Germinal Center Formation, Immunoglobulin Class Switching, and Autoantibody Production Driven by "Non α/β" T Cells

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Summary

The production of class-switched antibodies, particularly immunoglobulin (Ig)G1 and IgE, occurs efficiently in T cell receptor (TCR)α−/− mice that are congenitally devoid of α/β T cells. This finding runs counter to a wealth of data indicating that IgG1 and IgE synthesis are largely dependent on the collaboration between B and α/β T cells. Furthermore, many of the antibodies synthesized in TCRα−/− mice are reactive to a similar spectrum of self-antigens as that targeted by autoantibodies characterizing human systemic lupus erythematosus (SLE). SLE, too, is most commonly regarded as an α/β T cell–mediated condition. To distinguish whether the development of autoantibodies in TCRα−/− mice is due to an intrinsic de-regulation of B cells, or to a heretofore poorly characterized collaboration between B and "non-α/β T" cells, the phenotype has been reconstituted by transfer of various populations of B and non-α/β T cells including cloned γ/δ T cells derived from TCRα−/− mice, to severe combined immunodeficient (SCID) mice. The results establish that the reproducible production of IgG1 (including autoantibodies) is a product of non-α/β T cell help that can be provided by γ/δ T cells. This type of B–T collaboration sustains the production of germinal centers, lymphoid follicles that ordinarily are anatomical signatures of α/β T–B cell collaboration. Thus, non-α/β T cell help may drive Ig synthesis and autoreactivity under various circumstances, especially in cases of α/β T cell immunodeficiency.

The production of IgG and IgE antibodies in the mouse is regarded as largely dependent on CD4+ α/β+ Th cells (1). Such T cells provide cytokines to B cells with which they enter into antigen-specific, TCR-mediated cognate interactions (2). The generation of antibodies is associated with the development of specific anatomical structures, germinal centers (GCs)[1], in which a significant fraction of Ig class switching and most somatic mutations occur (3, 4). T cells seem to be required for these processes because antibody production (particularly IgG1) (5, 6) and GC formation (7, 8) are reduced in congenital athymic nu/nu mice, but return to normal levels after adoptive transfer of T cells (8–10). Likewise, SCID mice receiving highly purified B cells failed to develop GCs, whereas GCs were inducible after transfer of B and CD4+ T cells (11).

Despite these data, several observations raise questions about the T cell dependence of GC formation. In particular, whereas GC formation is clearly less efficient in nu/nu mice, it nonetheless occurs (7, 8, 10, 11), and in some colonies of nu/nu mice, IgG synthesis can be comparable to normal (12). This may be because the nu/nu mutation (13) is pleiotropic, with variable effects on α/β T cells and γ/δ T cells. Thus, whereas it is clear that CD4+ α/β T cells enhance GC formation, it is unclear whether GC development in the nu/nu mouse is wholly supported by the small number of CD4+ α/β T cells that "leak through" (11), or by other T cells.

Germane to this issue are recent data showing that TCRα−/− mice, congenitally devoid of α/β T cells, commonly display serum IgG and IgE levels comparable to or higher than those of α/β T cell–bearing littermates (14), and that among nonimmunized mice, more GCs develop than in TCRα+/− littermates (Dianda, L., A. Judge, W.

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[1]Abbreviations used in this paper: ANA, antinuclear antibody; CD40L, CD40 ligand; GC, germinal center; PNA, peanut agglutinin.

Pao, A. Hayday, I.C.M. MacLennan, and M.J. Owen, manuscript submitted for publication). These data suggest a reasonably efficient pathway of B cell maturation and Ig production that cannot involve α/β T cells, and for which a paradigm is currently lacking. Furthermore, many of the antibodies in TCRα−/− mice are reactive to self-antigens such as double stranded DNA (dsDNA) and small ribonucleoprotein particles (snRNPs), that are commonly the targets of autoantibodies in patients with SLE (16). This raises the issue of whether or not the initiation of SLE is always truly dependent on CD4$^+$ α/β$^+$ Th cells, as has most often been concluded. In fact, there are numerous reports of autoantibodies produced in α/β T cell–immunodeficient individuals, such as those with AIDS (17). The potential to develop autoantibodies in the absence of α/β T cells emphasizes the need to understand better the processes of non-α/β T cell–dependent B cell differentiation and GC formation.

To determine the mechanism of B cell maturation in the absence of α/β T cells, an initial question is whether the phenotype is B cell autonomous. For example, B cells that develop in TCRα−/− mice may lack downregulatory effects of α/β T cells, and therefore may be intrinsically hyperactive. This concept is analogous to that invoked for cases of human common variable immunodeficiency, in which B cell activation is enhanced by depletion of CD8$^+$ α/β T cells (1). Such a scenario may also explain the predisposition to SLE-like autoimmunity shown by NZBxNZW.F$_1$ mice, since aspects of that disease can be reconstituted in SCID mice by the adoptive transfer of B cells alone (18). Alternatively, the TCRα−/− mouse may constitute a prime example of T cell help provided by "non-α/β T cells." Suggestive of this, IL-4 production was detected in supernatants of T cells from TCRα−/− mice, and coculture of such T cells in the presence of pokeweed mitogen (which enhances B–T interactions) strongly stimulated B cell proliferation (14). However, these experiments did not determine the precise cellular requirements for the "TCRα−/− phenotype," collectively, the α/β T cell–independent production of class-switched Igs (commonly autoantibodies), and the formation of GCs.

Thus, to define the cells required for generation of the TCRα−/− phenotype, an adoptive transfer approach has been employed, whereby various combinations of B cells and TCRα−/−–derived non-α/β T cells, including cloned γ/δ T cells, have been transferred to lymphocyte-deficient SCID mice. Those studies, described here, indicate that B cell maturation characteristic of TCRα−/− mice can reproducibly be established and maintained outside of the TCRα−/− animal, but that it requires B cells plus non-α/β T cells. From the data presented, it is possible to conclude that non-α/β T cells, including γ/δ T cells, can help B cells, a finding that may be highly relevant to instances of autoimmunity, especially in cases of α/β T cell immunodeficiency. The potential differences between help provided by α/β T cells and non-α/β T cells, and the possible relevance of this to normal animal physiology, are discussed.

Materials and Methods

Mice. All mice used in this study were bred and maintained in our specific pathogen-free animal facilities at Yale University. TCRα−/− mice were generated by gene targeting (19). CB17.SCID, C57BL/6.SCID, NOD.SCID, TCRβ−/− and TCRδ−/− mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME); TCR(β×δ)−/− mice were generated locally. TCRα−/− mice used for transfers were backcrossed either five generations to BALB/c (H-2^d) or two generations to 129 (H-2^b), and selected on the basis of homozygosity at the MHC.

mAbs. The following mAbs were purchased from PharMingen (San Diego, CA): PE-conjugated anti-CD3 (2C11), anti-TCR-γ/δ (GL3), anti-CD8 (53-6.7); FITC-conjugated anti-TCRα/β (H57), anti-CD4 (RM4-5), and biotin-conjugated anti-CD90 (anti-Thy1.2; 53-2.1), anti-CD45R (B220, RA3-6B2), and anti-CD40 ligand (CD40L, MR1). Hybridoma culture supernatants were either maintained in this laboratory (2C11, H57, and GL3) or kindly provided by Dr. C. Janeway, Jr., Howard Hughes Medical Institute, Yale University (2.4G2, anti–Fc receptor; 212A.1, anti-I-A$^{b/d}$).

Depletion of T Cells. Total splenocytes from TCRα−/− mice were largely depleted of T cells by negative selection using magnetic beads. Splenocytes were incubated with mAbs 2C11, H57, and GL3 (all hamster IgG) for 30 min on ice. After washing, the cells were incubated with goat anti–hamster IgG-conjugated magnetic beads (Advanced Magnetics, Inc., Cambridge, MA) for 1 h on ice with gentle agitation. T-depleted splenocytes were then separated using a magnetic plate (two cycles). All mAbs used were supernatants that had been previously titered. T cell depletion was evaluated by FACS® analysis (Becton Dickinson & Co., Mountain View, CA).

Cell Staining and FACS® Analysis. Single cell suspensions (10^6 cells/ml) were incubated with PE- or FITC-conjugated antibodies at pretitered dilutions on ice for 30 min, followed by washing three times with PBS plus 1% FCS and 0.02% sodium azide. When biotin-conjugated mAbs were used, cells were incubated with fluorescence-conjugated streptavidin. Stained cells were fixed in PBS containing 1% paraformaldehyde and analyzed on a FACScan® (Becton Dickinson & Co.). Dead and nonlymphoid cells were excluded from analysis by selective gating based on forward and side scatter.

Establishment of γ/δ T Cell Clones. The detailed isolation and characterization of γ/δ T cell clones will be described elsewhere (Wen, L. et al., manuscript in preparation). In brief, splenocytes (2 × 10^6) from TCRα−/− mice were cultured in Click's medium containing 5 U/ml IL-2 (supernatant of EL4), 5% heat-inactivated FCS (Hyclone Laboratories, Inc., Logan, UT), and antibiotics (GIBCO BRL, Gaithersburg, MD). Clone G5 was derived from limiting dilution performed in 96-well microculture plates at a concentration of <1 cell/well. For maximum expression of CD40L, cloned γ/δ T cells were activated by PMA (10 ng/ml) and ionomycin (1 μM) (GIBCO BRL) for 6 h. Cytokine mRNA profiles of the γ/δ T cell clone were determined by reverse transcription (RT)-PCR (20).

Adoptive Cell Transfer and Cell Tracing. 15 × 10^6 splenocytes from TCRα−/− mice (3–5 mo-old) were injected intravenously into CB17.SCID, C57BL/6.SCID, and NOD.SCID mice (5–8-wk-old). A T-depleted cell population (10 × 10^6) derived from spleens of TCRα−/− mice was also used in the transfer experiments. To investigate the kinetics of GC formation, SCID mice reconstituted with splenocytes from TCRα−/− mice were killed at different time points after reconstitution. Direct evidence of re-

constitution was obtained by labeling the cells with the fluorescent dye DiI (Molecular Probes, Inc., Eugene, OR), at 37°C for 30 min before reconstitution. Transferred cells that labeled with the dye were easily observed on frozen sections by fluorescence microscopy. To investigate the role of γ/δ T cells, reconstitution was also performed using 5×10^6 cloned γ/δ T cells (see above) together with 10×10^6 splenocytes derived from TCR(β×δ)−/− mice (8–10-wk-old) as a source of B cells that lacks any type of T cell.

Quantitation of Ig Levels. SCID mice were bled and individual serum samples collected every 2 wk after reconstitution. Total levels of serum IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were determined by ELISA. Briefly, 96-well ELISA plates (Falcon) were coated with goat anti–mouse Ig (H+L chain) (Southern Biotechnology Associates Inc., Birmingham, AL) overnight at 4°C (5 μg/ml) in sodium carbonate buffer (pH 9.6). After three washes in PBS containing 0.1% Tween 20 (washing buffer), the plates were blocked with 1% BSA in PBS (blocking buffer) for 1 h at 37°C. Serum samples diluted in blocking buffer were added and incubated for 2 h at 37°C. After three washes with washing buffer, the plates were then incubated for 2 h with alkaline phosphatase (AP)–labeled goat anti–mouse Ig isotype antibodies (Southern Biotechnology Associates Inc.) diluted 1:500 in blocking buffer. After washing, the plates were developed with p-nitrophenyl phosphate (1 mg/ml) (Sigma Chemical Co., St. Louis, MO) as substrate. The reaction was stopped by 1 N NaOH. Plates were read on a microplate reader (Titertek) at OD 405.

Detection of Autoantibodies. Antinuclear (ANA) and anti-DNA (dsDNA) antibodies were detected by indirect immunofluorescence using HEp-2 cells (Quidel, San Diego, CA) and *Crithidia luciliae* (Chemicon, Temecula, CA) as antigens, respectively, and FITC-conjugated goat anti–mouse IgG (Sigma Chemical Co.). Anti-snRNP antibodies were detected by ELISA (21).

Detection of GCs. GC formation was examined in the spleens of TCRα−/− mice and reconstituted SCID mice using immunohistochemistry, as reported (22). Briefly, 6-μm-thick frozen spleen sections were rehydrated in PBS followed by treatment in PBS containing 0.15% H_2O_2 to inhibit endogenous peroxidase activity. The sections were then blocked with rat IgG (100 μg/ml) for 30 min to prevent nonspecific binding. T and B lymphocytes in GCs were identified by biotin-labeled mAb to CD90 (Thy1.2, 53-2.1) and CD45R (B220, RA3-6B2), respectively (PharMingen) followed by Streptavidin-conjugated with AP (Southern Biotechnology Associates Inc.). Sections were then incubated with substrate Napthol AS-MX phosphate/Fast blue BB base (Sigma Chemical Co.). GC B cells, characterized by binding of peanut agglutinin (PNA), were identified with PNA conjugated to horseradish peroxidase (E-Y. Laboratories, Inc., San Mateo, CA). Staining was visualized using the substrate 3-amino-9-ethyl-carbazole (Sigma Chemical Co.).

Histological Examination. Livers, kidneys, intestines, and lungs from reconstituted SCID mice were fixed in 10% buffered formalin, paraffin-embedded, and stained with hematoxylin and eosin. The sections were examined microscopically for lymphocyte infiltration to evaluate the presence of GVHD.

Results

Phenotypic Characterization of TCRα−/− Splenocytes Used for Reconstitution. Three scenarios could explain the basis for B cell maturation, Ig class switching, and autoreactivity seen in the absence of α/β T cells (the TCRα−/− pheno-

type): (*a*) the TCRα−/− phenotype was an autonomous property of TCRα−/− B cells that could be transferred to another animal using TCRα−/− B cells alone; (*b*) it was a property of cell–cell interactions in TCRα−/− mice that could be transferred to another animal using TCRα−/− splenocytes; and (*c*) it was the product of complex lymphoid–stromal interactions unique to the TCRα−/− mouse that could not be transferred to another animal. To distinguish among these possibilities, TCRα−/− splenocytes were adoptively transferred to SCID mice that were subsequently evaluated for Ig class switching, autoantibody production, and GC formation. For comparison, we examined SCID mice that were either mock reconstituted or reconstituted with T-depleted TCRα−/− splenocytes.

TCRα−/− splenocytes used for transfer were ∼15% CD3+ and >60% B220+ (Fig. 1 *a*), in general agreement with previous data (19, 23). A small subset of cells were B220+ and CD3+, also discussed recently (24). SCID mice analyzed 6 wk after engraftment with TCRα−/− splenocytes displayed a FACS® profile that was almost indistinguishable from the donor inoculum (Fig. 1 *b*). By contrast, essentially no CD3+ or B220+ cells were detected in the spleens of SCID mice mock reconstituted with PBS alone (Fig. 1 *c*). SCID mice that received T-depleted TCRα−/− splenocytes contained essentially no CD3+ cells; the stably engrafted population was ∼100% B220+ (Fig. 1 *d*).

No evidence of overt GVHD was found after comprehensive pathological investigation of several organs in the adoptively transferred mice. Other signs of GVHD, such as
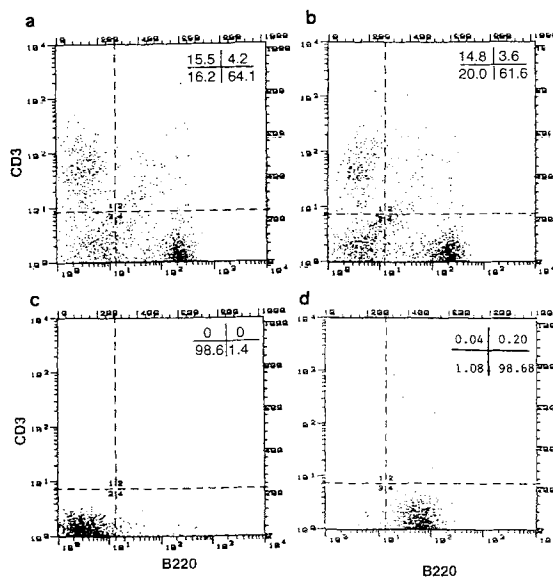

Figure 1. T (*CD3+*) and B (*B220+*) profiles of splenocytes from TCRα−/− mice and SCID mice, 6 wk after reconstitution with cells from TCRα−/− mice. (*a*) TCRα−/− splenocytes used for adoptive transfer; (*b*) spleen cells from SCID mouse reconstituted with TCRα−/− splenocytes; (*c*) spleen cells from SCID mouse reconstituted with PBS; and (*d*) spleen cells from SCID mouse reconstituted with TCRα−/− splenocytes predepleted of T cells. Y-axis, anti-CD3; x-axis, anti-B220. Percentages of cells in each quadrant are indicated by the numbers in the top corner of each panel.

diarrhea and skin lesions, were also not evident (data not shown). Since GVHD usually involves host B cells, which are absent in SCID recipient mice, this finding is not surprising. This issue is discussed further below.

Reconstitution of Ig Production in SCID Mice. Reconstituted SCID mice were assessed for serum Ig production. Circulating levels of Ig of all isotypes were measured every 2 wk after reconstitution until the experiments were terminated (usually at 20 wk after transfer). Igs of all isotypes examined could be readily detected in CB17.SCID mice (H-2^d) within 2 wk after reconstitution with TCRα−/− lymphocytes (H-2^b) (Fig. 2 A). Similarly, Igs of all isotypes examined were detectable in NOD.SCID mice (K^{d}D^{b}I-A^{g7}) reconstituted with TCRα−/− lymphocytes (H-2^b) (Table 1). To examine whether Ig production depended on mismatch of donor and recipient MHC, C57BL/6.SCID (H-2^b) and CB17.SCID (H-2^d) mice were each used as recipients

for splenocytes from H-2^b and H-2^d TCRα−/− donors, respectively. In each case, class-switched antibody production was observed (Fig. 2, B and C), although the levels were usually lower than in MHC-mismatched reconstitutions (Table 2 and discussed below).

By contrast, four of five mice that received T-depleted donor splenocytes produced exclusively IgM. When class-switched Ig levels were converted to micrograms per milliliter, the results (Table 2) confirmed that mice receiving TCRα−/− splenocytes invariably supported the synthesis of class-switched Ig, whereas this only occurred in one of five mice receiving T-depleted cells. As expected, no Ig was detected in sera from SCID mice mock reconstituted with PBS (Table 1).

Reconstitution of Spontaneous Autoantibody Production in SCID Mice. Ig production in TCRα−/− mice is commonly characterized by autoreactivity (14). The level of autoanti-
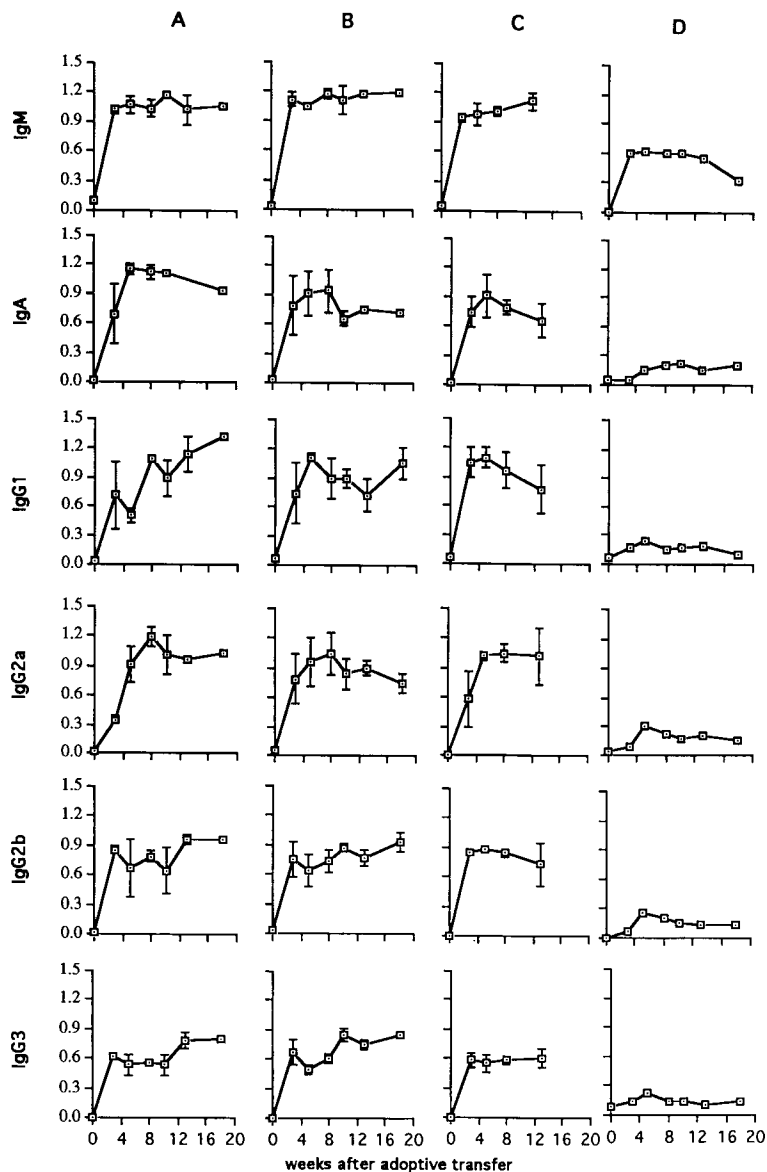

Figure 2. Ig production, detected by ELISA in the sera (diluted 1:100) of SCID mice reconstituted with TCRα−/− splenocytes (A–C), and in SCID mice reconstituted with TCRα−/− splenocytes predepleted of T cells (D), detected at 2, 5, 8, 10, 13, and 18 wk after transfer.

Table 1. *Reconstitution of TCRα−/− Phenotype in SCID Mice**

	IgM	Class switching	GC	ANA
CB17.SCID (H-2^d)				
reconstituted with:				
TCRα−/− splenocytes (H-2^b)	10/10[‡]	10/10[‡]	10/10[§]	5/10[∥]
B cells (H-2^b)	2/2	0/2	0/2	1/2
TCRα−/− splenocytes (H-2^d)	4/4	4/4	4/4	3/4
PBS	0/2	0/2	0/2	0/2
B6.SCID (H-2^b)				
reconstituted with:				
TCRα−/− splenocytes (H-2^b)	6/6	6/6	6/6	6/6
PBS	0/2	0/2	0/2	0/2
NOD.SCID (H-2^{g7})[¶]				
reconstituted with:				
TCRα−/− splenocytes (H-2^b)	6/6	6/6	4/6	ND
B cells (H-2^b)	3/3	1/3	0/3	ND
PBS	0/3	0/3	0/3	ND

*SCID mice (5–8 wk of age) on different backgrounds were reconstituted with different lymphocytes populations from TCRα−/− mice.
[‡]Serum IgM and class-switched Igs were detected by ELISA.
[§]GCs were detected on sections of spleens from the various SCID recipients.
[∥]ANA autoantibodies were detected using HEp-2 cells as substrate.
[¶]H-2 haplotype of NOD mice is K^{d}D^b/I-A^{g7}.

bodies was therefore examined in the sera of reconstituted SCID mice. 70% (14/20) of SCID mice reconstituted with total splenocytes produced IgG ANA (Table 1). Two major ANA staining patterns were observed, homogeneous and speckled, both of which are commonly produced by autoantibodies from MRL/lpr mice (data not shown). More-

over, several TCRα−/− reconstituted SCID mice had ANA titers as significant as those of MRL/lpr mice (1:1,280–1:2,560, data not shown). Whereas total Igs could be easily detected by 2 wk post-cell transfer, ANA was not readily detectable until 3–4 wk. Again, ANA were produced in both MHC-matched and -mismatched transfers (Table 1).

Anti-dsDNA was assayed by immunofluorescence against *C. luciliae*, and anti-snRNP antibodies assayed by ELISA. 3/14 (21%) of ANA-positive SCID sera were also positive for anti-dsDNA antibodies, albeit at low titer, and anti-snRNP autoantibodies were present in 4/14 (29%) of ANA-positive SCID sera tested (data not shown). One SCID mouse reconstituted with T-depleted splenocytes also developed detectable ANA, but exclusively of IgM isotype (Table 1).

GC Formation in SCID Mice Reconstituted with TCRα−/− Cells. Although GCs are considered a hallmark of α/β T cell help (3, 4), many of them can be found in nonmanipulated TCRα−/− mice (15). GCs were also detectable in 100% of CB17.SCID mice and B6.SCID mice reconstituted with total splenocytes from TCRα−/− mice (*n* = 20), and in 67% (four of six) of NOD.SCID mice likewise reconstituted (Table 1). The GCs were easily seen in the sections of spleens (Fig. 3, *a*, *b*, *e*, and *f*): analysis by staining serial sections with PNA and B220, or PNA and anti-CD90 (anti-Thy1.2), revealed a characteristic GC pattern in which B220$^+$ cells formed a mantle zone, bordering the PNA$^+$ area (Fig. 3, *b* and *f*), whereas T cells (CD90$^+$) were offset to one side (Fig. 3, *a* and *e*), toward the central arteriole. Consistent with the TCRα−/− mutation, the T cell zones were usually less densely populated than the corresponding regions of immunized BALB/c mice or TCRα+/− littermates (data not shown). When examined blind of genotype, GCs detected in reconstituted SCID mice could not be distinguished from those in TCRα−/− mice (data not shown). By contrast, GCs were absent in SCID mice reconstituted with either PBS, or T-depleted splenocytes

Table 2. *IgG Serum Levels in SCID Mice Receiving Splenocytes from TCRα−/− Mice*

	Number of mice	IgG2a >25 μg/ml	IgG2a >50 μg/ml	IgG1 >8 μg/ml	IgG1 >30 μg/ml
CB17.SCID (H-2^d)					
reconstituted with:					
TCRα−/−: total splenocytes (H-2^b)	10	+*	+	+	+
TCRα−/−: T-depleted cells (H-2^b)	2	−	−	−	−
TCRα−/−: total splenocytes (H-2^d)	4	+	−	+	−
PBS	2	−	−	−	−
B6.SCID (H-2^b)					
reconstituted with:					
TCRα−/−: total splenocytes (H-2^b)	6	+	−	+	−
PBS	2	−	−	−	−

*(+) indicates complete concordance.

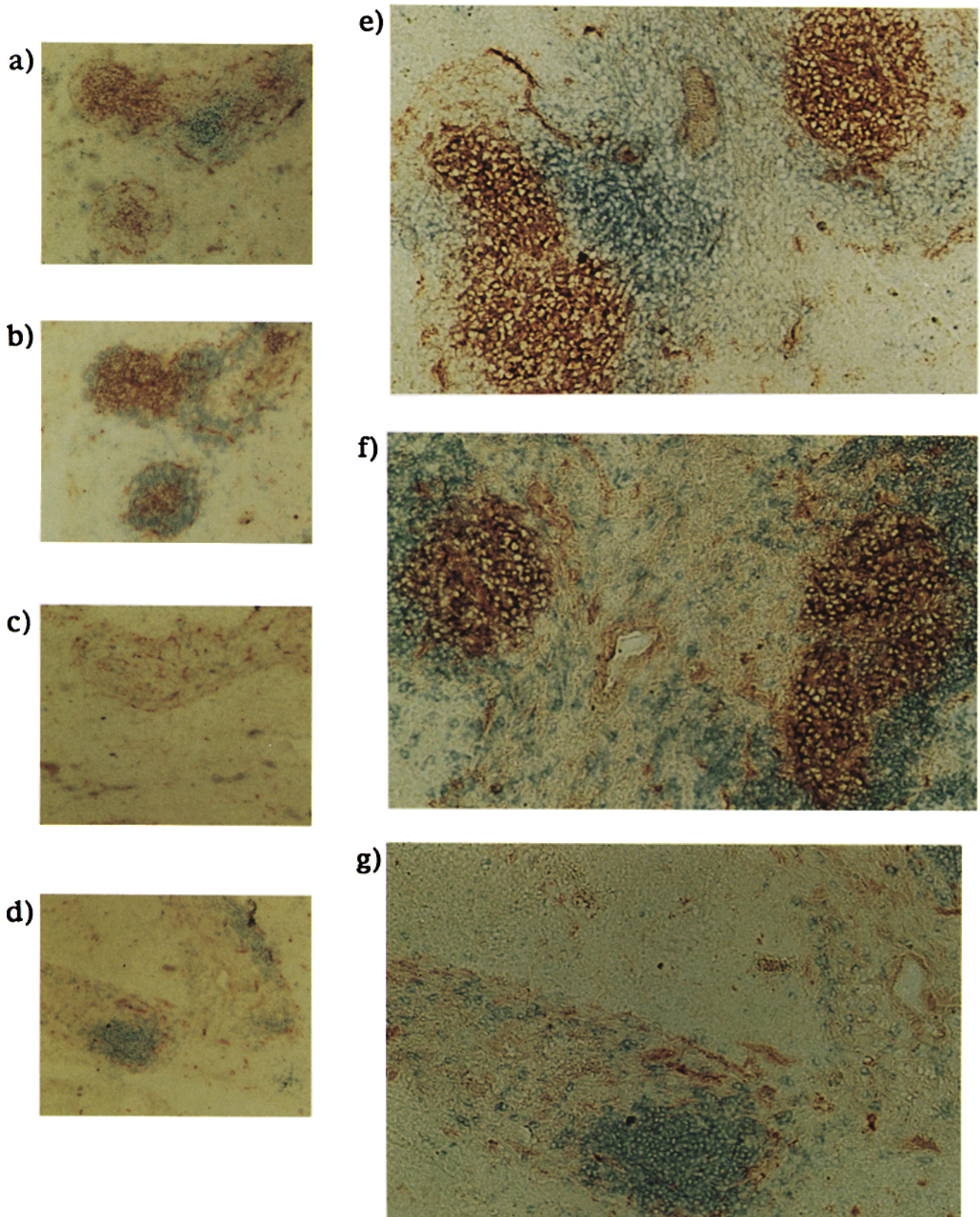**Figure 3.** GC formation in reconstituted SCID mice. GCs, detected with PNA, which stained red-brown, were found in SCID mice reconstituted with TCRα−/− splenocytes (*a, b, e,* and *f*); but not in SCID mice reconstituted with TCRα−/− splenocytes predepleted of T cells (*c, d,* and *g*). Characteristic GC patterns are shown at higher power (×100) (*e* and *f*). T cell zones around arterioles were detected in *a* and *e* with anti-Thy1.2, which stained blue. Primary follicular B cell areas were detected in *b, d, f,* and *g* with anti-B220 (*blue*). Originally: (*a–d*) ×40; (*e–g*) ×100.

(Table 1). In the latter case, areas of B220$^+$ cells could be detected in the spleens (Fig. 3, *d* and *g*), but neither PNA$^+$ areas, nor CD90$^+$ cells were ever detected (Fig. 3, *c*, *d*, and *g*). This is consistent with previous observations that B cells alone cannot support GC formation (11).

Kinetics of PNA$^+$ GC Formation. The formation of splenic GCs in conventional mice ordinarily occurs within 4–14 d after primary immunization (7, 25). After secondary immunization, it may occur as early as day 2 (7, 26). To examine the kinetics of GC formation in recipient SCID mice, animals from a group of SCID mice transferred with TCRα–/– splenocytes were killed at days 2, 6, 12, and 20 after trans-

fer. To trace the transferred cells, splenocytes were labeled with the lipophilic fluorescent dye, DiI, for 30 min before the transfer. This tracing experiment showed that reconstituted donor cells were clearly detectable as distinct anatomical aggregates by day 6 (Fig. 4 *a*). By day 12 after reconstitution, these cells were still observed, although the fluorescence was lighter (Fig. 4 *c*), probably because of decay of the dye, the death of a subset of the inoculum, and/or a reduction in cellular concentration of the dye in remaining viable cells as a result of cell division. Interestingly, although B220$^+$ aggregates were revealed at day 6 (Fig. 4 *b*), PNA$^+$ GCs were not apparent until day 12 after reconstitution (Fig. 4 *d*).
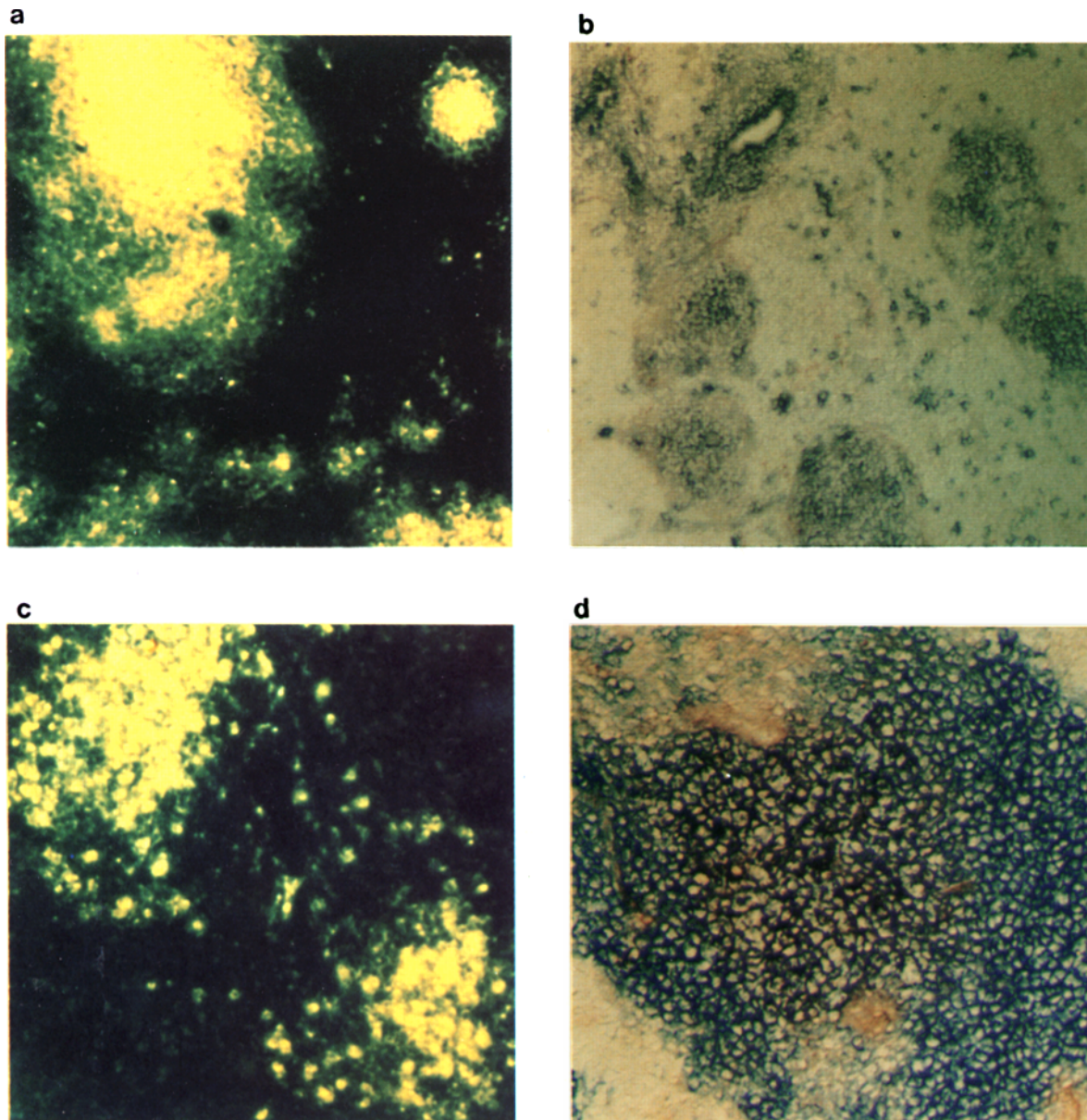

Figure 4. Kinetics of GC formation. Splenocytes from TCRα–/– mice were labeled with DiI, before transfer to SCID mice, and subsequently detected as follicular areas on day 6 (*a*) and day 12 (*c*) after transfer. GCs double stained with PNA (*red-brown*) and anti-B220 (*blue*) were absent from spleen sections on day 6 after transfer (*b*), but were notable in sections from mice on day 12 after transfer (*d*). B cells were stained with anti-B220 (*blue*) (*a–c*) ×40; (*d*) ×100.
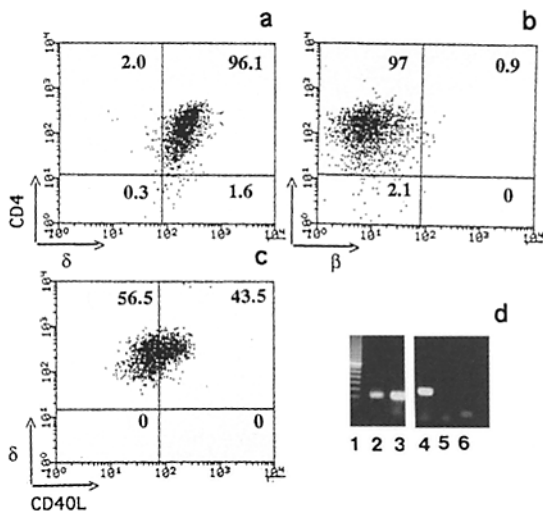
Figure 5. FACS® profiles of γ/δ T cell clone G5. Cloned γ/δ T cells (G5) were stained with PE-conjugated anti-CD4 (RM4-5) together with FITC-conjugated anti-TCRδ (GL3, a) or TCRβ (H-57, b). (c) Expression of CD40L (MR1) on clone G5 6 h after activation (10 ng/ml of PMA plus 1 μM ionomycin). Percentages of cells in each quadrant are indicated by the numbers in the top corner of each panel. The cytokine profile of G5 was assessed by RT-PCR, as shown in the ethidium bromide–stained gel (d). (Lane 1) 100-bp molecular weight marker; (lanes 2, and 3) amplification of IL-4 cDNA from G5 RNA (lane 2 is 1:2 dilution of lane 3 before loading); (lane 4) amplification of HPRT cDNA from G5 RNA; (lanes 5 and 6) attempted amplification of IFN-γ and TNFβ cDNA, respectively, from G5 RNA.

GC Formation Supported by γ/δ T Cells. TCRα−/− mice contain two types of T cell: TCRα⁻β⁺ and γ/δ T cells. Either or both of these may provide help to B cells and support GC formation. To determine which population was responsible, cloned TCRγ/δ⁺ and TCRα⁻β⁺ cell lines were derived for use in reconstitution experiments together with B cells from TCR(β×δ)−/− mice, that for genetic reasons cannot be contaminated with T cells. This approach was adopted because it proved difficult to absolutely deplete TCRα−/− splenocytes of either just γ/δ T cells or TCRα⁻β⁺ cells. Minor contamination by either population could affect interpretation of long-term adoptive transfer.

The growth of TCRα⁻β⁺ cell lines was too slow for transfer studies. However, it was possible to transfer a γ/δ T cell clone, G5 (see Materials and Methods and a detailed description of which will be provided elsewhere) (Wen, L., F.S. Wong, M.J. Owen, and A.C. Hayday, manuscript in preparation). G5 cells are TCRδ(⁺)β(⁻), CD4⁺ (Fig. 5, a and b). Almost half the cells were CD40L⁺ after treatment with PMA and ionomycin (Fig. 5 c). This result is important in assessing the capacity of G5 to support GC formation, since GC development is impaired in CD40L−/− mice (27, 28). Consistent with the phenotype of clone G5, and contrary to our previous assessment (14), we have shown that a subset of TCRα−/− γ/δ T cells, in a T cell–enriched population, stains positive for CD40L, directly ex vivo. The cytokine profile of G5 could be classified as Th2, since the cells expressed IL-4 mRNA (Fig. 5 d, lanes 2 and 3), but
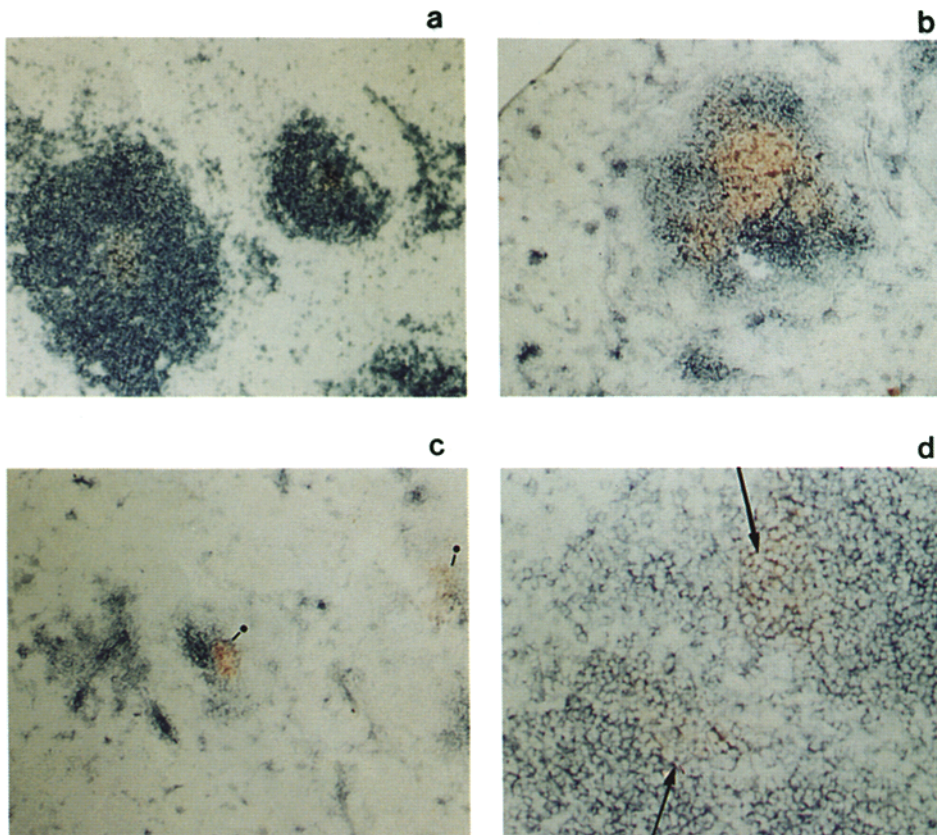

Figure 6. GC formation supported by γ/δ T cells. GCs, detected with PNA, in SCID mice reconstituted with G5 γ/δ T cells and splenic B cells derived from TCR(β×δ)−/− mice (a, c and d), and in SCID mice reconstituted with TCRα−/− splenocytes (b). (a, b, and d) Double stained for B220 (blue); (c) double stained with anti-CD90 (blue). (a–c) ×40; (d) ×100.

no mRNA for IFN-γ or TNFβ (Fig. 5 *d*, lanes *5* and *6*. Lane *4* shows amplification of the HPRT transcript cDNA, performed as a positive control for the PCR reaction). The capacity to amplify IFN-γ and TNFβ transcripts was shown by a parallel analysis of Th1 phenotype clones (data not shown).

All mice ($n = 3$) receiving G5 plus B cells from TCR ($\beta \times \delta$)$-/-$ mice examined after 6 wk scored positive for splenic GCs (Fig. 6); no GCs were detected in either mice ($n = 3$) receiving only B cells from TCR($\beta \times \delta$)$-/-$ mice or PBS ($n = 3$). The general architecture of the GCs appeared normal: PNA$^{(+)}$ clusters of cells (brown) rosetted by a mantle of B220$^{(+)}$, PNA$^{(-)}$ B cells (blue) (Fig. 6 *a*). The GCs were commonly considerably smaller than those developing in mice that received total splenocytes (Fig. 6 *b*). Reasons for this are considered in the Discussion, but one factor may be relatively poor engraftment of the γ/δ T cell clone after 6 wk. Staining for CD90 (blue, Fig. 6 *c*) detected T cells in the appropriate sites (offset to one side of the PNA$^{(+)}$ clusters (brown), denoted with a dotted line), but the numbers were small. The γ/δ T cell–supported GCs are shown at higher magnification in Fig. 6 *d*, where PNA$^{(+)}$ cells (brown, arrowed) are clearly visible surrounded by B220$^{(+)}$ cells (blue). These data provide direct evidence that GC formation can be driven by γ/δ T cells alone.

Discussion

The data presented here demonstrate a form of T cell help for B cells that does not require CD4$^+$ α/β T cells. Splenocytes from TCR$\alpha-/-$ mice, congenitally deficient in α/β T cells, sustained the production of autoreactive Ig of multiple subclasses upon adoptive transfer to lymphocyte-deficient SCID mice. In >90% of cases (24/26) there was accompanying formation of GCs, anatomical features that signify T–B collaboration. Generation of the TCR$\alpha-/-$ phenotype requires some kind of T cells, because the phenotype was not observed when CD3$^+$ cells were depleted from the transfer inoculum. Although transfers of splenocytes depleted of T cells facilitated stable engraftment of B cells, all but one of the recipient mice produced only IgM, and all failed to demonstrate GCs.

TCR$\alpha-/-$ splenocytes contain two types of potentially active T cells: γ/δ T cells and TCR$\alpha^-\beta^+$ cells (23, 29). Our earlier work indicated that γ/δ T cells could produce IL-4 (14), a finding since corroborated by studies of γ/δ responses to various pathogens (30). The evidence that such cells can play a causal role in a GC reaction is presented here by the use of a γ/δ T cell clone with a Th2-type phenotype to reconstitute a SCID mouse together with B cells from TCR($\beta \times \delta$)$-/-$ mice (such B cells being uncontaminated by T cells). Furthermore, recent data from our laboratories clearly demonstrate that B cell maturation and GC formation can be induced by particular conditions in TCR$\beta-/-$ mice (in which all T cells are TCRγ/δ^+), but not in TCR($\beta \times \delta$)$-/-$ mice, in which there are no T cells (Pao, W., L. Wen, A.L. Smith, and A.C. Hayday, unpublished observations).

Nonetheless, GCs in mice reconstituted with B cells plus cloned γ/δ T cells are reproducibly smaller than those induced with total TCR$\alpha-/-$ splenocytes, possibly for a few reasons. First, in the absence of other T cells (either TCR$\alpha^-\beta^+$ cells or polyclonal γ/δ T cells), the engraftment of cloned γ/δ T cells and the kinetics of GC formation supported by those cells may be different to those in mice reconstituted with total splenocytes. Second, TCR($\beta \times \delta$)$-/-$ B cells may be less responsive to T cell help than TCR$\alpha-/-$ B cells. Third, the kind of help that γ/δ T cells provide may be qualitatively different from that given by α/β T cells. Finally, γ/δ T cells may simply not be as efficacious at supporting the GC reaction as are other non-α/β T cells, notably TCR$\alpha^-\beta^+$ T cells. Consistent with this, immunohistochemical studies of GCs of TCR$\alpha-/-$ mice show that the larger number of T cells present are commonly TCR$\alpha^-\beta^+$ (15).

The contribution of TCR$\alpha^-\beta^+$ cells to non-α/β T cell help requires further elucidation. Although such cells have not yet been detected in normal mice or humans, there is no reason a priori to exclude their existence. Rearrangement of the TCRβ chain genes is epistatic to both TCRα gene rearrangement and expression (31); thus, the status of the TCRα locus should not affect the capacity of TCR$\alpha^-\beta^+$ cells to exit the thymus and enter the periphery. Possibly, both types of non-α/β T cell can provide B cell help, driving the GC reaction: γ/δ T cells may help B cells under some circumstances, whereas TCR$\alpha^-\beta^+$ cells may provide help under others, according to the environmental status of the mouse.

Recently, it has been shown that NK cells can provide B cell help (32, 33). However it is likely that non-α/β T cells are the active inducers of the TCR$\alpha-/-$ phenotype for at least three reasons. First, there is no compelling evidence that murine TCR$(-)$ cells with NK activity express CD3, and mice carrying various CD3 gene disruptions, e.g., CD3$\zeta-/-$, have unperturbed NK activity (34). Second, GC formation could be reconstituted with B cells plus cloned γ/δ T cells (discussed above). Finally, CD4$^+$ non-α/β T cells were invariably detected in TCR$\alpha-/-$ GCs by immunohistochemistry (15).

The defining difference between α/β T cells and non-α/β T cells is the nature of the TCR, and by extrapolation, antigen specificity. Conventional α/β T–B help is mediated by the TCR in an antigen-specific fashion, and the broad capacity of α/β T cells to recognize any one of myriad peptides presented by MHC facilitates skewing of the antibody repertoire toward the preeminent challenging antigen at any one time. Whether there is a subset of exogenous challenges to which non-α/β T cells skew the B cell repertoire remains to be clarified. However, the efficient development of high titer, highly diverse, antigen-specific responses may be a unique property of α/β T cells. This would explain why TCR$\alpha-/-$ mice fail to mount efficacious responses against a number of pathogens (35, 36, Roberts, S.J., A.L. Smith, A.B. West, L. Wen, M.J. Owen, and A.C. Hayday, manuscript submitted for publication), and why the relative frequency of B cells specific for nominal T-dependent exogenous antigens, such as oxazalone, is not

substantially increased by challenge of TCRα−/− mice (our unpublished data).

What determines the repertoire of B cells helped by non-α/β T cells? Polyclonal B cell activation may result from the recognition of native, class II MHC by γ/δ T cells (38). In numerous phylogenetic classes of animal, including various birds and mammals, γ/δ T cells interact with other cells that can express class II MHC, such as those of the intestinal epithelium (39). Specific interactions of γ/δ T cells with various activated B cells have been characterized by several laboratories, in each case with a seemingly different antigenic basis for the interaction (40–42). Alternatively, and not mutually exclusively, TCRα−β+ cells may recognize activated B cells either through MHC class II molecules and/or via endogenous superantigens, such as viral proteins (43). It is likewise possible that adoptively transferred non-α/β T cells are in part activated by MHC mismatches, since an apparent low level reactivity of non-α/β T cells to polymorphisms at nonclassical MHC loci was recently reported (44). Indeed, the highest levels of Ig synthesis were sustained in mice in which there was obvious genetic mismatch between graft and host. However, since there are no host B cells, particularly in NOD.SCID mice, the B cell activation documented here is not explained by a conventional GVHD, in which the host B cells expand in response to their recognition by donor T cells (45).

Alternatively, the antibody-antigen specificities seen in TCRα−/− mice may reflect antigen-specific responses of the non-α/β T cells, either directly, or as a result of cross-reactivity. For example, it has been demonstrated that human γ/δ T cells can respond to mycobacterial derivatives of thymidine that might render such cells cross-reactive to DNA (46). It is possible, therefore, that pathogens or autoantigen aggregates are recognized directly by γ/δ T cells, thereby initiating an immune response that includes the help of self-reactive B cells.

In terms of other molecules involved, available data suggest that GC formation in mice is dependent on the interaction of CD40 on B cells with CD40L on T cells (47, 48). We have determined that ∼10% of TCRγ/δ+ cells in TCRα−/− mice express either CD28 or CD40L (Wen, L., unpublished data). Moreover, as demonstrated in this paper, the expression of CD40L on γ/δ T cells increases significantly after activation. Thus, these costimulatory molecules may also be required to generate the TCRα−/− phenotype.

Irrespective of the precise mechanisms involved in T–B interactions, the data presented here indicate that there is a T cell–dependent pathway of B cell maturation that is not dependent on α/β T cells. These findings may explain why GC formation (7, 10, 11) and Ig class switching (5, 6, 12) have been observed in a low percentage of congenitally athymic nu/nu mice. Although these mice harbor highly variable numbers of CD4+ α/β T cells, nu/nu mice commonly contain a significant number of γ/δ T cells (49).

Non-α/β T cell help may be physiologically relevant in normal individuals. Help from γ/δ T and/or TCRα−β+ cells could also have significant ramifications for the production of autoantibodies, particularly, in cases of α/β T cell immunodeficiency. Rajagopalan et al. (50, 51) have reported that B cell help in patients with SLE could be provided by γ/δ T cells. Whether non-α/β T cells provide help in other cases of human health or disease remains to be investigated. Nevertheless, it is striking that overt transferable autoreactivity can be shown in this paper to be the result of a single genetic lesion that precludes α/β T cell function.

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