

The Journal of Immunology

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This information is current as of July 26, 2021.

J Immunol 2016; 196:1026-1035; Prepublished online 4 January 2016;
doi: 10.4049/jimmunol.1401059
http://www.jimmunol.org/content/196/3/1026

Supplementary Material http://www.jimmunol.org/content/suppl/2016/01/02/jimmunol.1401059.DCSupplemental

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Splenic Long-Lived Plasma Cells Promote the Development of Follicular Helper T Cells during Autoimmune Responses

Eunkyeong Jang,* Wang Sik Cho,* Yeon-Kyung Oh,* Mi-La Cho,† Jung Mogg Kim,‡ Doo-Jin Paik,* and Jeehee Youn*

Long-lived plasma cells (LLPCs) develop under the help of follicular helper T (Tfh) cells and reside mainly in the bone marrow. However, these cells are unusually abundant in the spleen of several autoimmune models including K/BxNsf mice, yet their pathogenic impact remains unknown. To investigate a previously unappreciated role of splenic LLPCs, we sorted splenic plasma cells (PCs) from K/BxNsf and K/BxN mice, corresponding to LLPCs and conventional short-lived PCs, respectively, and compared their phenotypes and ability to prime and induce the differentiation of naive CD4$^+$ T cells into effector cells in vitro and in vivo. We found that K/BxNsf PCs had lower levels of the Ag presentation machinery and costimulators than K/BxN PCs, and also a lower CD4$^+$ T cell priming capacity. Autoantigen-pulsed K/BxNsf PCs selectively polarized cognate CD4$^+$ T cells toward the expression of molecules necessary for Tfh development and function. As a result, the K/BxNsf PC-primed CD4$^+$ T cells were more effective in stimulating B cells to produce autoantigen-specific IgGs than K/BxN PCs or even dendritic cells. Adoptive transfer of K/BxNsf PCs, but not K/BxN PCs, to K/BxN mice increased numbers of Tfh cells in draining lymph nodes. These results propose that abnormal accumulation of LLPCs in the spleen of autoimmune models drives the differentiation of autoantigen-primed CD4$^+$ T cells to Tfh cells. This positive feedback loop between splenic LLPCs and Tfh cells may contribute to the persistence of humoral autoimmunity. ***The Journal of Immunology*****, 2016, 196: 1026–1035.**

Systemic autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and Sjögren's syndrome, are characterized by an abundance of autoantibodies. These Abs initiate complement- and FcR-mediated inflammatory responses in target tissues, which in turn extends the breakdown of self-tolerance and ultimately precipitates symptoms of autoimmunity (1). In this way, autoantibodies play a crucial role in the development of chronic autoimmune diseases.

Long-lasting plasma cells (PCs), which secrete high-affinity Abs, are produced mainly during the germinal center (GC) response. GCs are sites responsible for the activation and maturation of B cells and are formed within B cell follicles in secondary lymphoid organs in response to T-dependent Ags (2). The GC response is governed by a specialized subset of CD4$^+$ Th cells known as follicular helper T (Tfh) cells. The transcriptional repressor protein B cell lymphoma (BCL)-6 is known to instruct naive CD4$^+$ T cells to commit to Tfh fates and produce their distinctive surface markers, such as CXCR5 and ICOS, and signature cytokines, such as IL-21 and IL-4 (3, 4). Tfh cells then signal B cells that recognize cognate Ags through surface and secreted molecules including CD40L, ICOS, and IL-21. As a result of this Ag-specific signaling, some B cells undergo affinity maturation and Ab class switching and differentiate into either memory B cells or PCs. Most PCs either die within 1 wk in the secondary lymphoid organs, which apparently provide only few survival niches for them, or leave the organs in search of other survival niches, such as bone marrow (BM) (5–7). Expression of CXCR4 promotes the recruitment of PCs into BM niches and contributes to the positioning of PCs near BM stromal cells expressing CXCL12. After entering the BM, PCs terminally differentiate to end-stage Ab-secreting cells and attain a lifespan of months or even decades. Tfh cells are, therefore, essential for the formation of affinity-matured, postswitched, long-lived PCs (LLPCs), which are sought to reside mainly in the BM.

Contrary to this canonical picture, we and others have presented evidence that substantial numbers of LLPCs accumulate in the spleen and lymph nodes (LNs) (especially in the extrafollicular and medullary regions) in certain autoimmune models such as the NZB/W F_1, NZM2410, and K/BxNsf mice (8–10). The K/BxNsf mice are male K/BxN congenic mice bearing a Foxp3-mutant *scurfy* allele and exhibit more severe symptoms of arthritis than their K/BxN littermates (10, 11). Most importantly, we found that the vast majority of splenic PCs in K/BxNsf mice are LLPCs (10). This phenomenon has been linked to a BM homing failure because of the cells' unresponsiveness to CXCL12. Despite their ectopic residence, the splenic LLPCs become refractory to treatment with cyclophosphamide and mitomycin C, just like the BM-residing

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Received for publication April 25, 2014. Accepted for publication November 23, 2015.

This work was supported by grants of the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT, and Future Planning (Grant NRF-2014R1A1A1038189 [to E.J.]) and the Korean Health Technology R&D Project, Ministry of Health and Welfare, Korea (Grant HI13C0016 [to J.Y.]).

The microarray data presented in this article have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc-GSE66947) under accession number GSE66947.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BCL, B cell lymphoma; BM, bone marrow; CD62L, L-selectin; DC, dendritic cell; dLN, joint-draining lymph node; GC, germinal center; GPI, glucose-6-phosphate isomerase; LLPC, long-lived PC; LN, lymph node; MHC II, MHC class II; PC, plasma cell; PD-1, programmed death-1; SLPC, short-lived PC; Tfh, follicular helper T; Treg, regulatory T.

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LLPCs. In addition, we found that splenic LLPCs in K/BxNsf mice can escape immune complex-mediated cell death by down-regulating the FcγRIIb receptor on their surface. This mechanism may account for their survival under autoantibody-rich conditions. Their resistance to cell death, along with their persistent production of Abs, may explain why LLPC-related diseases are deemed incurable.

Traditionally, the only role of PCs is thought to be Ab secretion (12). Although their immediate B-lineage progenitors mediate diverse functions such as Ag presentation, cytokine and chemokine secretion, and lymphoid organization, PCs were thought to have lost these capacities (13). However, a recent study showing that conventional PCs can act as APCs and inhibit the development of Tfh cells (14) challenges this view and implies that the role of PCs may be more diverse than previously thought. It is not yet known whether splenic LLPCs can act as APCs, and if so, what their role would be in regulating humoral immunity. In this study, we demonstrate that, unlike splenic conventional PCs, splenic LLPCs in K/BxNsf mice can behave as APCs that specifically promote the development of Ag-specific Tfh cells. We propose that positive feedback between Tfh cells and splenic LLPCs could provide a novel mechanism by which ongoing humoral autoimmunity is autonomously amplified.

Materials and Methods

Mice

KRN TCR transgenic mice on a C57BL/6 background (15) were originally donated by Dr. D. Mathis (Harvard Medical School, Boston, MA) and kept in our animal facility. The mice were crossed with NOD mice to generate K/BxN mice. The majority of $CD4^+$ T cells in the K/BxN mice express a TCR that specifically recognizes $pGPI_{282–294}$, a peptide derived from glucose 6-phosphate isomerase, in the context of $I\text{-}A^{g7}$ (15). K/BxNsf mice were generated as described previously (10). Breeders of OT II mice were purchased from The Jackson Laboratory, and their progeny were maintained in our facility. All mice were housed in a pathogen-free barrier facility at Hanyang University, and the study was approved by the Institutional Animal Care and Use Committee.

Gene expression arrays

$B220^+CD138^+$ PC populations from the K/BxNsf and K/BxN mice were sorted by FACS Aria III (BD Biosciences). Total RNA was purified with TRIzol reagent (Life Technologies), and gene expression profiles were assayed using an Illumina Mouse Ref-8 version 2 Expression Bead Chip (Illumina). The array data were analyzed using Illumina Genome Studio version 1.5.4 (Gene Expression Module version 2009.2). The microarray data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE66947 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE66947).

Abs and FACS

Single-cell suspensions of spleen and LN cells were stained as described previously (16). Samples were analyzed by FACS Canto II (BD Biosciences). The following mAbs and reagents were purchased from BD Biosciences or eBioscience: anti-CD86 (GL1), anti–$I\text{-}A^b$ (M5/114.15.2), anti-CD74 (In-1), anti-B220 (RA3-6B2), anti-CD4 (RM4-5), anti-CD25 (7D4), anti-CD138 (281-2), anti–L-selectin (CD62L) (MEL-14), biotinylated anti-CXCR5 (2G8), anti-CD11c (N418), anti-CD44 (IM7), anti-CD40L (MR1), anti-IgG1 (P3), anti-programmed death-1 (PD-1) (RMP-30), anti–BCL-6 (K112-91), 7-aminoactinomycin D, and Annexin V. The mAbs were used as FITC, PE, PerCP, allophycocyanin, or allophycocyanin-Cy7 conjugates.

Fluorescence microscopy

Mouse spleens were embedded in OCT compound and snap-frozen in liquid nitrogen. Cryosections (7 μm thick) were fixed with acetone, blocked with 10% goat serum, and stained with an appropriate combination of mAbs. Anti–GL-7-FITC and anti–CD138-PE mAbs were purchased from BD Biosciences. Anti–CD4-PerCP-Cy5.5, biotinylated anti–PD-1, streptavidin-PE, and anti–B220-allophycocyanin were purchased from eBioscience. Anti–CD4-Alexa 647 and anti–B220-Alexa 488 were purchased from Caltag Laboratories. Fluorescence images were acquired using an LSM 510 confocal microscope (Zeiss).

In vitro $CD4^+$ T cell priming

To prepare APCs, PCs ($B220^+CD138^+$), dendritic cells (DCs) ($B220^-CD3^-CD11c^+$), and B cells ($B220^+CD138^-$) from the spleens of K/BxN and K/BxNsf mice at ~8 and 5 wk of age, respectively, that exhibited similar signs of disease, were sorted by FACS Aria III (BD Biosciences). To purify naive $CD4^+$ T cells, erythrocyte-depleted spleen cells from the same K/BxN mice were first sorted by MACS to collect all $CD4^+$ cells and then sorted further by FACS to collect $CD25^-CD62L^+CD44^{lo}$ cells. The purity of the sorted naive $CD4^+$ T cells routinely exceeded 99%. These cells were labeled with 3 μM CFSE (Molecular Probes) and cultured with APCs at a cell ratio of 1:1 in the presence or absence of 10 μM $pGPI_{282–294}$ (Peptron). Alternatively, naive $CD4^+$ T cells sorted from OT II mice and 2 μg/ml $pOVA_{323–339}$ (Peptron) were used instead of those from K/BxN mice and $pGPI_{282–294}$, respectively. In some experiments, 20 μg/ml anti-MHC class II (MHC II) mAb (M5/114.15.2; eBioscience) or isotype control Ab was added to the culture, and Transwells with 5-μm pores (Corning) were used to physically separate the T cells from the APCs. After 4 d of culture, $CD4^+$ T cells were assayed by FACS to determine the extent of CFSE dilution and other phenotypes.

In vitro B cell activation

After 4 d of culture for in vitro T cell priming as described above, activated, dividing $CD4^+$ T cells ($B220^-CD138^-CD11c^-CD4^+CD44^{hi}CFSE^{lo}$) were sorted from individual wells. The cells were then added to a culture of naive B cells ($GL7^-CD138^-CD11c^-B220^+$) purified from K/BxN mice at a cell ratio of 1:1. The cultured cells were stimulated with 2 μg/ml soluble anti-CD3 mAb and 10 μM $pGPI_{282–294}$. To block IL-21, IFN-γ, and IL-4 signaling, 10 ng/ml soluble IL-21R-Fc fusion protein (R&D Systems), 10 μg/ml anti–IFN-γ mAb and 10 μg/ml anti–IL-4 mAb, respectively, or their isotype control mAbs (BD Pharmingen) were added. After 6 d of culture, supernatants were collected and assayed by ELISA to measure levels of GPI-specific IgG Abs, as described previously (16). Serum collected from 7 wk-old K/BxN mice was serially diluted and used as the standard. On the basis of the standard curve, the Ab titers were given in arbitrary units per milliliter. To examine proliferative responses, purified naive B cells from K/BxN mice were labeled with 3 μM CFSE prior to coculture with T cells. Three days later, the division profiles of the CFSE-positive cells were analyzed by FACS.

Cell delivery to mice

PCs ($B220^+CD138^+$) from spleens of 8-wk-old K/BxN or 5-wk-old K/BxNsf mice were sorted by FACS Aria III. Four-week-old K/BxN mice were injected intravenously with 8×10^5 PCs or with PBS. Joint-draining LNs (dLNs; a pooling of axillary, inguinal, and popliteal LNs) and spleens from the recipient mice were assayed postmortem by FACS after 4 d and by immunofluorescence microscopy after 7 d.

RT-PCR

Quantitative RT-PCR was performed to characterize activated, dividing $CD4^+$ T cells ($B220^-CD138^-CD11c^-CD4^+CD44^{hi}CFSE^{lo}$) and PCs ($B220^+CD138^+$). Total RNA from the sorted cells was isolated using TRIzol reagent (Life Technologies). cDNA was generated using a QuantiTech Reverse Transcription Kit (Qiagen). Real-time PCR was carried out with SYBR Green Quantitative PCR Master Mix (Applied Biosystems). The mRNA expression levels of each gene were normalized to the mRNA expression levels of β_2-microglobulin or β-actin, and differences between samples were normalized by the cycle threshold method (ΔC_t). Primer sequences were as follows: $I\text{-}A^b$, 5′-GCG GGC CGA GCT GGA CAC G-3′ (forward) and 5′-CGC ACT TTG ATC TTG GCT GGG TAG-3′ (reverse); ICOS, 5′-CCT GAG ACT GTC CAG CAG-3′ (forward) and 5′-GTG GGT CAA AAA TGG ACA-3′ (reverse); PD-1, 5′-GTG CCT GGA AAT GGA GAG AT-3′ (forward) and 5′-TAG GTG CTG GGC CCA TAG TA-3′ (reverse); and β_2-microglobulin, 5′-TGA CCAGCT TGT ATG CTA TC-3′ (forward) and 5′-CAG TGT GAG CCA GGA TAT AG-3′ (reverse). The primers for CD74, H-2O, H-2M, CIITA, cathepsin H, CD80, CD86, BCL-6, IL-21, Batf, RORγt, IL-17, IFN-γ, GATA3, IL-4, Foxp3, CXCR5, and β-actin were as described previously (14, 17, 18).

Statistical analysis

Results are reported as the mean ± SEM of each sample. Unpaired, two-tailed Student *t* tests were used to determine statistical significance. A p value <0.05 was considered statistically significant.

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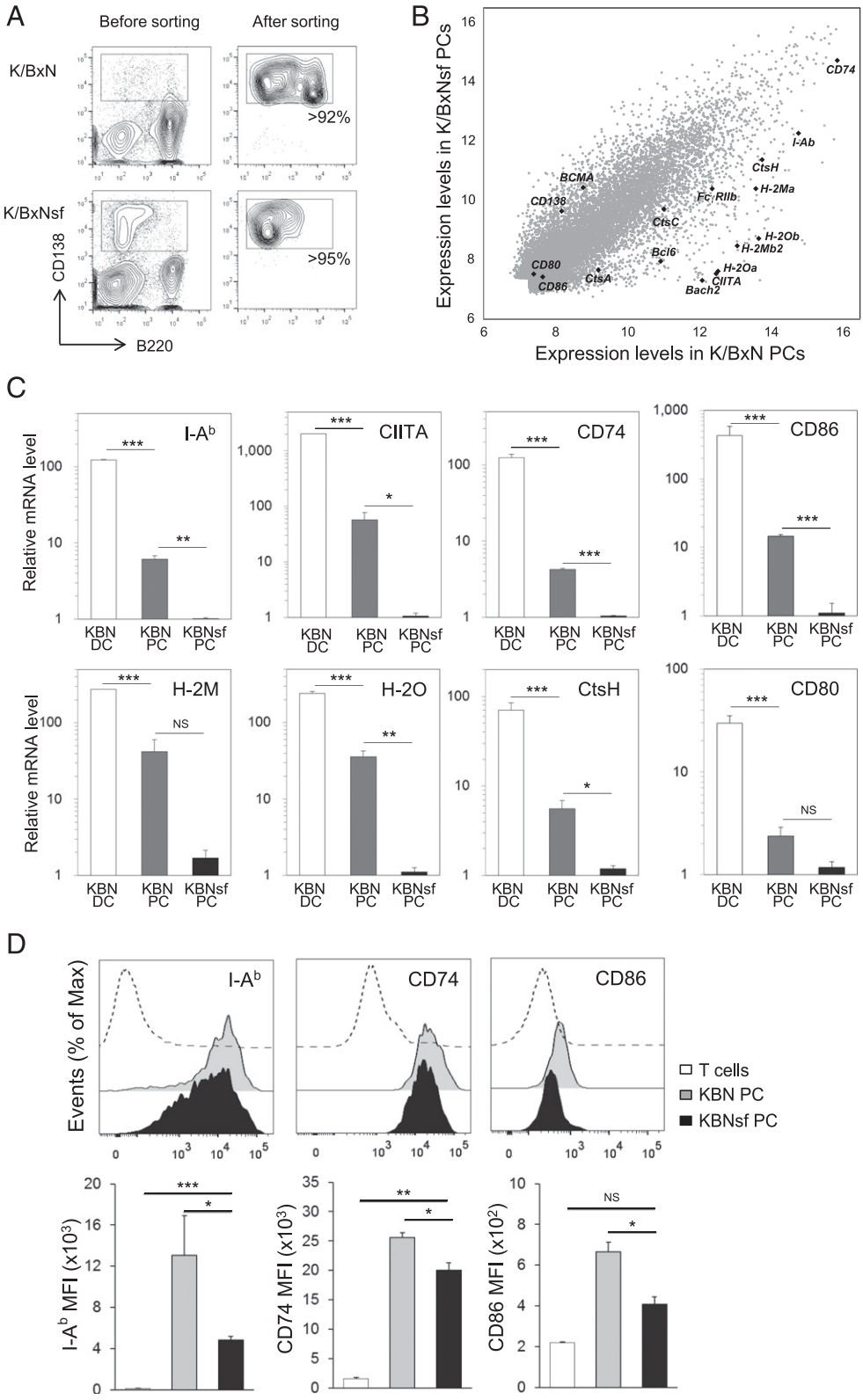

FIGURE 1. Splenic LLPCs express lower levels of costimulatory molecules and the intracellular machinery for Ag presentation than conventional PCs. (**A**) PCs from K/BxNsf and K/BxN mice were sorted (purity is shown). (**B**) The transcriptomes of the two sorted PC populations, analyzed by cDNA microarray, are displayed as a pairwise scatter plot. (**C**) CD11c$^+$ DCs from K/BxN mice and PCs from K/BxN and K/BxNsf mice were sorted, and transcripts were assayed by quantitative RT-PCR. The levels of transcripts encoding the indicated genes were normalized to the level of β_2-microglobulin. The graphs show the mean ± SEM of five to eight experiments. (**D**) Intracellular expression of CD74 and surface expression of I-A^b and CD86 on DCs from K/BxN mice and PCs from K/BxN and K/BxNsf mice. Representative FACS profiles (*upper*) and mean ± SEM MFI (n = 3–5 mice/group) are shown. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, Student t test. CtsH, cathepsin H.

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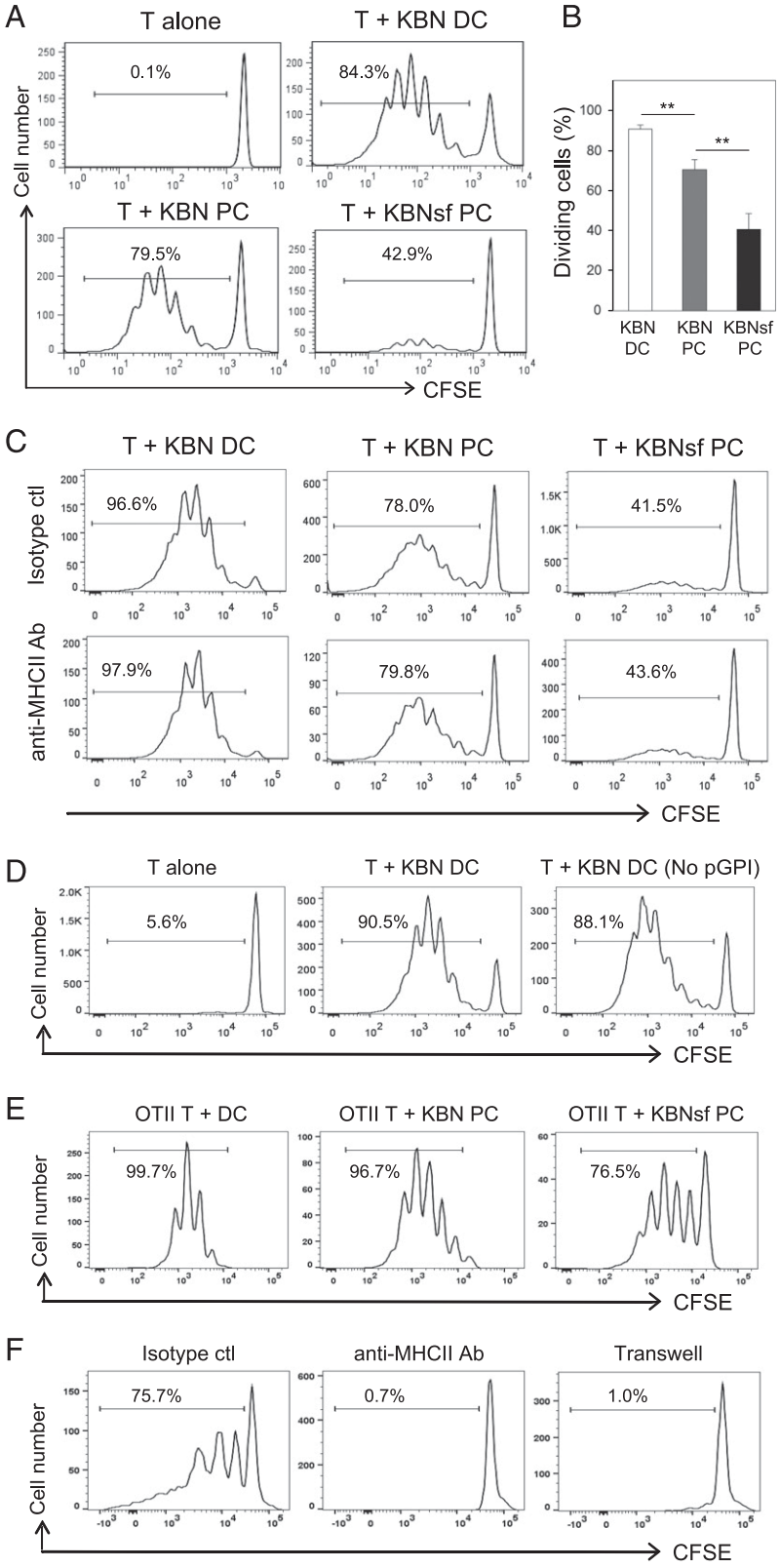

FIGURE 2. Splenic LLPCs retain the ability to prime $CD4^+$ T cells. (**A–D**) Naive $CD4^+$ T cells ($CD25^-CD44^{lo}CD62L^{hi}$) sorted from K/BxN mice were labeled with CFSE and cocultured with K/BxN DCs, K/BxN PCs, or K/BxNsf PCs for 4 d in the presence of $pGPI_{282–294}$. In some experiments, anti-MHC class II mAb or isotype control Ab was added (C), and $pGPI_{282–294}$ was not added (D) to the culture. (**E** and **F**) Naive $CD4^+$ T cells ($CD25^-CD44^{lo}CD62L^{hi}$) sorted from OT II mice were labeled with CFSE and cocultured with K/BxN DCs, K/BxN PCs, or K/BxNsf PCs for 4 d in the presence of $pOVA_{323–339}$. Anti-MHC II mAb or isotype control Ab was added to the culture, or T cells and PCs were separated by transwells, as indicated. (A, C, and D–F) Representative FACS profiles gated on $CD4^+$ T cells with the percentage of proliferating cells. (B) Percentages of proliferating $CD4^+$ T cells; mean ± SEM of five experiments. $^{**}p < 0.01$, Student *t* test.

Results

Splenic LLPCs express lower levels of molecules related to Ag presentation and costimulation than conventional PCs and DCs

We demonstrated previously that, although ~90% of PCs emerging in autoimmune K/BxN mice divided and turned over rapidly, their scurfy congenic line (known as K/BxNsf) developed unconventional PCs (10). We found that >95% of the entire PC population residing in the K/BxNsf spleens and LNs met the criterion for LLPCs (surviving in vivo more than two weeks without division) (10), whereas it was reported to be <75% in other models (8, 9). The development of K/BxNsf LLPCs resulted from the combination of two conditions (i.e., the deficiency of $Foxp3^+$ regulatory T [Treg] cells together with the

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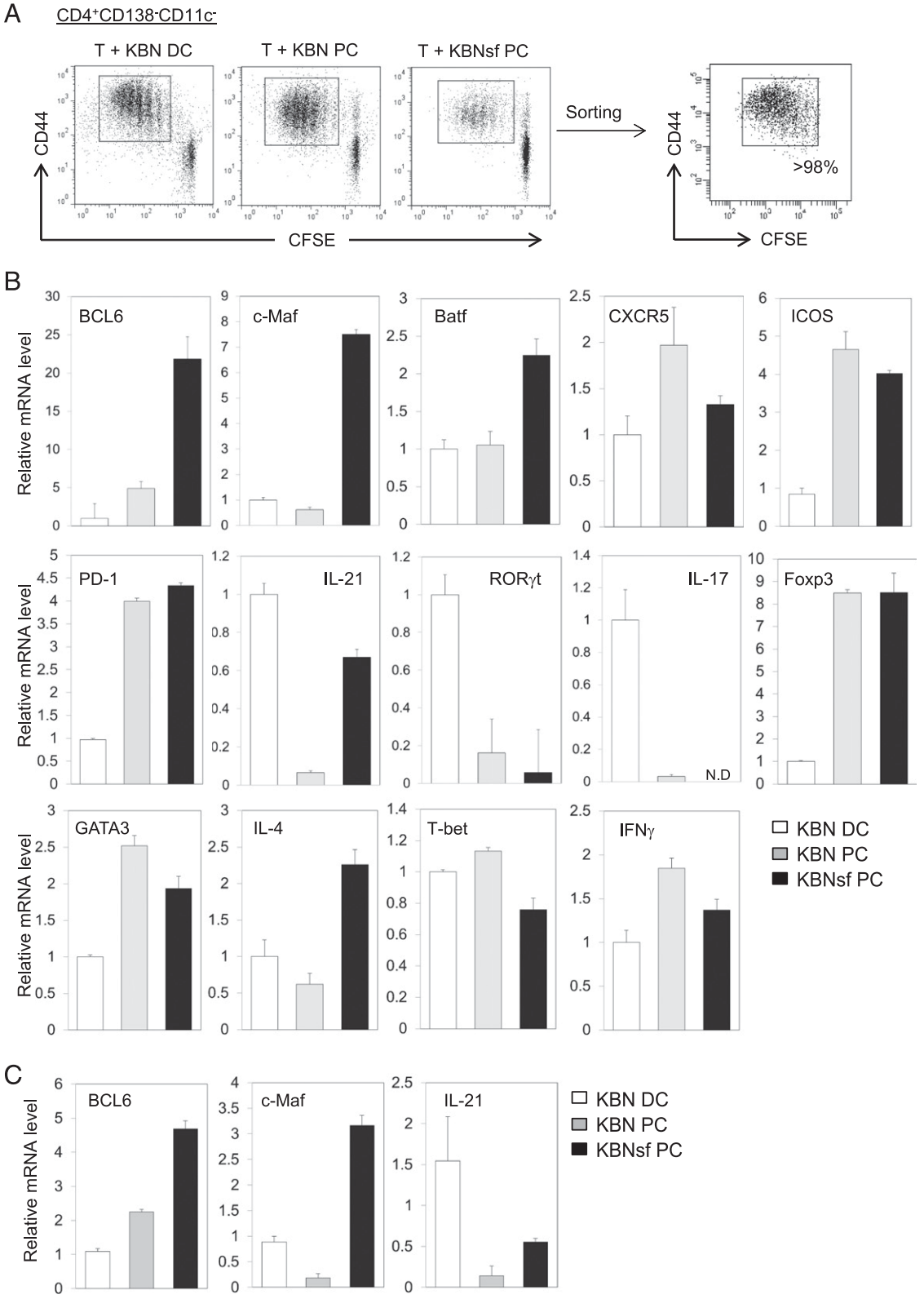

FIGURE 3. Splenic LLPCs selectively induce the expression of Tfh-related genes. (**A**) CFSE-labeled naive $CD4^+$ T cells ($CD25^-CD44^{lo}CD62L^{hi}$) from K/BxN mice were cocultured with K/BxN DCs, K/BxN PCs, or K/BxNsf PCs for 4 d in the presence of $pGPI_{282–294}$, and the $CD4^+CD44^+CFSE^{dim}$ cells were sorted (>98% purity). (**B**) The sorted cells were assayed by quantitative RT-PCR. (**C**) OT II cells primed with $pOVA_{323–339}$-pulsed APCs were examined as (A) and (B). The expression level of each mRNA was normalized to the levels of β-actin mRNA. The data represent three independent experiments.

genetic background of the K/BxN strain) because the number of splenic LLPCs fell when Treg cells were reconstituted in K/BxNsf mice and they did not appear in scurfy C57BL/6 mice (Supplemental Fig. 1). In this study, we took advantage the scurfy model to purify splenic LLPCs from K/BxNsf mice without genotoxic labeling.

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To define the phenotypic and functional characteristics of splenic LLPCs that distinguish them from conventional PCs, we sorted splenic PCs from K/BxNsf and K/BxN mice (Fig. 1A) and compared their transcriptomes by cDNA microarray analysis. The PC fractions included $B220^{hi}CD138^{+}$ early PCs (also called plasmablasts) and $B220^{lo}CD138^{+}$ mature PCs, and these two populations equally produced IgG_1 Abs in both strains (Supplemental Fig. 2). The resulting pairwise scatter plot has $r = 0.852$ and revealed a variety of differentially expressed genes (Fig. 1B, Supplemental Tables I, II). Consistent with our previous findings (10), the expression of FcγRIIb transcripts was lower in the K/BxNsf PCs. Furthermore, transcripts encoding factors important for the survival and maintenance of BM LLPCs, such as BCMA and CD138 (19, 20), were more abundant, whereas those encoding factors antagonizing Blimp-1, such as BCL-6 and Bach2 (21, 22), were less abundant. These results suggest that the K/BxNsf PCs have features consistent with LLPCs.

Because conventional PCs were previously shown to act as APCs when pulsed with foreign Ags (14), we looked for genes involved in Ag presentation and T cell priming in our two PC populations. We found that the levels of transcripts for MHC II $I\text{-}A^{b}$ and its transactivator CIITA were lower in the K/BxNsf PCs (Fig. 1B). Transcripts encoding the intracellular machinery for Ag processing, including invariant chain (CD74), peptide editing complexes H-2O and H-2M and lysosome-associated carboxypeptidase cathepsin H, were also less abundant in the K/BxNsf PCs, as were transcripts encoding the costimulator CD86, whereas transcripts encoding CD80 were equally abundant in the two sets of PCs.

To validate the microarray data we carried out quantitative RT-PCR. The levels of transcripts encoding $I\text{-}A^{b}$, CIITA, CD74, H-2O, H-2M, cathepsin H, and CD86 were indeed significantly lower in the K/BxNsf PCs than in the K/BxN PCs (Fig. 1C). The levels of H-2M and CD80 transcripts also tended to be lower in the K/BxNsf PCs, although not significantly so. The PCs from the K/BxN mice expressed all these molecules at significantly lower levels than the $CD11c^{+}$ DCs from the K/BxN mice. We also observed that the K/BxNsf PCs produced significantly lower levels of $I\text{-}A^{b}$, CD74, and CD86 proteins than the K/BxN PCs but still higher than the T cells in both strains (Fig. 1D). This suggests that the short-lived PCs (SLPCs) that develop under chronic autoimmune conditions retain the potential to act as APCs, albeit to a lesser extent than conventional APCs, and that this ability is compromised along with the apparent acquisition of a longer lifespan.

Splenic LLPCs have a lower capacity for $CD4^{+}$ T cell priming than conventional PCs

To examine whether splenic LLPCs were able to prime T cells, we purified naive $CD4^{+}$ T cells from K/BxN mice, labeled them with CFSE, and cocultured them with K/BxNsf PCs, K/BxN PCs, or K/BxN DCs as syngeneic sources of APCs, in the presence of $pGPI_{282–294}$. After 4 d of culture, the extent of dilution of the CFSE-labeled cells was measured. The K/BxN DCs, which are a source of professional APCs, were found to stimulate the proliferation of syngeneic $CD4^{+}$ T cells most strongly. The fraction of dividing cells primed with the K/BxNsf PCs was approximately half of that primed with the K/BxN PCs (Fig. 2A, 2B). These results demonstrate that splenic LLPCs retain a robust ability to prime $CD4^{+}$ T cells, despite their compromised potency.

To determine whether this effect was dependent on Ag presentation on MHC II molecules, we added anti-MHC II neutralizing Ab to the coculture. Interestingly, this Ab did not interfere with the APC-provoked proliferation of $CD4^{+}$ T cells, regardless of the type of APC used (Fig. 2C). Moreover, the $CD4^{+}$ T cells from K/BxN mice equally responded to APCs in the presence or absence of exogenous $pGPI_{282–294}$ (Fig. 2D). These results suggest that K/BxNsf PCs presented endogenous self-peptide on MHC II molecules in vivo and did not require the in vitro pulsing with the same peptide to prime the cognate $CD4^{+}$ T cells. Under this view, the system using $pGPI_{282–294}$ and K/BxN T cells seemed to be inappropriate to address this question regarding MHC II dependence.

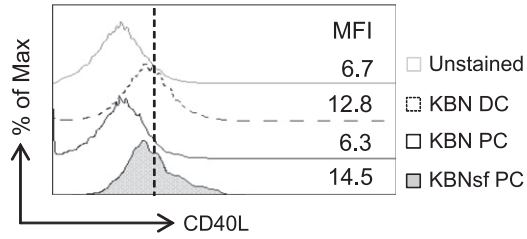

FIGURE 4. Phenotypes of $CD4^{+}$ T cells primed with splenic LLPC CFSE-labeled naive $CD4^{+}$ T cells ($CD25^{-}CD44^{lo}CD62L^{hi}$) from K/BxN mice were cocultured with K/BxN DCs, K/BxN PCs, or K/BxNsf PCs in the presence of $pGPI_{282–294}$. Expression of CD40L on $CD4^{+}CD44^{+}CFSE^{dim}$ cells on day 4 after culture is shown. The numbers are the mean fluorescence intensities of each histogram. The data represent three independent experiments.

We adopted another system in which T cell priming relies on the presentation of exogenous foreign Ag peptide on MHC II molecules. K/BxNsf PCs, K/BxN PCs, or K/BxN DCs were pulsed with a peptide derived from OVA ($pOVA_{329–339}$) and cocultured with $CD4^{+}$ T cells purified from OT II mice. Similar to the results with K/BxN T cells in response to $pGPI_{282–294}$, all three types of APCs elicited the proliferation of OT II cells (Fig. 2E). Importantly, the proliferative responses were efficiently inhibited by the addition of anti-MHC II Ab to the culture and by the Transwell block of physical contact between K/BxNsf PCs and OT II cells (Fig. 2F). Thus, these results demonstrate that splenic LLPCs serve as APCs in an MHC II– and contact-dependent manner.

Splenic LLPCs selectively promote the differentiation of Tfh-like cells in vitro

Many studies have shown that the types and activation states of APCs can influence the developmental fates of Th subsets (23). In particular, conventional PCs were shown to negatively regulate the development of Tfh cells (14). In this study, we raised the question whether splenic LLPCs could play a similar role. We purified proliferating cells with an activated phenotype ($CD44^{+}CFSE^{dim}$) from among all the $CD4^{+}$ T cells that had been primed with $pGPI_{282–294}$-pulsed PCs or DCs (Fig. 3A) and measured their expression of Tfh-specific genes (Fig. 3B). Surprisingly, transcripts encoding BCL-6—the Tfh master transcription factor and its downstream effectors c-Maf and Batf—were more abundant in the cells primed with K/BxNsf PCs than in those primed with K/BxN PCs. Also, unexpectedly, the levels of these three transcripts were not significantly different in cells primed with PCs or DCs derived from K/BxN mice. The levels of CXCR5 transcripts did not differ significantly between the cells primed with these three APC populations. The cells primed with two types of PCs contained similar levels of ICOS and PD-1 transcripts, which were higher those primed with the DCs.

To determine whether the action of splenic LLPCs was Tfh-specific, we also examined the ability of the three sources of APCs to induce other subsets of Th cells by measuring the expression of master regulators and signature cytokines specific for each subset. In both populations, transcripts encoding RORγt and IL-17 were more abundant in the $CD4^{+}$ T cells activated by DCs

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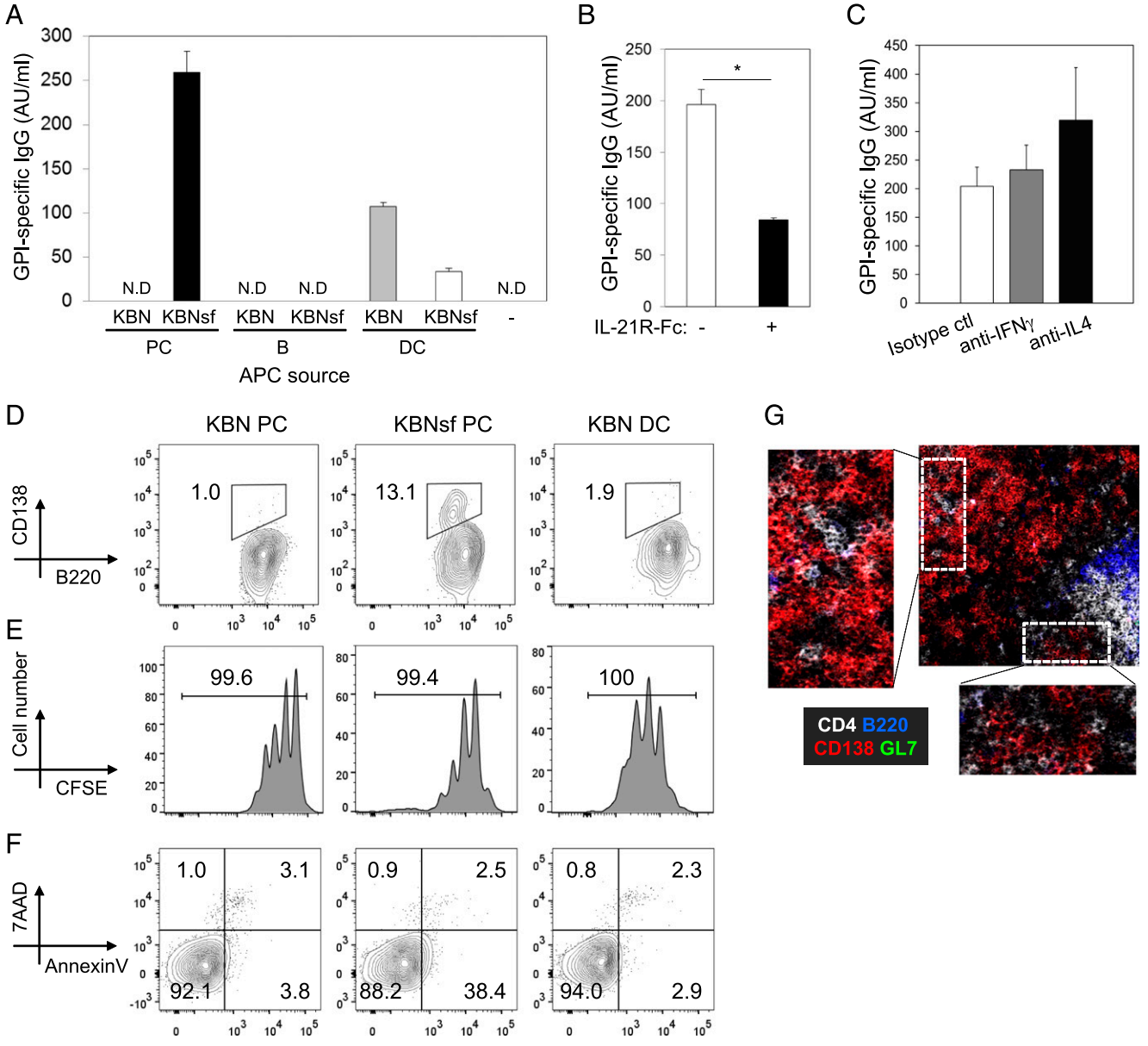

FIGURE 5. $CD4^+$ T cells primed with autoantigen-pulsed splenic LLPCs are the most efficient at inducing PC differentiation in vitro. (**A**–**C**) CFSE-labeled naive $CD4^+$ T cells ($CD25^-CD44^{lo}CD62L^{hi}$) from K/BxN mice were cocultured with $pGPI_{282–294}$-pulsed APCs for 4 d, and $CD4^+CD44^+CFSE^{dim}$ cells were then sorted and cocultured with naive B cells from K/BxN mice. IL-21R-Fc, anti–IFN-γ, anti–IL-4 mAbs, or isotype control mAb was added to cultures containing $CD4^+$ T cells that had been activated by K/BxNsf PCs ($*p < 0.05$) (B and C). After 6 d of culture, supernatants were assayed to measure the anti-GPI IgG titers. (**D**) CD138 expression in $B220^+$ cells activated as in (A) was assessed by FACS. (**E**) CFSE-labeled B cells were cultured as in (A) and assayed by FACS. (**F**) B cells cultured as in (A) were stained with Annexin V and 7-aminoactinomycin D, then assayed by FACS. All the FACS profiles were gated on $B220^+$ cells. (**G**) Cryosections from K/BxNsf spleen were stained with anti–CD4-PerCP-Cy5.5 (white), anti–B220-APC (blue), anti–CD138-PE (red), and anti–GL-7-FITC (green) and observed by fluorescence microscopy. Original magnification ×200; two extrafollicular sites are further magnified. Data are representative of two (B–F) or three (A and G) independent experiments.

than in those activated by PCs. IL-21, which can be produced by both Th17 and Tfh cells, was expressed in activated $CD4^+$ T cells primed with K/BxNsf PCs and with K/BxN DCs but not in cells primed with K/BxN PCs. Similarly, IL-4, which can be produced by Tfh cells as well as Th2 cells, increased slightly upon priming with K/BxNsf PCs, despite the absence of any obvious increase in GATA3. The abundance of transcripts encoding Th1-specific molecules—such as IFN-γ and T-bet and the Treg cell marker Foxp3—did not differ significantly between the cells primed by K/BxNsf PCs and those primed by K/BxN PCs. Thus, splenic LLPCs seem to induce naive $CD4^+$ T cells to commit preferentially to Tfh differentiation rather than to Th17 cells, whereas conventional DCs elicit the opposite response. SLPCs derived from K/BxN mice appear to be extremely poor inducers of both Th subsets, despite their capacity to prime $CD4^+$ T cells. Th1 and Th2 cells seem not to be efficiently induced regardless of the type of APC in this system derived from autoimmune mice. In support with this hypothesis, we obtained consistent results in the levels of BCL-6, c-Maf, and IL-21 when we used the OT II/ $pOVA_{329–339}$ system (Fig. 3C).

We detected CD40L on the surface of primed T cells, a key molecule that can influence differentiation and/or function of Tfh cells. CD40L was elevated on the surface of $CD4^+CD44^+CFSE^{dim}$ T cells primed with K/BxNsf PCs or K/BxN DCs but could not be

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detected on those primed with K/BxN PCs (Fig. 4). These results suggest that the mechanism underlying this effect of splenic LLPCs includes upregulation of CD40L on the surface of $CD4^+$ T cells.

Splenic LLPCs induce cognate $CD4^+$ T cells to help B cells differentiate into Ab-secreting cells in the most efficient manner

We investigated whether Tfh-like cells polarized as a result of priming with K/BxNsf PCs could actually function as Tfh cells. To this end, $CD4^+$ T cells cocultured with each population of $pGPI_{282–294}$-pulsed PCs or DCs were sorted and cultured with syngeneic naive B cells together with TCR stimulation. B cells cocultured with K/BxNsf PC-primed T cells produced ~2.5-fold higher levels of GPI-specific IgG Abs than those cocultured with K/BxN DC-primed T cells (Fig. 5A). By contrast, these Abs were not detected after coculture with T cells primed with K/BxN PCs or B cells derived from either strain. They were not produced unless T cells were added to the culture, confirming that their production was T cell dependent. Furthermore, their production was inhibited by the addition to the B–T cell coculture of soluble IL-21R-Fc, which acted as a block to IL-21 signaling, but it was not inhibited by neutralizing mAbs to IFN-γ and IL-4 (Fig. 5B, 5C). In agreement with this, more $B220^+$ cells gave rise to $CD138^+$ cells when cocultured with K/BxNsf PC-primed $CD4^+$ T cells than when cocultured with K/BxN PCs and DCs (Fig. 5D). The $CFSE^{dim}$ and Annexin V^+/7-$AAD^{+/-}$ factions of $B220^+$ cells did not differ under any conditions of cultures, indicating that the K/BxNsf PC-primed T cells did not affect the proliferation and survival of B cells (Fig. 5E, 5F). These results demonstrate that cognate interaction of $CD4^+$ T cells with splenic LLPCs was the most efficient at inducing the development of Tfh cells, which functioned in an IL-21–dependent manner to help B cell differentiation. Our observation that PCs are in contact with $CD4^+$ T cells at extrafollicular sites in K/BxNsf spleens (Fig. 5G) also supports this idea.

Adoptive transfer of splenic LLPCs into autoimmune mice promotes Tfh cell development

To see whether the positive feedback loop between splenic LLPCs and Tfh cells could be replicated in vivo, we adoptively transferred splenic PCs purified from K/BxNsf or K/BxN mice to K/BxN mice during a very early phase of autoimmune arthritis. The vehicle control mice spontaneously developed Tfh cells in their dLNs, as had been shown previously (16). Infusion with K/BxNsf PCs resulted in increased proportions and numbers of Tfh cells in the dLNs (Fig. 6A–C). By contrast, infusion with K/BxN PCs reduced numbers of Tfh cells. We also observed that the GCs of K/BxNsf PC-infused mice exhibited more numerous $CD4^+PD\text{-}1^+$ T cells than those of PBS-infused mice (Fig. 6D). This experiment provides robust evidence that splenic LLPCs, unlike conventional splenic PCs, promote the development of Tfh cells in mice with chronic autoimmunity.

Discussion

Contrary to the traditional view that PCs are specialized only for Ab secretion, it was recently shown that they can function as competent APCs and thereby negatively regulate the development of Tfh cells (14). Consistent with this, we found in the current study that SLPCs that developed during autoimmune responses in the spleen of K/BxN mice acted as extremely poor inducers of Tfh cells in vitro, and they even reduced the number of Tfh cells when infused into syngeneic recipients in which autoimmune responses were ongoing. Most importantly, we found that splenic LLPCs derived from a congenic line of K/BxN mice had functional characteristics distinct from the conventional SLPCs of K/BxN mice. The former retained the ability to promote the proliferation of $CD4^+$ T cells, despite their compromised potency. After priming by LLPCs, naive $CD4^+$ T cells gave rise preferentially to Tfh cells at the expense of alternative fates such as Th17 cell

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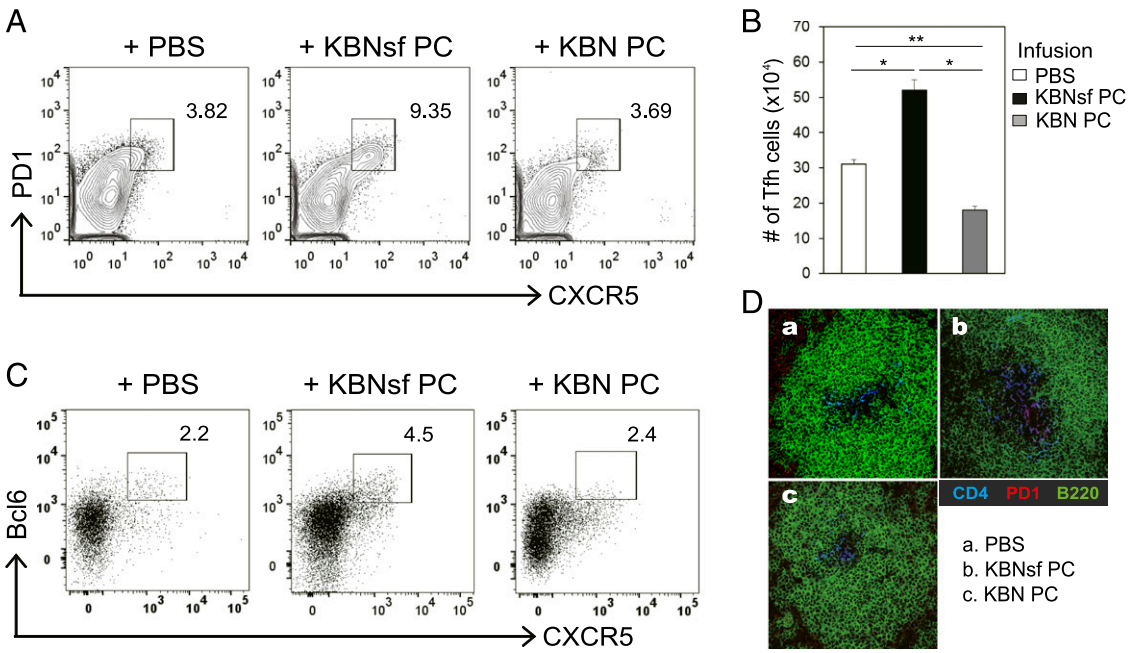

FIGURE 6. Infusion of splenic LLPCs promotes the development of Tfh cells in autoimmune mice. (**A**–**C**) PCs ($B220^+CD138^+$) from spleens of 8-wk-old K/BxN or 5-wk-old K/BxNsf mice were sorted and injected i.v. into 4-wk-old K/BxN mice. PBS was injected as a vehicle control. Four days after infusion, cells extracted from dLNs were analyzed by FACS. (A) Representative FACS profiles with percentages of Tfh cells ($PD1^{hi}CXCR5^{hi}$) among the $CD4^+$ T cells are shown. (B) Absolute numbers (mean ± SEM) of $PD1^{hi}CXCR5^{hi}$ Tfh cells in the dLNs. $*p < 0.05$ and $**p < 0.01$, Student t test. (C) Representative FACS profiles with percentages of Tfh cells ($BCL\text{-}6^{hi}CXCR5^{hi}$) among the $CD4^+$ T cells are shown. (**D**) Immunofluorescence staining of spleen cryosections with anti–CD4-Alexa 647 (blue), anti–PD-1-PE (red), and anti–B220-Alexa 488 (green) 7 d after infusion. Original magnification ×200.

formation. This effect could be recapitulated in vivo because adoptive transfer of K/BxNsf LLPCs resulted in expansion of the Tfh cell population of the recipients. Therefore, our findings identify a novel role of splenic LLPCs as positive regulators of Tfh cell development.

We did not analyze in detail the molecular mechanisms underlying the action of splenic LLPCs. However, we suggest that their effect on Tfh polarization might be related to their reduced capacity to prime $CD4^+$ T cells and thereby their reduced IL-2 signaling. Indeed, it is well known that IL-2 signaling inhibits Tfh differentiation by inducing Stat5-dependent expression of Blimp-1, a repressor of BCL-6 (24–26). Alternatively, IL-2 signaling may promote the expression of T-bet, which forms complexes with BCL-6 and prevents BCL-6 from repressing the expression of Blimp-1 (27). We hypothesize that the effector T cells primed by LLPCs providing weak TCR/costimulation signals express IL-2 and/or its receptor at the level lower than those primed by strong signals, and this favors the expression of BCL-6.

The process of Tfh cell development involves upregulation of surface CD40L expression, and CD40L signaling is necessary for T cell helping of B cells (3). We found that CD40L was elevated on the surface of $CD4^+CD44^+CFSE^{dim}$ T cells primed with K/BxNsf PCs or K/BxN DCs but could not be detected on T cells primed with K/BxN PCs. This result suggests that T cells primed with LLPCs or DCs, but not with SLPCs, can provide CD40L signals to B cells. Consistent with this, only $CD4^+$ T cells primed with LLPCs or DCs, not those primed with SLPCs, helped cognate B cells to differentiate to Ab-secreting cells.

In addition to CD40L induction, only $CD4^+$ T cells primed with LLPCs or DCs, but not those primed with SLPCs, produced IL-21. It is unclear whether IL-21 is sufficient to drive Tfh differentiation from naive $CD4^+$ T cells because contradictory results have been reported (18, 28, 29). Nevertheless, we found previously that IL-21R–deficient ($IL\text{-}21R^{-/-}$) K/BxN mice contained greatly reduced numbers of Tfh cells and autoantibody-secreting cells (16), suggesting that IL-21 plays a role in maintaining Tfh responses in this autoimmune arthritic model. In this context, our finding, in the experiment using IL-21R-Fc, that the effect of LLPC-primed Tfh-like cells is largely mediated by IL-21 further supports the efficacy of LLPCs as promoters of Tfh responses.

We also observed that K/BxNsf PCs induced the expression of BCL-6 and c-Maf but not CXCR5 in $CD4^+$ T cells. This uncoupling may indicate that the Tfh differentiation program involves two successive phases (30). During the early phase, the precursors of Tfh cells would express CXCR5 and then migrate into B cell follicles. Once in the follicles, they would complete the second phase of the program in response to BCL-6. Therefore, CXCR5 and BCL-6 may serve as early and late checkpoints, respectively. It is known that these two molecules are regulated somewhat independently from each other. For example, achaete-scute homolog 2 or Wnt agonists can upregulate CXCR5 but not BCL-6 (30, 31). In this regard, we speculate that K/BxNsf PCs mainly influence the later phase of the Tfh program (via BCL-6), rather than the earlier phase.

Like conventional APCs, K/BxNsf PCs could prime K/BxN $CD4^+$ T cells to proliferate in the absence of exogenous cognate peptide and even in the presence of neutralizing Ab to MHC II. This is consistent with previous investigations demonstrating in vivo activation of KRN T cells by endogenous GPI (32) and in vitro proliferation of K/BxN $CD4^+$ T cells in response to autologous APCs that were not pulsed with exogenous GPI peptides (33). Thus, our result implies that LLPCs present in K/BxNsf spleens display endogenous self-peptides in the context of MHC class II on their surface and act in a manner of APCs.

We found that, unlike LLPCs, conventional DCs induce naive $CD4^+$ T cells to commit preferentially to Th17 rather than Tfh cell differentiation. This effect may reflect the fact that BCL-6 represses the expression of other Th subset–specific transcription factors including RORγt (34, 35). It is worth noting that our findings regarding the impact of DCs on Tfh development conflict with previous reports according to which Ag presentation by DCs is necessary and sufficient to initiate the Tfh cell program, including the induction of BCL-6 expression (36, 37), and DCs are more efficient at BCL-6 induction by $CD4^+$ T cells than PCs (14). Many functional aspects of autoAg presentation by DCs during chronic autoimmune responses seem to be altered if the DCs are exposed to transient responses upon immunization with exogenous Ags. We suspect that these changes may be responsible for the diverse outcomes of T cell regulation by DCs. Furthermore, there is evidence that potent humoral immune responses to influenza virus are dependent not on the presence of $CD11c^+$ DCs, but rather on the responses of other tissue-residing, nonprofessional APCs (38, 39). That finding is in line with our evidence that DCs are less potent than LLPCs in terms of induction of Tfh cell differentiation. Although we found that DCs were poor inducers of BCL-6, they were more able to recruit $CD4^+$ T cells to help B cells than conventional PCs. This may be due to the activity of Th17 cells polarized by DCs because Th17 cells are effective B cell helpers (40, 41).

In summary, we propose a novel function of splenic LLPCs, namely that autoantigen presentation by these cells plays a crucial role in driving the differentiation of cognate $CD4^+$ T cells into Tfh cells in chronic autoimmune conditions. Our results thus provide insight into how splenic LLPCs can sustain humoral autoimmunity.

Acknowledgments

We thank Dr. Diane Mathis for providing the KRN mice, Dr. Seok-Rae Park for valuable discussion, Dr. Julian Gross for editorial assistance, and the Analytical Instrumental Center (Seoul) at Hanyang University for technical assistance with FACS Aria.

Disclosures

The authors have no financial conflicts of interest.

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