect of IL-6 on B cells (57–59), because the numbers of GC B cells were equivalent between IL-6-deficient and -sufficient mice, and IgG titers were normal at day 15. Ab defects have been previously reported in IL-6-deficient mice (20, 22, 71). These results demonstrate that there is an IL-6-independent mechanism for T_{FH} cell development and Bcl6 upregulation in vivo. One additional possibility that may explain

these disparate results, and those obtained upon challenge of IL-21-deficient mice (8, 9), is that IL-6 and IL-21 may be functionally redundant with respect to the upregulation of Bcl6 and consequently T_{FH} cell development. This notion finds support in the observation that protein immunization of STAT3-deficient mice revealed a defect in T_{FH} cell formation (9). This hypothesis would explain the incongruent results seen in vitro and in vivo while also suggesting a reason why different model systems may reveal distinct roles for IL-21 or IL-6. Taken together with our data demonstrating that B cells are more critical for maintenance of T_{FH} cells, we would argue that the precise signal, or more likely, combination of signals, that drives initial upregulation of this transcriptional repressor in vivo remains to be defined.

On the basis of the data presented in this paper, we propose a model in which CD4 T cells upregulate Bcl6 levels upon activation in the T cell zone, with consequent repression of PSGL1 expression (Supplemental Fig. 3). Diminished expression of PSGL1 removes a T zone retention signal via a loss of ability to bind CCL19 and CCL21 in concert with reduced CCR7 expression (72, 73), allowing Bcl6⁺PSGL1^{lo} cells to move toward the B cell follicle (74). At the T-B cell border, additional signals from B cells promote T_{FH} cell differentiation and expansion by promoting increased levels of Bcl6 and consequently upregulation of PD-1 and CXCR5 (17, 18). The upregulation of CXCR5 allows T_{FH} cells to move into the B cell follicle where they promote survival and maturation of B cells in the GC via CD40L, IL-21, and other activating signals (48, 75).

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Disclosures

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