Cutting Edge: Germinal Centers Can Be Induced in the Absence of T Cells

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Cutting Edge: Germinal Centers Can Be Induced in the Absence of T Cells

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Immunization of mice containing mutations that inactivate the TCR Cβ and Cδ genes with the T cell-independent (TI) type 2 Ag (4-hydroxy-3-nitrophenyl)acetyl-Ficoll induces clusters of peanut agglutinin-binding B cells in the spleen. These clusters are histologically indistinguishable from germinal centers (GCs) typical of T cell-dependent immune responses. They are located in follicles, and contain mature follicular dendritic cells, immune complex deposits, and B cells that display the phenotypic qualities of conventional GC B cells. However, the kinetics of this TI GC response differ from T cell-dependent GC responses in being rapidly induced and of short duration. Moreover, the Ab V genes expressed in TI GCs have not undergone somatic hypermutation. Therefore, T cells may be required for B cell differentiation processes associated with the intermediate and latter stages of the GC reaction, but they are dispensable for the induction and initial development of this response. The Journal of Immunology, 2001, 167: 15–20.

However, previous studies have raised questions regarding the source and amount of T cell help necessary for the GC response. Repeated immunization of mice with targeted inactivation of the TCR α or Cβ genes induces GCs (10, 11), demonstrating that conventional αβ T cells are not required. Immunization of T cell-deficient mice with certain TI type 2 (TI-2) Ags can induce GCs (12, 13), suggesting that cognate T cell-B cell interaction is not obligatory. GCs have also been induced in athymic nu/nu mice by TD and TI-2 Ags (14, 15). Importantly, however, nu/nu mice have T cells, and TI-2 Ags can stimulate thymus-independent T cells to produce factors that regulate the B cell response (16). Thus, the question of whether the GC reaction is TD has remained unresolved. To stringently address this issue, we used mice that completely lack CD3+ T cells due to targeted inactivation of both the TCR Cβ and Cδ genes (17, 18).

Materials and Methods

Mice and immunizations

C57BL/6 and C57BL/6-Tcrb tm1 Mom Tcrd tm1 Mom (TCR βδ-/-) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions. Mice were immunized i.p. with 100 μg (4-hydroxy-3-nitrophenyl)acetyl (NP) or aminooxy carboxymethyl-Ficoll (Biosearch Technologies, Novato, CA) in PBS.

Immunohistochemistry

Immunohistochemistry was performed as described (19). The following reagents, sometimes in combination, were used: rat anti-B220 (clone 6B2), polyclonal mouse anti-rat IgG-alkaline phosphatase, and HRP-polyclonal donkey anti-mouse IgG (all obtained from Jackson Immunoresearch, West Grove, PA); biotin-anti-IgD (clone 2B17-170), anti-CD3-biotin (clone 145-2C11), and biotin-anti-CD23-35 (clone 8C12) (all obtained from PharMingen, San Diego, CA); HRP-anti-CD4 (clone GK1.5; made in our laboratory); biotin-anti-Ki67 (clone TEC-3; Dianova, Hamburg, Germany); HRP-peanut lectin (agglutinin) (PNA) and biotin-PNA (both obtained from Sigma, St. Louis, MO); follicular dendritic cells (FDC-M1 and FDC-M2 (gifts of Dr. M. Kosco-Vilbois, Sereno Pharmaceutical Research Institute, Geneva, Switzerland); biotin-anti-α (clone L213; made in our laboratory); biotin-NP-chicken γ-globulin (CGG; made in our laboratory); GL7 (PharMingen); streptavidin-alkaline phosphatase (Dako, Glostrup, Copenhagen, Denmark); biotin-polyclonal mouse-anti-rat Ig (Jackson Immunoresearch); MOMA-2-FITC (PharMingen); and streptavidin-PE (Molecular Probes, Eugene, OR).

Microdissection of GC and DNA amplification and sequencing

PNA+ follicular clusters were microdissected from spleen sections using a micromanipulator (Carl Zeiss, Thornwood, NY)-controlled capillary pipette, processed, and subjected to PCR as described previously (19). Two rounds of PCR each included 40 cycles (95°C for 1 min, 56°C for 30 s, 72°C for 3 min). The first round primers were FwspR1 (5'-GGAATTCCT GCCTGGAAATTGGAGATTGGA), which hybridizes to a region between the complementarity-determining region (CDR)1 and CDR2 of most J558 VH genes (17, 18), and internal to the J H 3-4Int site. PCR products were cloned into pCRdIIIback (5'-GACTTCAAGCTTCAGTGACTTGC-3') and sequenced.
the pBluescript vector (Stratagene, La Jolla, CA) and sequenced as described (19).

**Results**

**PNA**<sup>+</sup> B cell clusters appear in the splenic follicles of T cell-deficient mice early during a TI-2 response

We have previously shown that TCR<sup>bd</sup><sup>−/−</sup> mice lack CD3<sup>+</sup> T cells (20). In addition, detailed analysis of the B cell compartment in these mice revealed normal levels of a variety of cell surface markers associated with activation and proliferation, but a lower surface (s)IgD-to-sIgM ratio, perhaps indicative of a somewhat less mature stage of differentiation (20). TCR<sup>bd</sup><sup>−/−</sup> mice, as well as C57BL/6 (B6) mice were immunized with NP<sub>32</sub>-Ficoll, and spleens were analyzed at various time points thereafter for PNA<sup>+</sup> B cell clusters via immunohistochemistry. Such clusters were detected within the follicles of a fraction of TCR<sup>bd</sup><sup>−/−</sup> mice in the day 2–4 time frame. Five of eleven mice sacrificed at day 2 and one of six mice sacrificed at both day 3 and day 4 revealed such clusters. In the spleens that contained such clusters, multiple clusters were observed per section, most of which were small (10–15 cell diameters). At the day 4 time point, the clusters observed in the one spleen were infrequent and small. Spleens obtained from B6 mice at all time points also revealed PNA<sup>+</sup> follicular clusters. Because naive B6 mice have small numbers of splenic GCs, whether some of these GCs were the result of background immune responses could not be determined. However, that all the PNA<sup>+</sup> clusters observed in TCR<sup>bd</sup><sup>−/−</sup> mice were induced by immunization was supported by the finding that of spleens isolated from 15 naive TCR βδ<sup>−/−</sup> mice analyzed, none revealed such clusters.

The PNA<sup>+</sup> B cell clusters in T cell-deficient mice have the phenotypic characteristics of TD GCs

Detailed immunohistochemical analyses of the follicular PNA<sup>+</sup> clusters observed in TCR βδ<sup>−/−</sup> mice was performed, and representative results are shown in Fig. 1. The location of these clusters was analogous to those that arise during TD immune responses, as illustrated in Fig. 1A (anti-B220-blue, anti-CD4-red), Fig. 1D (PNA-red, NP-CGG-blue), Fig. 1G (PNA-red, anti-λ-blue), and Fig. 1J (PNA-blue). Most B cells in these clusters lacked sIgD (Fig. 1K). They also stained brightly with the GL7 mAb, a more recently used marker of GC B cells in TD responses (Fig. 1H). The B cells in these clusters also appeared to be proliferating, as most stained with an Ab to the Ki67 nuclear proliferation Ag (Fig. 1I).

This was consistent with these clusters having arisen 2 days after immunization. However, the B cells in these follicular clusters did not stain detectably with NP-CGG (Fig. 1D), suggesting that either they expressed B cell receptors (BCRs) with low or no affinity for NP, had substantially down-regulated surface BCR levels, or both. Although there was no uniform cellular staining within these clusters with either NP-CGG or an anti-λ mAb, these reagents gave rise to reticular patterns of staining in a number of such clusters, indicating deposition of immune complexes (data not shown).

As expected, no staining with anti-CD3 was observed in any region of the TCR βδ<sup>−/−</sup> spleens (data not shown). Staining with

**FIGURE 1.** Immunohistochemical characterization of the location and phenotype of TI GCs. A–G, Results obtained from a single series of tandem spleen sections from a TCR βδ<sup>−/−</sup> mouse immunized 2 days earlier with NP-Ficoll. A, Anti-B220 (blue) and anti-CD4 (red); B, anti-CD21/35; C, FDC-M1 (blue); D, PNA (red) and NP-CGG (blue); E, PNA (blue); F, FDC-M2 (blue); G, PNA (red) and anti-λ (blue). H–J, Results obtained from a different series of tandem sections from the spleen of a different TCR βδ<sup>−/−</sup> mouse at day 2 after immunization with NP-Ficoll. H, GL7 (blue); I, anti-Ki67 (blue); J, PNA (blue). K, Results of staining of a third follicular region in this spleen containing a PNA<sup>+</sup> follicular cluster stained with PNA (red) and anti-IgD (blue). Results are representative of those obtained from five mice. The original magnification of the images in A–G was ×100, and in H–K ×250.
anti-CD4 did reveal occasional positive cells in the PNA⁺ clusters and numerous such cells surrounding central arterioles. These CD4⁺ cells may be a class of CD4 expressing dendritic cells (DCs) (21). Intense reticular staining that overlapped the PNA⁺ areas was obtained using anti-CD21/35 (Fig. 1B), indicating the presence of well developed FDC networks. This was confirmed using the FDC-M1 and FDC-M2 mAbs (Fig. 1, C and F). Because strong FDC-M1 staining is not observed in the primary follicles of T cell-sufficient mice (22), the FDCs in the follicular PNA⁺ clusters in TCR βδ⁻/⁻ mice appear to be mature. Finally, preliminary immunofluorescence analyses using the anti-macrophage/monocyte mAb MOMA-2 revealed percentages of strongly staining cells in the PNA⁺ clusters in TCR βδ⁻/⁻ spleens similar to those observed in TD splenic GCs induced by SRBC immunization of B6 mice (data not shown). Whether these cells correspond to conventional tingible body macrophages will require further studies.

Although previous studies have failed to identify focal regions of B cell proliferation in the follicles of B6 mice immunized with NP-Ficoll, the extrafollicular Ab-forming cell (AFC) response in such mice has been reported to be initially located in the junction zones between the red pulp and T cell zones (23). We also observed this distribution of strongly NP-staining and λ-expressing cells at days 2–4 following immunization with NP-Ficoll in B6 mice (Fig. 2). In contrast, cells that stained with NP-CGG and anti-λ in NP-Ficoll-immunized TCR βδ⁻/⁻ mice were seen exclusively in the middle of white pulp regions, surrounding the central arterioles. This area corresponds to the same region where extensive CD4 staining is observed (Fig. 1A).

The Ab V genes expressed in TI GCs have not undergone somatic hypermutation and are homogeneous

To determine whether V gene hypermutation was occurring in the TI GCs of TCR βδ⁻/⁻ mice, J558 V₅₆ genes and their associated J₇ and 3' flanking sequences were PCR amplified and cloned from microdissected PNA⁺ splenic follicular clusters. Somatic hypermutation acts in the DNA just 3' of rearranged V(D)J genes nearly as efficiently as in adjacent V₅₆ sequence, and analysis of these regions provides an unambiguous assay for this process. V₅₆ clones obtained from three splenic GCs from B6 mice immunized with NP-CGG and sacrificed 12 days later contained characteristic single-base changes indicative of hypermutation at a frequency of 0.5% (Table I). In contrast, 41 clones from nine TI GCs sampled from two TCR βδ⁻/⁻ mice immunized with NP-Ficoll and sacrificed 2 days later contained no base changes in J₇ and 3' flanking sequences (over 13 kb of sequence total). Analysis of GC V₅₆ clones from six GCs from two B6 mice immunized with NP-Ficoll and sacrificed 2 days later revealed mainly clones that contained no mutations, but two of the GCs yielded clones containing one and two mutations (average mutation frequency, 0.2%).

FIGURE 2. The early splenic extrafollicular anti-NP-Ficoll response in T cell-deficient mice surrounds central arterioles. B6 and TCR βδ⁻/⁻ mice were immunized with NP-Ficoll, and spleens were taken and processed for histology as described in Materials and Methods. Examples of parallel sections stained with PNA (red), and anti-λ (blue) or NP-CGG (blue) are shown for days 2, 3, and 4. Arrows indicate the location of central arterioles that stain weakly with PNA. Left, Strong anti-λ and NP staining in the junction regions between the T zone (periarteriolar lymphoid sheath) and the red pulp in B6 spleens, close to the marginal zone (which also stains with PNA). Right, Similar levels of staining observed surrounding the central arterioles in TCR βδ⁻/⁻ spleens. This region also contains many CD4⁺ cells (Fig. 1A). The original magnification of all images was ×250.
Although the TI GCs observed in TCR \(\beta^\delta/\beta^\delta\) and B6 mice did not stain with NP-CGG, many of the V genes recovered from these GCs were members of a subfamily of \(J558\ V_H\) genes that is used in the responses to NP-Ficoll (24) and NP-CGG (2) in T cell-sufficient mice (Fig. 3). Furthermore, all VH clones obtained from individual TI GCs were identical, suggesting

<table>
<thead>
<tr>
<th>GC Source and No.</th>
<th>(J_H) Segment</th>
<th>No. PCR Clones</th>
<th>No. Mutations</th>
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<tbody>
<tr>
<td>B6, NP-CGG, day 12</td>
<td>(J_H) 2</td>
<td>5</td>
<td>Three shared in all five clones&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>46-6</td>
<td>(J_H) 2</td>
<td>5</td>
<td>Three in both clones&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>46-7</td>
<td>(J_H) 2</td>
<td>5</td>
<td>Three in the (J_H) 2 clone&lt;sup&gt;a&lt;/sup&gt;, one in The (J_H) 1 clone&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>46-9</td>
<td>(J_H) 2 and (J_H) 1</td>
<td>3</td>
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</tbody>
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TCR \(\beta^\delta/\beta^\delta\), NP-Ficoll, day 2<sup>d</sup>

<table>
<thead>
<tr>
<th>Clones</th>
<th>(J_H) Segment</th>
<th>No. PCR Clones</th>
<th>No. Mutations</th>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>4</td>
<td>(J_H) 2</td>
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<td>(J_H) 3</td>
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<td>(J_H) 3</td>
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<tr>
<td>48-1</td>
<td>(J_H) 1</td>
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<td>0</td>
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B6, NP-Ficoll, day 2<sup>d</sup>

<table>
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<th>Clones</th>
<th>(J_H) Segment</th>
<th>No. PCR Clones</th>
<th>No. Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(J_H) 2</td>
<td>5</td>
<td>One shared by three of four clones&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>5</td>
<td>(J_H) 2</td>
<td>4</td>
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<tr>
<td>9</td>
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<td>47-1</td>
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<tr>
<td>47-4</td>
<td>(J_H) 3</td>
<td>5</td>
<td>0</td>
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<tr>
<td>47-7</td>
<td>(J_H) 2</td>
<td>4</td>
<td>Two shared by all four clones&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mutations in \(J_H\) flanking sequence.

<sup>b</sup>One mutation in \(J_H\) segment and two in flanking sequence.

<sup>c</sup>One mutation in \(J_H\) segment.

<sup>d</sup>Data obtained from two different mice.

Although the TI GCs observed in TCR \(\beta^\delta/\beta^\delta\) and B6 mice did not stain with NP-CGG, many of the V genes recovered from these GCs were members of a subfamily of \(J558\ V_H\) genes that is used in the responses to NP-Ficoll (24) and NP-CGG (2) in T cell-sufficient mice (Fig. 3). Furthermore, all \(V_H\) clones obtained from individual TI GCs were identical, suggesting

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**FIGURE 3.** The \(V_H\) genes in the TI GCs of TCR \(\beta^\delta/\beta^\delta\) mice are members of a \(J558\ V_H\) subfamily associated with the anti-NP response of B6 mice. The \(V_H\) region sequences obtained from the individual TI GCs of two TCR \(\beta^\delta/\beta^\delta\) mice (knockout-1–22; and 48-3 and 48-8) are compared with the sequence of the B6 anti-NP \(V_H\) gene \(V_H 186.2\) in codon form, as well as the \(V_H 130\) gene previously shown to encode anti-NP Abs in B6 mice. The location of CDR2 is underlined. Nucleotide identity to the \(V_H 186.2\) sequence is indicated by a dash. Nucleotide differences are shown explicitly. The 5’ PCR primer site is indicated by italic letters.
these GCs were nucleated by B cells that were members of a single or limited number of clones.

**Discussion**

The salient differences between the TI GC response we describe here and the GC response to TD Ags are their kinetics and the activity of hypermutation. Such differences may be functionally interrelated. The TD GC response occurs over a period of at least 2 wk, and expressed V genes in newly formed TD GCs have few or no mutations (2, 3). This suggests that the B cells that nucleate TD GCs undergo an initial period of growth before the induction of hypermutation. Therefore, the lack of mutations in V genes expressed by B cells in TI GCs may simply result from the transient nature of this response.

Alternatively, T cells may act directly on B cells to initiate hypermutation. This notion is supported by studies showing that T cell help is required for the induction of hypermutation in B cells and B cell lines in vitro (25–27). It is also possible that TI GCs are formed by B cells that are intrinsically incapable of activating hypermutation. Because NP-Ficoll is a TI-2 Ag, the B1 or MZ subsets, which respond particularly well to this type of Ag, might be predominant participants in the TI GC response. B1 cells may be incapable of inducing hypermutation (28). Whether primary MZ B cells can be recruited into the hypermutation/memory B cell pathway is not known.

Although the lack of T cells in TCR βδ−/− mice did not noticeably alter the microenvironmental locale or histological characteristics of the GC reaction, the location of the anti-NP extrafollicular response differed dramatically from that of T cell-sufficient mice. This has been previously reported by MacLennan and colleagues (23). They also presented evidence suggesting that the extrafollicular AFC response in the spleen is supported by CD11c<sup>bhi</sup>, DEC-205<sup>bhi</sup> DCs that in normal mice are located in the junction zone between the red pulp and T cell areas but, in the spleens of TCR βδ−/− mice, surround the central arterioles (23). Interestingly, a class of splenic CD11c<sup>bhi</sup>, DEC-205<sup>bhi</sup> CD4<sup>+</sup> DC has recently been described by Shortman and colleagues (21). It is tempting to speculate that these two DC types are one in the same and, in the absence of T cells, their microenvironmental locale is altered. Indeed, we observed extensive clusters of CD4<sup>+</sup> cells immediately surrounding central arterioles (Fig. 1A), precisely in the region where the extrafollicular anti-NP-Ficoll response was observed in TCR βδ−/− mice.

A curious aspect of the TI GC response is its inconsistency. Moreover, the spleens of mice that do mount this response contain numerous (4–5 per section) GCs, and all immunized mice mount an extrafollicular response. This indicates that a stochastic variable determines whether the TI GC response takes place. We can only speculate about what this variable might be. Perhaps nucleation of GCs by naive B cells is inefficient, but is efficient if B cells that are already activated but have not yet committed to the AFC pathway are recruited into the GC pathway. In T cell-sufficient mice, such GC precursor B cells may be routinely generated via T cell-B cell interactions outside of follicles (2, 4). In T cell-deficient mice, stimulation by autoantigens, environmental TI Ags, or inflammatory mediators might occasionally generate GC precursor B cells. Even in pathogen-free conditions mouse colonies may be undergoing qualitatively and quantitatively diverse background immune responses. B cells expressing BCRs that are cross-reactive with the TI immunogen and the Ag that initially stimulated them would be expected to be particularly good TI GC precursors, but might be rather rare.

This was suggested by our observation that TI GCs did not stain detectably with NP-CGG (Fig. 1D), yet expressed V<sub>H</sub> family genes previously found to be used in the NP response of B6 mice (2, 24) (Fig. 3). The highly conjugated form of NP-Ficoll used for immunization would be expected to efficiently cross-link BCRs with even low affinity for NP. Moreover, identical V<sub>H</sub> clones were always obtained from individual TI GCs, indicating that each GC was formed by a single or very limited number of clones. We are presently investigating whether the BCRs expressed in TI GCs have measurable affinity for NP or Ficoll.

Recently, it has been found that GCs spontaneously develop in the mesenteric lymph nodes of FDC-deficient LTβ<sup>−/−</sup> mice (29). Thus, the two proposed accessory cells for the B cell GC response, CD4<sup>+</sup> T cells and FDCs, are dispensable, under certain conditions, for at least the early phases of the histologically defined GC reaction. It will be important to expand studies of the similarities and differences in the B cell responses that take place in GCs induced in the presence and absence of T cells or FDCs. Such studies will likely provide new insights into the role of these accessory cells in the initiation or promotion of the steps in memory B cell development that take place in this microenvironment.

**Acknowledgments**

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**References**


