T Helper Cells in Murine Germinal Centers Are Antigen-specific Emigrants That Downregulate Thy-1

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Summary

After immunization, activated splenic T cells proliferate in periarteriolar lymphoid sheaths (PALS) and subsequently migrate to the lymphoid follicle where they enter nascent germinal centers. Analysis of TCR V(D)J gene rearrangements indicates extensive emigration, frequently involving more than a single white pulp region. These migrants constitute a unique set of T helper cells that express antigen-specific αβ TCR, CD3, and CD4, but little or no Thy-1, a differentiation antigen present on the great majority of peripheral murine T lymphocytes. The origin of $CD4^+Thy\text{-}1^-$ follicular T cells appears to be the Thy^+ population in the PALS, as both sets commonly share identical V(D)J rearrangements.

Collaborative interactions between T and B lymphocytes are necessary for the production of antibody responses to most protein antigens and for the generation of memory B cells in germinal centers (GCs)[1] (1). These collaborations depend upon activation signals delivered by the Ig and TCR complexes (2, 3) and by contact-dependent, costimulatory interactions that modulate the effects of lymphocyte activation (4–7). During primary responses, these cognate interactions first occur when antigen-activated T and B lymphocytes meet in the T cell zones of secondary lymphoid tissues (8, 9). Later, selected T and B cells migrate into the tissue's B cell areas and renew their collaborations to initiate the GC reaction (10–14). Disruption of early T-B collaboration by agents that block costimulation results in greatly diminished antibody responses and the virtual absence of splenic GCs (15–17). Injection of anti-costimulator antibodies at later times (days 6–10 postimmunization) does not affect the titer or isotype of serum antibody but does interfere with the established GC reaction, either by inducing the premature emigration of centrocytes (anti-CD86) or by inhibiting Ig hypermutation and B cell differentiation into the memory compartment (anti-CD96) (18). Thus, while the initial cognate encounters between T and B cells are necessary for mature humoral responses and B cell memory, they are not sufficient for them; in some fashion, T-B collaboration in GCs is unique.

In primary responses, GC T cells are universally $CD4^+$ and most bear αβ TCR specific for the eliciting antigen (1, 10–13). T cells are a minor (≈5–10%), but requisite, component of the GC cell population; in the absence of T cell help, GCs are not formed (19, 20). However, the relationship between $CD4^+$ T cells and the GC reaction is complex. Adoptive transfer experiments in rats and mice demonstrate that a histologically typical GC reaction to thymus-dependent antigens may be sustained by a level of $CD4^+$ T cells insufficient to support serum antibody responses or the generation of specific plasmacytes (20). Interestingly, these GCs support little or no Ig hypermutation but larger supplements of $CD4^+$ T cells proportionately increase mutation frequencies (21). Despite its central role in humoral immunity, little is known about this important T cell set. In human tonsil, GC T cells are phenotypically distinct helper-inducer cells ($CD4^+$, $CD45R0^+$) that express CD57, a marker shared by human NK cells (22), and early (CD69) but not late (CD25, CD71), markers of cell activation (23). This unique phenotype is thought to reflect the specialized role of GC T cells in driving Ig hypermutation in memory B cell precursors. In contrast, the surface phenotype of murine GC T cells has been thought to be unremarkable and the lack of a distinctive surface phenotype has hampered the study of these crucial T helper cells in mice. Here we demonstrate that in immunized mice, GC T cells share a common clonal origin with the antigen-specific, $CD4^+$ T cells in the periarteriolar lymphoid sheath (PALS; the splenic T cell zone). However, GC and follicular T cells are distinct from all other peripheral αβ TCR^+, $CD4^+$ cells in that they express little or no Thy-1, a 25-kD differentiation antigen that marks most cells in the murine T lineage (24, 25).

[1] *Abbreviations used in this paper:* AP, alkaline phosphatase; CGG, chicken gamma globulin; GCs, germinal centers; GPI, glucose phosphatidyl-inositol; HRP, horseradish peroxidase; NP, alum-precipitated (4-hydroxy-3-nitrophenyl)acetyl; PALS, periarteriolar lymphoid sheaths; PNA, peanut agglutin; PP, Peyer's patch; PPC, pigeon cytochrome *c*.

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Materials and Methods

Antigens, Mice, and Immunization. Female B10.A, C.B-17, and C57BL/6 mice (SPF, 6–10 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME), maintained in autoclaved microisolator cages, and provided with sterile bedding, food, and water. Mice were immunized with a single intraperitoneal injection of 50–100 μg of alum-precipitated (4-hydroxy-3-nitrophenyl)acetyl (NP; Cambridge Research Biochemicals, Cambridge, UK) conjugated to pigeon cytochrome *c* (PCC; Sigma Chemical Co., St. Louis, MO) or chicken gamma globulin (CGG; Accurate Chemical & Scientific Corp., Westbury, NY) (8, 13).

Immunohistology. Spleens and ileal Peyer's patches (PPs) were flash frozen in OCT embedding media; serial, 6-μm-thick frozen sections were cut in a cryostat microtome, thaw mounted onto poly-l-lysine-coated slides, air-dried, fixed in ice-cold acetone for 10 min, and stored at −80°C (8). Three color immunolabeling of $CD4^+$, $V\alpha11$ TCR^+ T cells and peanut agglutin (PNA)-binding GC cells has been described elsewhere (13). Briefly, histologic sections were incubated with the rat anti-CD4 antibody, GK1.5, and PNA conjugated to horseradish peroxidase (HRP; E-Y Laboratories, San Mateo, CA). This incubation was followed by goat anti–rat Ig coupled with alkaline phosphatase (AP; Southern Biotechnology Associates, Birmingham, AL). After washing, sections were further incubated with a biotinylated third reagent, e.g., antibody RR8-1 (PharMingen, San Diego, CA), specific for mouse $V\alpha11^+$ TCR. Bound AP and HRP were then visualized using naphthol AS-MX, phosphate/fast blue BB and 3-aminoethyl carbazole, respectively. Labeled sections were then treated in 1 M HCl for 20 min, rinsed, and incubated with streptavidin-AP. Second-round AP activity, i.e., bound RR8-1, was then detected with fast red. Other biotinylated third antibody reagents used in this study were purchased from PharMingen: anti-Thy1.2 (clones 30-H12 and 53-21.); anti-CD3 (clone 145-2C11); anti-αβ TCR (clone H57-597); anti-γδ TCR (clone GL-3); anti-CD8 (clone 53-6.7); anti-$V\beta3^+$ TCR (clone KJ25); anti-Ly-6A/E (clone E13-161.7); anti-CD48 (clone HM48-1); and anti-NK1.1 (clone PK136). Anti-Bcl-2 antibody (clone 4C11) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Double immunolabeling was performed as described (8). Cells of the KLH-specific line, HDK-1 (the gift of J. Cerny, University of Maryland, Baltimore, MD), were harvested 1 d or 2 wk after stimulation with APC, KLH, and IL-2. Cytospin preparations of activated and resting cloned T cells were fixed as for spleen sections and stained for Thy-1.2 as above. By histology, the expression of Thy-1.2 is equivalent on recently stimulated and resting HDK-1 cells (not shown).

Microdissection of Cells from Tissue Sections. Spleen sections were stained with anti-$V\alpha11^+$ TCR antibody and PNA to visualize $V\alpha11^+$ T cells and germinal centers. For dissecting GC T cells or T cells in the PALS, ~40 labeled cells were picked from each site as described (13). To analyze single cells, individual cells were isolated by a sharpened micropipette driven by a hydraulic micromanipulator (Narishige, Tokyo, Japan) and then aspirated into a microinjector fixed to a second micromanipulator. Photographs taken before and after single cell dissections allowed precise localization of these cells in the spleen.

BrdUrd Labeling and Detection. Immunized and control mice described above were given a single intraperitoneal injection of 2 mg BrdUrd (10 mg/ml) 2 h before killing. Cells which had incorporated BrdUrd into their DNA were detected in frozen tissue sections which had been processed for immunohistology (above). Sections were then treated for 30 min with 1 M HCl at 60°C to expose and partially degrade the DNA. This treatment also served to terminate enzymatic reactions which had taken place previously without displacing the precipitates produced by peroxidase or alkaline phosphatase. Sections were then washed and incubated with the mouse monoclonal antibody BU20a (DAKO A/S, Denmark) which specifically binds BrdUrd incorporated into cellular DNA. Bound BU20a was revealed by sequential incubations with biotinylated goat anti–mouse IgG antibody and streptavidin-AP. The AP activity was visualized using fast red TR/naphthol AS-MX (Sigma).

PCR Amplification. Cell matter from one or more lymphocytes was incubated with proteinase K overnight at 37°C and after heat-inactivation of the enzyme at 96°C for 10 min, the lysate was subjected to two rounds of PCR amplification. All amplifications of template DNA were by the *Pfu* polymerase (Stratagene Inc., La Jolla, CA) using nested primers specific for particular TCR V or J exons. The sequences of PCR primers used for Vα11-Jα84 and Vβ3-Jβ1.2 rearrangements and PCR conditions have been given elsewhere (13). For amplification of DNA from single cells, a first-round PCR was carried out in a volume of 50 μl containing 50 ng rRNA and other reagents as described (13). The amplification program consisted of one cycle at 95°C for 2 min, 55°C for 4 min, 72°C for 2 min, followed by 38 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, finally 1 cycle of 94°C 1 min, 55°C 1 min, and 72°C for 7 min. 2-μl aliquots of the first-round reaction mixture were reamplified for an additional 40 cycles with the same conditions. For multiplex PCR, first round amplifications were carried out in a single tube containing two sets of external primers specific for α- and β-chain rearrangements using the PCR program described above. Second-round amplifications were carried out as separate reactions, using a second internal set of primers specific for either Vα11 or Vβ3 rearrangements. 5-μl aliquots of each reaction were analyzed by electrophoresis on 1% agarose gels.

Sequence Analysis. Amplified DNA was extracted in phenol/chloroform, precipitated in ethanol, digested with restriction endonucleases, and ligated into pBluescript SK^+ as described (13). Competent DH5α bacteria were transformed by electroporation and recombinant colonies screened with ^{32}P-labeled oligonucleotides specific for Vα11 or Vβ3 rearrangements (13). DNA inserts from positive clones were sequenced in both directions.

Results

Immunohistology of Antigen-specific Follicular and GC T Cells. T cell responses to PCC in B10.A mice are clonally restricted; more than 70% of the $CD4^+$ T cells elicited by PCC bear TCR encoded by the Vα11 and Vβ3 gene families (26, 27). Further, T cells that enter the lymphoid follicles and GCs after immunization are antigen-specific (10–13). Thus, we immunized B10.A mice with NP-PCC to characterize systematically the phenotype of $V\alpha11^+V\beta3^+$ T cells in the PALS and GCs.

The numbers of $V\alpha11^+V\beta3^+$ T cells increased dramatically in response to NP-PCC, first in the PALS and later in the follicles and GCs. These increases are concomitant with local cellular proliferation. At day 2 of the response, 2-h pulses with BrdUrd labeled cells in the PALS with little incorporation in follicles. By day 10, proliferating cells are present both in the PALS and lymphoid follicles; GCs may be identified by their particularly intense labeling at later times

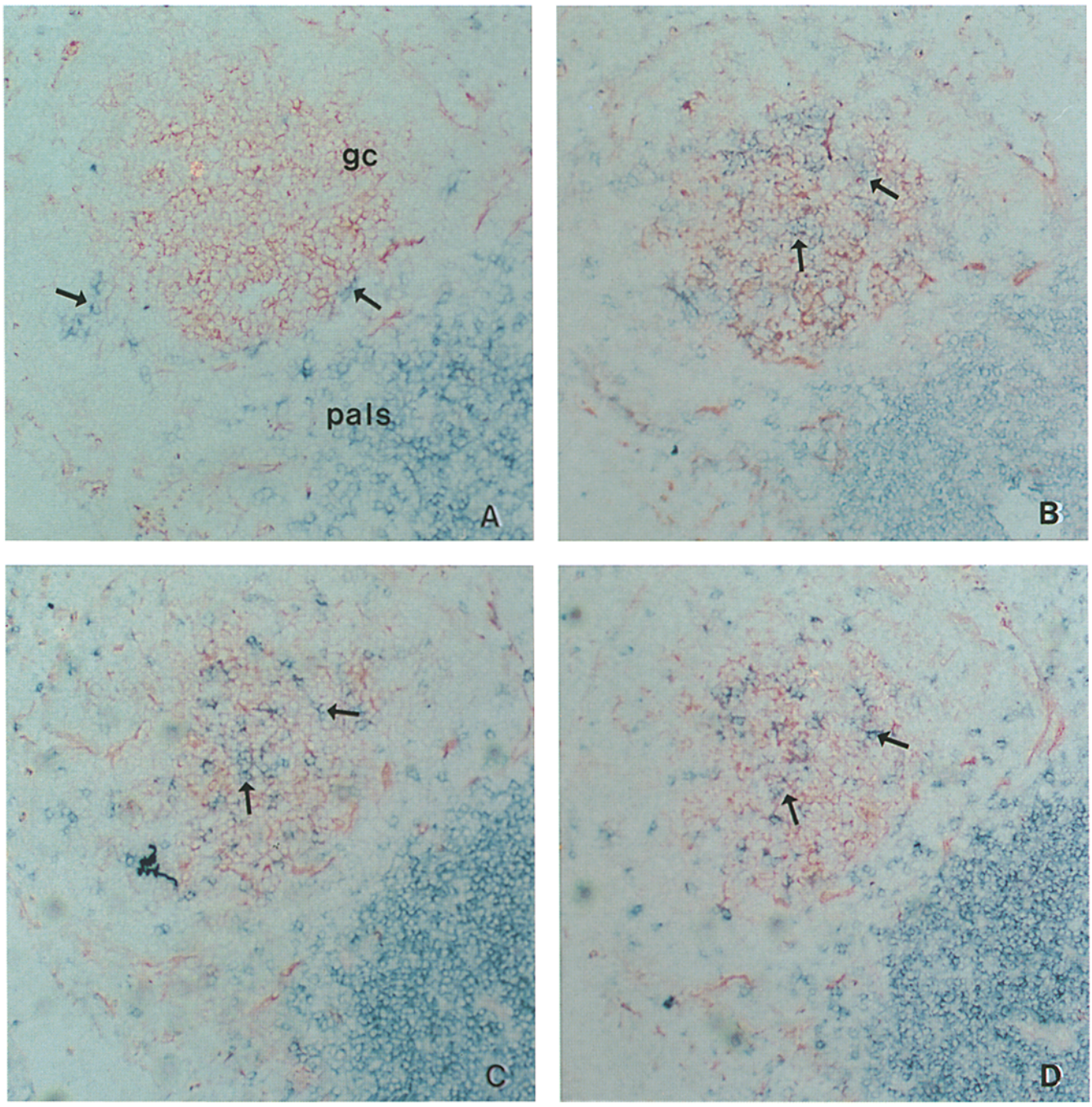

Figure 1. Phenotype of T cells in germinal centers induced with NP-PCC. Immunization and immunohistology are described in Materials and Methods. Four serial sections were stained with PNA-HRP conjugate and biotinylated anti-Thy-1.2 (*A*), or anti-CD4 (*B*), or anti-αβ TCR (*C*), or anti-CD3 (*D*). Germinal centers (*gc*) are visualized as red areas, and T cells within periarteriolar lymphoid sheath (*pals*) are stained blue. Arrows indicate Thy-1.2$^+$, CD4$^+$, αβ TCR$^+$, or CD3$^+$ T cells in the GC or follicle. 200×.

postimmunization (data not shown). By day 16 postimmunization, 40–60% of GC T cells carried Vα11$^+$Vβ3$^+$ TCR, implying specificity for PCC (26).

As shown in Table 1, at day 10–16 of the primary response to NP-PCC, virtually all CD3$^+$ cells in the follicles and GCs bear αβ TCR and express CD4. Although the PALS contains substantial numbers of CD3$^+$ lymphocytes that express the CD8 coreceptor or γδ TCR (Table 1), these cells do not enter the follicular spaces after immunization with NP-PCC, suggesting that only conventional CD4$^+$ helper T cells normally enter primary GCs. GC T cells, like GC B cells, do not express detectable levels of the anti-apoptotic protein, Bcl-2 (not shown). However, to our surprise, >95% of CD4$^+$ lymphocytes in the GCs did not express detectable levels of Thy-1 (Fig. 1 *A*) even though they bore αβ TCR and CD3 (Fig. 1, *C* and *D*). This Thy-1$^-$ T cell population was not a limited consequence of the response of B10.A mice to PCC, as C57BL/6

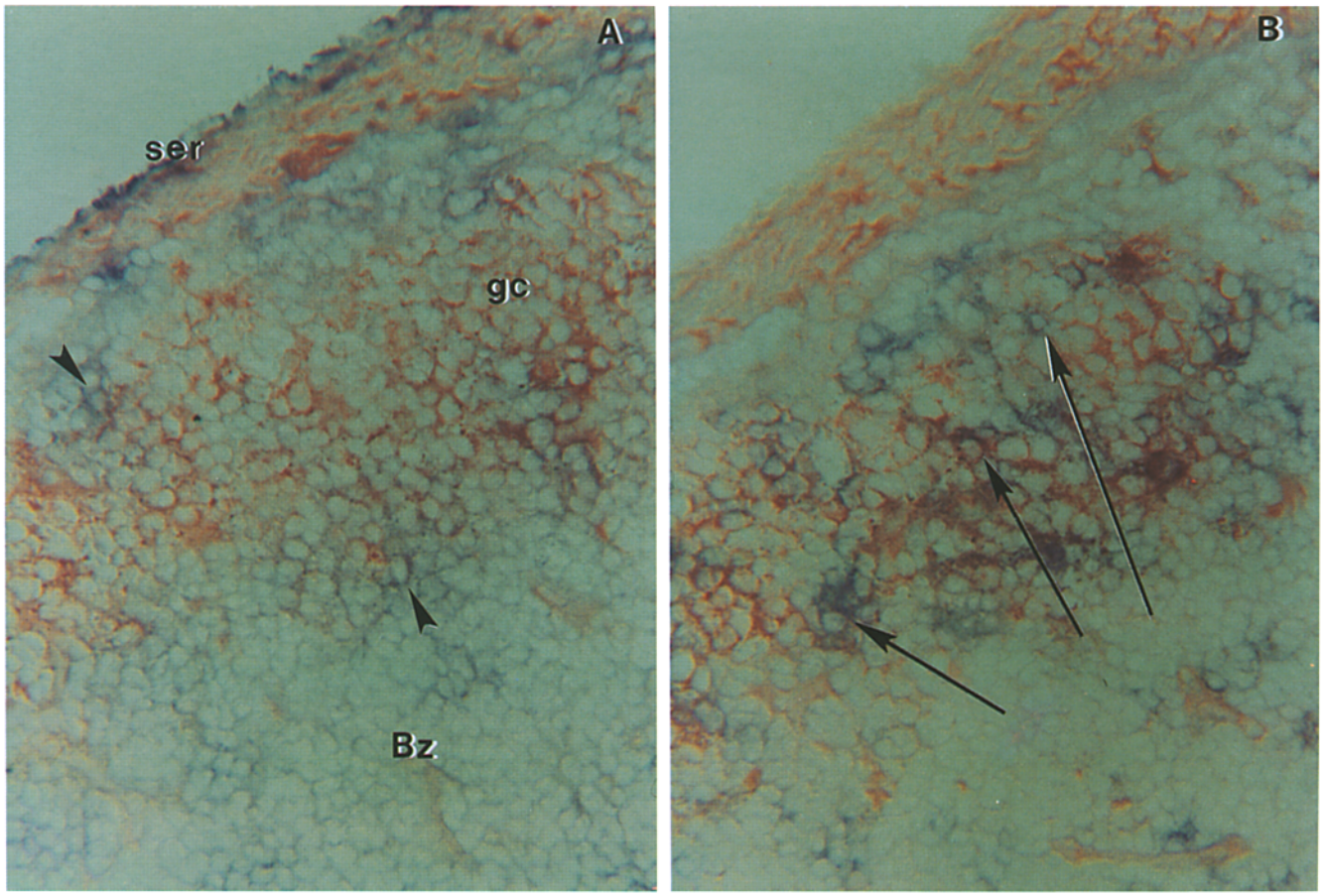

Figure 2. Phenotypes of T cells in PPs. Ileal PPs were taken from normal B10.A mice and immunohistology was carried out as described in Materials and Methods. Two adjacent sections were stained with PNA-HRP and anti-Thy-1.2 (*A*) or anti-CD4 (*B*). PNA^+ GC cells are stained red and Thy-1.2^+ or $CD4^+$ cells are blue. Thy-1.2^+ T cells (*A*, *arrowheads*) in the B cell zone (*Bz*) can only be found at the margins of the germinal center (*gc*) while $CD4^+$ cells (*B*, *arrows*) are frequent within the GC. The serosal surface (*ser*) of the gut is indicated. 200×.

and C.B-17 mice immunized with NP-CGG also developed GCs that contained Thy-1^- T cells. Indeed, the ileal PPs of unimmunized B10.A (Fig. 2) and C57BL/6 mice contained prominent PNA^+GCs that held large numbers of $CD4^+$, Thy-1^- T cells. Nor was the Thy-1^- phenotype dependent upon the antibody reagents used to detect the Thy-1 antigen; histologic analyses with two independent Thy-1-specific antibodies, 30-H12 and 53-2.1 yielded identical results (data not shown).

As the Thy-1 molecule is anchored to the cell surface via

Table 1. *Phenotypes of Germinal Center T Cells*

	$CD3^+$	$CD4^+$	$CD8^+$	$\alpha\beta^+$	$\gamma\delta^+$	Thy-1^+
Total no. cells	1163	1125	20	1130	0	41
Cells/GC						
X ± SD	117 ± 76	113 ± 80	2 ± 2	113 ± 79	0 ± 0	4 ± 6
Range	52–291	38–303	0–7	37–302	–	0–18
% $CD3^+$						
X ± SD	–	94 ± 6	2 ± 2	95 ± 9	0 ± 0	3 ± 3
Range	–	81–104	0–5	83–110	–	0–6

Cells were enumerated in serial, triple-stained histologic sections through 10 germinal centers (GCs) at day 12 postimmunization. Antibodies used to identify specific T cell markers were: CD3, 145-2C11; CD4, GK1.5; CD8, 53-6.7; αβ TCR, H57-597; γδ TCR, GL-3; and Thy-1-2, 30-H12. Phenotypes of $CD3^+$ cells in the PALS on the same sections were as follows: $CD4^+$ ≈63%; $CD8^+$ ≈30%; $\alpha\beta^+$ ≈98%; $\gamma\delta^+$≈2%; Thy-1.2^+ ≈98%.

	Vα11			N		Jα84			White Pulps		
	A	A	E			T	S	S			
	GCT	GCT	GAG			ACT	TCA	AGT	PALS	GC	CDR3 (aa)
1.	---	---	---			---	---	---	7	1,2,6	8
2.	---	---	--A			---	---	---	3,5	1,3,4,5	8
3.	---	---	---	GCA		---	---	---	1,3,6	1,4,5	9
4.	---	---	-CA			---	---	---	n.f.	6,7	8
5.	TG-	--C	-CA			---	---	---	n.f.	1	8
6.	---	---	--A	GCA		---	---	---	n.f.	4	9
7.	CG-	--C	-CC	GCA		---	---	---	1	n.f.	9
8.	---	---	---	GCC	CTC	---	---	---	6	n.f.	10
9.	---	---	--C	T		del.	---	---	7	1	-
10.	---	---	-G-	GCG	GG	del.	del.	G--	1	1,3,5	-
11.	---	---	---	C		---	---	---	1	n.f.	-
12.	---	--G	-CC	A		---	---	---	3	n.f.	-
13.	---	--G	-GC	A		---	---	---	5	n.f.	-
14.	---	---	ACC	A		---	---	---	7	n.f.	-
15.	---	---	---	GT		---	---	---	5	n.f.	-
16.	---	---	---	GAG	G	G--	---	---	4	n.f.	-

Figure 3. Junctional sequences of unique Vα11-Jα84 rearrangements recovered from the spleen of a B10.A mouse 12 d after immunization with NP-PCC. DNA was recovered from cells isolated from individual GCs or PALS from two adjacent histologic sections. PCR amplification for Vα11-Jα84 was carried out as described in Materials and Methods. The underlined germline sequence and the one-letter code designating the translated amino acid are shown on the top. Sequences are organized as productive (sequences 1–8) or non-productive (9–16) rearrangements. Dashes indicate identity to the germline Vα11-Jα84 sequence and only those nucleotides that differ from the reference sequence are illustrated. Individual PALS or GCs have been numbered for identification and the predicted lengths of the CDR3 loops encoded by each productive VJ joint is given. Deletions (*del.*) and not found (*n.f.*) are indicated where appropriate. The numbering of PALS and GCs is arbitrary and does not refer to the PALS and GCs in Fig. 4. These sequence data are available from GenBank under accession numbers U63758-U63773.

a glucose phosphatidyl-inositol (GPI) bridge (28), downregulation of Thy-1 on activated, GC T cells could be non-specific, the result of generalized GPI hydrolysis mediated by phospholipase C (29). To determine if the expression of other GPI-anchored proteins was also diminished on GC T cells, biotinylated antibodies specific for Ly-6 (30) and CD48 (31), were used to label sections of immune B10.A spleens. Of these three GPI-anchored proteins, only Thy-1 was downregulated in GCs (data not shown).

In human GCs, T helper cells express the NK cell marker, CD57 (22). To see if murine GC T cells expressed a similar marker, NK-1.1, we stained immunized spleen sections with antibody specific for the NK-1.1 antigen which is encoded by a member of the mouse NKR-P1 gene family and expressed on NK cells of C57BL/6 mice (32). However, 12–16 d postimmunization with NP-CGG, the GC T cells of C57BL/6 mice were NK-1.1$^-$.

Antigen-activated T Cells in the PALS and GC Share Common Clonal Origins. Lymphocytes descended from a common precursor share V(D)J joint sequences that are often sufficiently complex to serve as unique identifiers of clonality (33). Shared V(D)J joint, or CDR3, sequences between PALS-associated plasmacytes and nearby GC B cells have been used to show that these distinct populations share common founders (14). We exploited the CDR3 diversity of TCR α- and β-chain rearrangements to identify the clonal relatedness of Vα11$^+$Vβ3$^+$ T cells in the PALS and GCs of B10.A mice immunized with NP-PCC. Fig. 3 shows the joint sequences of Vα11-Jα84 DNA rearrangements recovered by microdissection at day 12 postimmunization. 6 white pulp areas comprising 7 GCs and 6 PALS distributed over half of the splenic length were sampled and the endogenous rearrangements amplified and sequenced. 16 unique VJ junctions were recovered, 13 from PALS and 8 from GCs; 8 sequences represented productive (P), in-frame joints (Fig. 3, sequences 1–8). Identical junctions were frequently recovered from GCs in widely separated regions of white pulp, suggesting extensive migration by antigen-activated T cells. In contrast, only PALS 3 contained distinctive VJ joints present in other PALS. Most VJ junctions recovered from GCs were also present in PALS (Fig. 3). This distribution of joint sequences is consistent with the independent activation and early proliferation of PCC-specific T cells in the PALS followed by their migration to multiple follicular sites. The similar distribution of one non-productive (nP) rearrangement (Fig. 3, sequence 10), presumably carried as an unselected, passenger gene, militates against the possibility that shared CDR3 sequences reflect instead, independent expansion of unrelated PCC-specific clones.

As expected (10–13), VJ joint sequences recovered from GCs provide evidence for antigen-driven selection. The ratio of P/nP joints recovered from PALS was 1 (8:8), indicating that only 6–8% of PALS T cells bore Vα11$^+$ TCR while the P/nP ratio in GCs was 3.5 (14:4) suggesting that ≥25% of GC T cells expressed Vα11 rearrangements (13). This analysis conservatively estimates selection since it gives equal weight to rare and frequent junctions. By histology, ≈50% of CD4$^+$ GC T cells expressed Vα11$^+$Vβ3$^+$ TCR at day 12 of the response and the joints most frequently recovered from GCs (Fig. 3) encode 8 or 9 residue CDR3 loops containing ETSS or EATSS motifs present in PCC-specific T cell lines and hybridomas (26, 27, 34). Indeed, in primary GCs the frequency of Vα11 rearrangements compatible with PCC-specific TCR is greater than that seen in activated Vα11$^+$Vβ3$^+$ T cell populations recovered by flow cytometry from lymph nodes (35).

This high degree of specificity was also evident when single GC T cells were isolated and amplified for both Vα11/Jα84 and Vβ3/Jβ1.2 rearrangements by multiplex

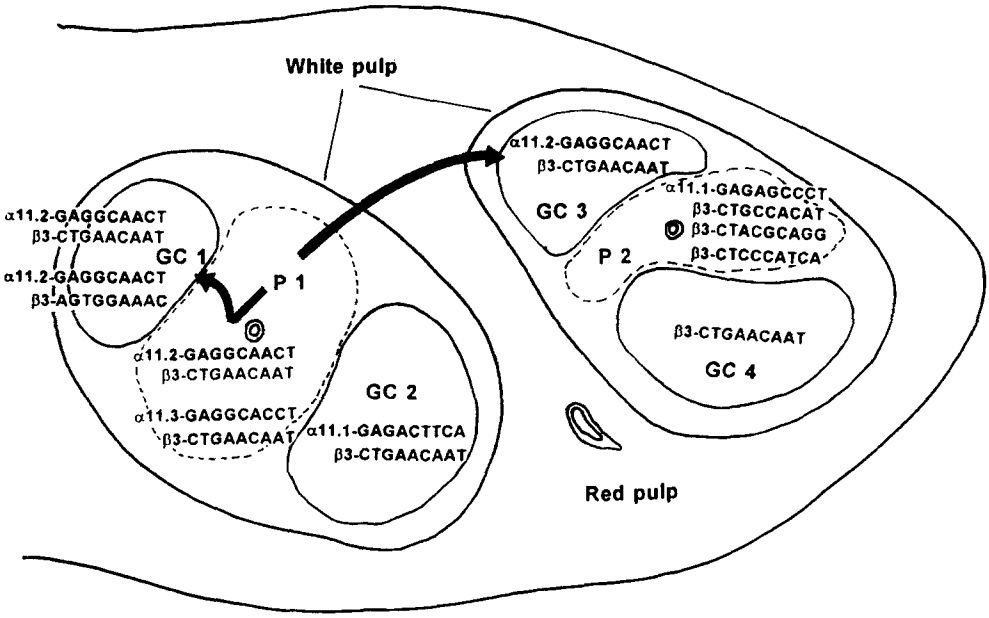

Figure 4. Recovery of identical TCR αβ rearrangements from two white pulp areas. DNA was recovered from regions that included two PALS (*P1* and *P2*) and four GCs (*GC1–GC4*). This numbering is arbitrary and does not refer to the PALS and GCs in Fig. 3. Multiplex PCR for both Vα11-Jα84 and Vβ3-Jβ1.2 rearrangements revealed several TCR pairings; the identified junctional sequences are illustrated at the sites of their recovery. Arrows indicate the inferred migration of a Vα11-Jα84/Vβ3-Jβ1.2 clone (Vα11.2-GAGGCAACT; Vβ3-CTGAACAAT) from P1 to the associated GC1 and the distal GC3. These sequence data are available from GenBank under accession numbers U63774-U63782.

PCR. More than 80% (5/6) of single T cells from which a Vα11/Jα84 rearrangement was recovered were also positive for rearranged Vβ3/Jβ1.2 gene segments, while in the PALS only 20% (1/5) of T cells positive for Vα11/Jα84 rearrangements also had Vβ3/Jβ1.2 (data not shown). Fig. 4 illustrates the paired recovery of identical Vβ3 and Vα11.2 V(D)J rearrangements (Vα11.2- GAG GCA ACT; Vβ3-CTG AAC AAT) by multiplex PCR from two separate white pulp areas. Both sequences encode V(D)J motifs present in PCC-specific T cells and were jointly recovered from a single PALS and two GCs. This distribution is consistent with the antigen-specific expansion in PALS 1 of a single T cell carrying these rearrangements and the subsequent migration of its daughter cells to colonize GC 1 and GC 3 (Fig. 4).

Discussion

Helper T cells are specialized for collaborative interactions with other immunocytes. Most bear αβ TCR and express CD4 on their surface. Distinctive patterns of secreted cytokines further define the helper class; type-1 helper T cells secrete IL-2, TNF-β, and IFN-γ and are believed to dominate T-T collaboration in inflammatory responses while type-2 cells produce IL-4, -5, -6, and -10 and are associated with T-B interactions leading to prominent antibody responses (36–38).

The GC reaction depends upon T cell help (18–20), although surprisingly, fewer T cells are required to support histologically typical GCs than that necessary for primary serum antibody responses (20). T-B collaboration leading to the formation of GCs requires costimulatory signals and occurs in two phases, an early phase in the outer PALS and a late phase within the GC itself (8, 9, 18). Disruption of late phase collaborations by a variety of means abrogates the GC reaction and later anamnestic antibody responses but permits a transient primary antibody response and normal T cell priming (18, 19). Thus at least for relatively short periods (30–90 d postimmunization), T cell priming and memory are independent of the GC reaction and entry of antigen-specific B cells into the memory compartment. Indeed, much, if not all, antigen-driven T cell proliferation and differentiation in vivo can occur in the absence of B lymphocytes altogether (39–40).

Here we have shown that the great majority of T cells in GCs possess a unique αβ TCR^+, $CD4^+$, Thy-1^- phenotype. To our surprise, this phenotype had been previously described as rare (≈3%) T cell subset in normal spleen that was expanded (≈40% of $CD4^+$ cells) after infection with the LP-BM5 murine leukemia virus (MuLV)(41) and as a significant minority (≈17%) of $CD4^+$cells in PPs (42). Significantly, retroviral infections induce large, chronic GCs in spleen (43) and each PP generally contains 1-2 GCs maintained by exposure to food and environmental antigens, and the gut flora (44). We have further demonstrated that the origin of Thy-1^- GC T cells is the Thy-1^+ T cell population in the PALS: antigen-induced T cell proliferation first occurs in the PALS and the later emigration of this population results in the frequent recovery of identical TCR α- and β-chain rearrangements from multiple GCs. Thus, αβ TCR^+, $CD4^+$, and Thy-1^- cells do not represent some minor T cell lineage (41, 42) but a specific differentiation state of activated Thy-1^+ helper T cells that have entered the B cell areas of secondary lymphoid tissues. Indeed, the appearance of follicular T cells and nascent GCs coincides with that time when antigen-specific T cells return to the thoracic duct (45).

A small minority of GC T cells retain Thy-1 on their surface (Table 1). These cells may be uninvolved in the GC reaction or they could constitute a second, distinct GC

population. Nonetheless, the high frequency of GC T cells bearing antigen-specific TCR requires that most cells fall within the Thy-1$^-$ subset.

Our conclusion that shared CDR3 sequences represent cellular migration rather than convergent selection is supported by multiplex PCR analyses of Vα and Vβ rearrangements and by the PALS→GC distribution of a nP, and thus unselectable, Vα11 rearrangement (Fig. 3). This dissemination of T cells contrasts with the limited migrations of activated B cells. Ig heavy chain VDJ rearrangements are shared only between B cells in the outer PALS and adjacent GCs (8). Thus, while there is little or no B cell trafficking, T cells appear to travel from one white pulp region to others.

Other histological studies have used antibodies specific for Thy-1 to demonstrate T cells within GCs (e.g., references 10, 11). If GC T cells are Thy-1$^-$, how was this possible? Harriman et al. (42) used flow cytometry to quantitate the amount of Thy-1 present on T cells in PPs, lymph nodes, and spleen. They note that in the PP, cells that bear αβ TCR, CD3, and CD4 may completely lack Thy-1 while similar cells in other tissue were "Thy-1^{dull}". In fact, deliberate overstaining with biotinylated anti-Thy-1 antibodies does reveal increased numbers of Thy-1$^+$cells in GCs but never more than 20–30% of the αβ TCR$^+$ CD4$^+$ population (data not shown). Thus, it may be that only in chronic GCs is Thy-1 fully downregulated. Nonetheless, Thy-1$^-$ or Thy-1^{dull} GC T cells may be clearly distinguished by histology and flow cytometry, permitting this important T cell subset to be recognized and studied.

The physiological significance of diminished Thy-1 expression by GC T cells is unknown. Monoclonal antibodies to Thy-1 induce transmembrane signals leading to T cell activation (46, 47) and removal of GPI-linked molecules from the surface of CD4$^+$ T cells inhibits their responsiveness to mitogen but not anti-CD3 antibody (29). Downregulation of Thy-1 expression may represent a response to minimize non-cognate T-B interactions that could reduce the stringency of antigen-driven selection in GCs (1).

The Thy-1 T cells prevalent in the spleens of mice infected with the LP-BM5 MuLV (41) mostly fell into the fraction I subset of CD4$^+$ T cells defined by Hayakawa and Hardy (48). This subset produces IL-2 but little or no IL-4 after in vitro culture in the presence of concanavalin A. Freshly isolated CD4$^+$, Thy-1$^-$ cells from PPs could be activated by soluble but not insoluble anti-CD3, secreted more IL-2 than IL-4, and produced undetectable amounts of IL-5 (42). These observations suggest that the CD4$^+$ Thy-1$^-$ T cells in GCs may represent a specialized type-1 helper cell (36) that does not itself drive secondary antibody responses (48) but is required for generation of B cell memory (18, 19). This notion is lent support by a recent report that interferons could drive resting B cells to express a partial GC phenotype in vitro (49). GC T cells may not be significant in the antigen-driven reactivation of memory B cells and may not persist long after the GC reaction ends. Indeed, to the best of our knowledge, Thy-1 expression is undiminished in memory T cell populations, consistent with the irrelevance of the GC reaction to T cell priming and memory (18, 19, 39, 40). Given the power of high parameter flow cytometry to identify activated, antigen-specific T cells (35), it should now be possible to determine the physiology and natural history of this important helper T cell class.

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