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Complementary Effects of TNF and Lymphotoxin on the Formation of Germinal Center and Follicular Dendritic Cells

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The formation of germinal centers (GC) around follicular dendritic cells (FDC) is a critical step in the humoral immune responses that depend on the cooperative effects of B cells and T cells. Mice deficient in either TNF or lymphotoxin (LT) fail to form both GC and FDC network in B cell follicles. To test a potential complementary effect of TNF and LT, a mixture of bone marrow cells from TNF$^{-/-}$ mice and LT$\alpha^{-/-}$ mice was transferred into irradiated LT$\alpha^{-/-}$ mice or TNF$^{-/-}$ mice. Interestingly, the formation of both GC and FDC clusters in B cell follicles was restored in such chimeric mice, suggesting that TNF and LT from different cells could complement one another. To identify the exact contributions of each subset to the complementary effect of TNF and LT, different sources of T and B cells from LT$\alpha^{-/-}$ mice or TNF$^{-/-}$ mice were used for reconstitution. Our study demonstrates that either T or B cell-derived TNF is sufficient to restore FDC/GC in the presence of LT-expressing B cells. However, TNF itself is not required for GC reactions if the FDC network is already intact. Thus, the development and maintenance of these lymphoid structures depend on a delicate interaction between TNF and LT from different subsets of lymphocytes. The Journal of Immunology, 2001, 166: 330–337.

Germinal centers (GC) around follicular dendritic cells (FDC) provide a dynamic microenvironment where Ag-specific B cells efficiently undergo clonal expansion, somatic hypermutation, and generation of memory cells or plasma cells (1–4). Recent studies suggest that stromal cells in the B cell follicles may release the chemokines necessary for attracting rare Ag-specific T and B cells, so that a coordinate effort can efficiently occur in follicles (5). Inside these GC, Ag-specific B cells efficiently take up Ags, receive signals from FDC, and then present the processed Ags to rare Ag-specific CD4$^+$ T cells. In return, activated T cells support further proliferation and differentiation of B cells with the help of FDC. The dynamic interaction between lymphocytes and FDC is also essential for induction and maintenance of GC (1–4). Recently, TNF/lymphotoxin (LT) ligand and their receptors have been implicated in the formation of B cell follicles, T/B cell segregation, FDC clustering, and GC formation (1, 2, 6–11). The lack of either TNF or LT activity results in the absence of FDC clusters and GC. The cytokines expressed by the stromal cells in the B cell follicles may release the chemokines necessary for attracting rare Ag-specific T and B cells, so that a coordinate effort can efficiently occur in follicles (5).

TNF, LT$\alpha$, and LT$\beta$ are structurally homologous cytokines, and their genes are closely clustered within a few kilobases of the MHC. TNF is produced by both hematopoietic and nonhematopoietic cells in homotrimERIC form (TNF-$\alpha_2$). LT$\alpha$ exists as either a soluble homotrimer (LT$\alpha_3$) or a membrane heterotrimer with LT$\beta$ and is detected on activated lymphocytes. TNF binds to TNFR-I and TNFR-II on both lymphoid and nonlymphoid cells, whereas membrane LT$\alpha_1$/LT$\beta_2$ binds to LT$\beta R$ on nonlymphoid cells (1, 12, 13). This raises the possibility of different types of cell-cell interactions between the TNF- and LT-producing and -receiving cells.

Both TNF$^{-/-}$ mice and LT$\alpha^{-/-}$ mice lack FDC network in primary B cell follicles and fail to form GC after immunization (1, 9). The relative contributions of LT and TNF in the formation of secondary lymphoid tissues are still controversial (1, 14, 15). LT$\alpha$/TNF$\alpha$ double-deficient mice showed an almost identical phenotype as LT$\alpha^{-/-}$ mice, suggesting the impaired TNF production in LT$\alpha^{-/-}$ mice (6, 14). LT$\alpha^{-/-}$ mice were reported to have 50–65% reduction of TNF production compared to wild-type (wt) mice (14, 16). Alexopoulou et al. proposed that the suppressed expression of the TNF gene in the LT$\alpha^{-/-}$ mice is due to nearby transcriptional interference by a phosphoglycerate kinase-neo selection cassette within the targeted LT$\alpha$ locus. Remarkably, they showed that transgenic TNF mice on an LT$\alpha^{-/-}$ background had ectopic GC but weakly restored FDC network (14). Furthermore, a recent study has shown that mice deficient in both TNF and LT$\beta$ exhibit altered microarchitecture in the spleen, which is more severe than that in LT$\alpha^{-/-}$ mice (17). They proposed that reduced expression of TNF, rather than LT$\alpha$, was responsible for the altered lymphoid follicles in LT$\alpha^{-/-}$ mice (14). However, recent evidence has also revealed that the action of LT in the formation of GC and FDC clusters is independent from TNF. 1) Although wt mice treated with LT$\beta R$-Ig fail to form GC and FDC, this is not seen in the mice treated with TNFR-Ig (8, 15). 2) LT$\beta R^{-/-}$ mice do not form FDC or GC. Deletion of LT or TNF should have no impact on the expression of the LT$\beta R$ gene, because the LT$\beta R$ gene is not clustered with the LT or TNF gene (18, 19). Therefore, it is possible that both LT and TNF play essential roles in the formation of GC and FDC in an independent or complementary fashion.

To further study the relative contributions of LT and TNF to the development of FDC and GC, we generated various forms of chimeric mice from LT$\alpha^{-/-}$ mice or TNF$^{-/-}$ mice. Our data showed that either T or B cell-derived TNF in the context of B cell-derived
LT is sufficient to form FDC/GC. In addition, LT-deficient cells could make enough TNF for reconstituted TNF-deficient mice to generate FDC/GC. Thus, our study suggests that the development and maintenance of secondary lymphoid structures depend on a delicate complementary effect between TNF and LT to from different subsets of lymphocytes.

### Materials and Methods

#### Animals

LT−/− mice (backcrossed to C57BL/6 mice for seven generations) and their wild-type littermates were bred under specific pathogen-free conditions in the University of Chicago animal facilities (20). TCR−/− B cells (anti-CR1 Ab, 8C12) were used as described previously (20). In this condition, anti-CR1 Ab (8C12) staining was presented using this Ab for FDC clusters is also brighter than that of FDC-M1, an primary and secondary follicles better than B cells, and the staining for T cells was determined by both 8C12 and FDC-M1, and GC were restored in the mice as indicated and were injected i.v. into recipients that had been lethally irradiated with 108 SRBC. BM cells from wt and TNF−/− mice were reciprocally transferred into lethally irradiated wt or TNF−/− mice. All the reconstituted mice were also immunized with 108 SRBC 10 days before harvest of the spleen. The spleens of reconstituted mice were collected 6, 10, 14, and 24 wk after BMT. Within the first 10 wk after BMT, the ability to form FDC clusters and GC was unchanged in wt mice reconstituted with TNF-deficient BM, but such ability gradually decreased. By 24 wk, FDC clusters and GC were hardly detected in the wt mice reconstituted with TNF−/− BM (Fig. 1). The slow loss of FDC/GC after BMT may explain why the 3-wk treatment of TNF-R-Ig failed to block the formation of FDC/GC in wt mice. In contrast, wt mice treated with LTβR-Ig lost their FDC clusters in <3 days, and wt mice reconstituted with LTα−/− BM more rapidly lost their ability to form FDC within 2–3 wk after BMT (8, 21). It is likely that TNF may act in different pathways than LT in both the development and the maintenance of FDC clusters. In contrast to the slow disappearance of FDC and GC in wt mice reconstituted with TNF−/− BM cells, irradiated TNF−/− mice reconstituted with wt BM showed a rather rapid restoration of B cell follicles and FDC/GC (<6 wk after BMT (Fig. 1). It takes 3–4 wk to allow lymphocytes to mature and repopulate peripheral lymphoid tissues after BMT and then another week to respond to immunization. Therefore, the microenvironments for the formation of B cell follicles and FDC clusters are not developmentally fixed in TNF−/− mice and can be restored soon (<2 wk) after TNF-expressing T and B cells mature and repopulate the spleen. Our BMT data also suggest that TNF derived from hematopoietic cells, but not from nonhemopoietic cells, determines the formation of FDC/GC.

#### Enrichment for T and B cells

Nylon wool columns (Polysciences, Warrington, PA) were used to enrich spleen cell suspensions for T or B cells. Spleenocytes were incubated on nylon wool columns with 10% FCS in DMEM for 1 h at 37°C. Nonadherent cells were collected with 10% FCS in DMEM at 37°C. Enriched T cells in the fraction of nonadherent cells were further purified by panning on tissue culture dishes coated with goat anti-mouse Ig H/L chain (Southern Biotechnology Associates, Birmingham, AL), which yielded 75–85% T cells with <5% B cell contamination. The adherent cells on the nylon wool were eluted by cold PBS. Panning on tissue culture dishes coated with anti-Thy1.2 Ab reduced contamination of T cells to <5%.

#### Transfer of spleenocytes, thymocytes, and bone marrow (BM) cells

Thymocyte (104) or spleenocyte (5 × 104) suspensions were prepared from the mice as indicated and were injected i.v. into recipients that had been irradiated with 750 rad 3 h earlier. When indicated, 105 SRBC were injected i.v. soon after lymphocyte transfer. Recipients were irradiated with 1050 rad (200 rad/min) and were reconstituted with 5 × 106 donor BM cells in either a single preparation or a mixed preparation of 2.5 × 105 BM cells from each donor. Without reconstitution, no mouse would survive at such a high dose of radiation. Six to 8 wk (or as indicated) after BM transplantation (BMT), recipients were immunized i.p. with 108 SRBC. Sera or spleen were collected 10 days after immunization for ELISA or immunohistology, respectively.

#### Ag-specific IgG

Specific Abs were measured and analyzed as previously described (21). For measurement of anti-SRBC Abs, 96-well Falcon plates (Becton Dickinson, Lincoln Park, NJ) were coated with SRBC (150 μl of 0.1% SRBC in PBS/well). Diluted mouse serum was then added and incubated at 4°C for 1 h. Bound Abs were detected using 100 μl of 1/2000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG-specific antiserum (Southern Biotechnology Associates), followed by addition of the alkaline phosphatase substrate p-nitrophenyl phosphate (Sigma) at 1 mg/ml. The mean OD at 405 nm from triplicate wells was compared with a standard curve of various titrated dilutions of a standard anti-SRBC immune serum. To calculate the relative units, we used linear regression analysis. The results are reported as the mean ± SEM.

#### Immunohistology

Spleens were harvested, embedded in OCT compound (Miles, Elkhart, IN), and frozen at −70°C. Frozen sections (6–10 μm thick) mounted on slides were fixed in cold acetone. Endogenous peroxidase was quenched with 0.2% H2O2 in methanol. After washing in PBS, the sections were stained by first incubating with FITC-conjugated anti-B220 (for B cells), biotinylated anti-complement receptor, CR1 (8C12, PharMingen, San Diego, CA), FDCM-1(2) (provided by Dr. Marie Kosco-Vilbois, Geneva, Switzerland), or biotinylated peanut agglutinin (PNA; Vector, Burlingame, CA), all at a 1/50–100 dilution for 1 h. After washing, HRP-conjugated rabbit anti-FITC (1/20; Dako, Glostrup, Denmark) and alkaline phosphatase-conjugated streptavidin (1/20; Vector) were incubated for 1 h. Color development was performed with an alkaline phosphatase reaction kit (Vector) and with 3,3′-diaminobenzidine (Sigma, St. Louis, MO). 8C12, an anti-CR1 Ab, binds to FDC clusters in both primary and secondary follicles better than B cells, and the staining using this Ab for FDC clusters is also brighter than that of FDC-M1, an anti-FDC Ab, in this condition. Therefore, 8C12 staining was presented throughout this study. We used both 8C12 and FDC-M1 to visualize FDC clusters. We have not seen discordance between the two types of staining in B cell follicles in this study. Therefore, we used 8C12 to present FDC and FDC/GC to represent secondary B cell follicles in this study.

#### Determination of follicular structure by BM-derived cells in a TNF-dependent fashion

Like LTβR-Ig, TNF-R-Ig treatment failed to block the formation of FDC clusters and GC in wt mice after immunization with SRBC (8, 15). It is possible that the action of TNF may not be blocked by TNF-R-Ig or that prolonged blockage of TNF activity is required for the prevention of FDC clustering. Therefore, we performed BMT to evaluate the long term effect of TNF-expressing cells on the formation of B cell follicles and FDC network. BM cells from wt and TNF−/− mice were reciprocally transferred into lethally irradiated wt or TNF−/− mice. All the reconstituted mice were also immunized with 108 SRBC 10 days before harvest of the spleen. The spleens of reconstituted mice were collected 6, 10, 14, and 24 wk after BMT. Within the first 10 wk after BMT, the ability to form FDC clusters and GC was unchanged in wt mice reconstituted with TNF-deficient BM, but such ability gradually decreased. By 24 wk, FDC clusters and GC were hardly detected in the wt mice reconstituted with TNF−/− BM (Fig. 1). The slow loss of FDC/GC after BMT may explain why the 3-wk treatment of TNF-R-Ig failed to block the formation of FDC/GC in wt mice. In contrast, wt mice treated with LTβR-Ig lost their FDC clusters in <3 days, and wt mice reconstituted with LTα−/− BM more rapidly lost their ability to form FDC within 2–3 wk after BMT (8, 21). It is likely that TNF may act in different pathways than LT in both the development and the maintenance of FDC clusters.

In contrast to the slow disappearance of FDC and GC in wt mice reconstituted with TNF−/− BM cells, irradiated TNF−/− mice reconstituted with wt BM showed a rather rapid restoration of B cell follicles and FDC/GC (<6 wk after BMT (Fig. 1). It takes 3–4 wk to allow lymphocytes to mature and repopulate peripheral lymphoid tissues after BMT and then another week to respond to immunization. Therefore, the microenvironments for the formation of B cell follicles and FDC clusters are not developmentally fixed in TNF−/− mice and can be restored soon (<2 wk) after TNF-expressing T and B cells mature and repopulate the spleen. Our BMT data also suggest that TNF derived from hematopoietic cells, but not from nonhemopoietic cells, determines the formation of FDC/GC.

#### Either TNF-expressing B cells or T cells could help TNF−/− mice restore the formation of FDC and GC

We and others have demonstrated that LT from B cells, but not that from T cells, is essential for the formation of FDC (22, 23). To define which TNF-expressing cells were responsible for delivering the essential signal in comparison with LT-expressing cells, we analyzed the structures of spleen follicles from chimeric TNF−/− mice that had been lethally irradiated and reconstituted with mixed BM cells from TNF−/− mice and either BCR−/− or TCR−/− mice (Fig. 2). Such a mixture of BM is used to provide a selective source of TNF from either T or B cells as well as retain all cellular components (T and B cells in particular) required for GC. Six weeks after reconstitution, B cell follicles, FDC clusters (determined by both 8C12 and FDC-M1), and GC were restored in TNF−/− mice that had received the mixed BM cells from TNF−/−
mice and TCR\textsuperscript{−/−} mice (Fig. 2). This suggests that TNF from T cells is not essential to complement TNF-deficient lymphocytes to form secondary B cell follicles.

In contrast to LT\textalpha\textsuperscript{−/−} mice, TNF\textsuperscript{−/−} mice that received both TNF-deficient and BCR-deficient BM exhibited the formation of B cell follicles, FDC clusters, and GC (Fig. 2 and Table I). It appears that T cell-derived TNF may provide sufficient help to TNF-deficient T/B cells to form FDC clusters. Interestingly, additional TNF-expressing macrophages from RAG-1-deficient mice have no impact on the restoration of either FDC or GC (Table I). Therefore, TNF from either T or B cells is sufficient to assist TNF-deficient lymphocytes to form FDC clusters and GC.

**Complementary effect of LT\textalpha and TNF in the formation of GC and FDC**

Neither LT-deficient cells nor TNF-deficient cells alone can form FDC clusters and GC, even when they are provided with a wt microenvironment (15, 21) (Fig. 1). To test whether LT\textalpha\textsuperscript{−/−} TNF\textsuperscript{+} cells and TNF\textsuperscript{−/−} LT\textalpha\textsuperscript{−/−} cells together could restore FDC clusters and GC, a mixture of BM cells from TNF\textsuperscript{−/−} and LT\textalpha\textsuperscript{−/−} mice (1/1 ratio) was infused into lethally irradiated TNF\textsuperscript{−/−} mice. Six to 10 wk after BMT, the mice were immunized with SRBC. Ten days following the immunization, the spleens were collected, and immunohistochemistry was performed. Interestingly, primary B cell follicles, FDC clusters (determined by both 8C12 and FDC-M1), and GC (PNA clusters in B cell follicles) were all restored, most likely accomplished by a complementary effect between TNF from LT-deficient cells and LT from TNF-deficient cells (Fig. 3A). LT\textalpha\textsuperscript{−/−} mice have a more profound defect in the spleen, generating no PNA\textsuperscript{+} cells or IgG in response to SRBC, a strong T-dependent Ag (22, 23). To investigate whether the complementary effect also occurred in an LT\textalpha\textsuperscript{−/−} recipient, lethally irradiated LT\textalpha\textsuperscript{−/−} recipients received a mixture of BM cells from TNF\textsuperscript{−/−} and LT\textalpha\textsuperscript{−/−} mice. Although these reconstituted mice still showed altered spleen architecture with ectopic central arteriole and unsegregated T/B cell zones, these mice did restore FDC clusters and form GC in response to T-dependent Ags (Fig. 3A). The data suggest that a subset of LT-deficient cells and another subset of TNF-deficient cells can complement one another, providing enough TNF and LT signal to support the formation of FDC and GC. Our data also demonstrate that cells from LT\textalpha\textsuperscript{−/−} mice make enough TNF to support TNF-deficient cells in the formation of FDC clusters and GC.

To test whether the above irradiated LT\textalpha\textsuperscript{−/−} mice reconstituted with a mixture of BM cells from LT\textalpha- and TNF-deficient mice could restore IgG responses to T-dependent Ag, the mice were immunized with SRBC 6 wk after BMT. The immunized mice showed full recovery of their ability to produce Ag-specific IgG.
These data indicate that the complementary effect of LTα and TNF derived from different subsets of cells is sufficient to restore not only FDC clusters but also GC and IgG response.

Role of T cell- or B cell-derived TNF in the formation of FDC and GC

TNF can be produced by cells other than T and B cells. However, TNF-deficient lymphocytes failed to form FDC and GC when they were transferred into RAG-1−/− mice lacking T and B cells (Fig. 4). Therefore, lymphocyte-derived TNF is essential for the formation of FDC and GC. To further address whether T cell-derived TNF is sufficient to support FDC in TNF−/− mice, a mixture of wt mice (a source of T cell-derived TNF) and splenocytes from TNF−/− mice was transferred into RAG-1−/− mice. FDC clusters formed within 10 days after the transfer (Fig. 4), suggesting that wt T cells complement TNF-deficient B cells allowing the formation of FDC clusters. Furthermore, the formed

Table I. **Cellular and molecular components essential for the formation of FDC/GC complex**

<table>
<thead>
<tr>
<th>Donor BM Cells</th>
<th>Recipient</th>
<th>Chimeric Mice Cells Expressing LT/TNF</th>
<th>FDC</th>
<th>GC</th>
<th>IgG</th>
<th>Reference</th>
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<tbody>
<tr>
<td>wt</td>
<td>wt</td>
<td>T, B, NK</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>LTα−/−</td>
<td>LTA−/−</td>
<td>T, B, NK</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>LTA−/− + RAG−/−</td>
<td>LTA−/−</td>
<td>NK</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>21</td>
</tr>
<tr>
<td>LTA−/− + BCR−/−</td>
<td>LTA−/−</td>
<td>NK, T</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>22, 23</td>
</tr>
<tr>
<td>LTA−/− + TCR−/−</td>
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<td>NK, B</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>22, 23</td>
</tr>
<tr>
<td>LTA−/− + TNF−/−</td>
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<td>NK, T, B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Fig. 3</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Fig. 1</td>
</tr>
<tr>
<td>TNF−/− + RAG</td>
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<td>NK</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
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<td>NK, T</td>
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<td>−</td>
<td>+</td>
<td>Fig. 2</td>
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<tr>
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<td>TNF−/−</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Fig. 2</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>LT−/− T</td>
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<td>Fig. 4</td>
</tr>
<tr>
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<td>RAG−/−</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>Fig. 4</td>
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</tbody>
</table>

*The protocols for BM or thymocytes (Thy) transfer were described in Materials and Methods of relevant references. +, Detectable; +/−, weak positive; −, undetectable.

b The recipient except RAG-1−/− mice were lethally irradiated (1050 rad) and received BM cells (see references for detail). RAG-1−/− mice do not receive radiation.

c Donor cells are the mixture of TNF-deficient splenocytes plus wt or LT-deficient thymocytes (Fig. 4).
FDC clusters were functional because they always support B cells to form GC in B cell follicles (left panel in Fig. 4). The data suggest that B cell-derived TNF is not absolutely required for the formation of FDC and GC.

T cells from wt mice express both TNF and LT (13–15). To determine whether T cell-derived TNF alone is sufficient to help the formation of FDC and GC, a mixture of LT-deficient thymocytes (from LTα−/− mice) and TNF-deficient splenocytes (from TNF−/− mice) was transferred into RAG-1−/− mice together with SRBC. Both GC and FDC were then formed 10 days after transfer, although a smaller FDC network and PNA clusters were detected compared with the transfer of wt T cells (Fig. 4). Ten days after transfer, the formation of PNA clusters was closely located around the FDC network when wt T cells were provided, whereas about half the PNA clusters were not colocalized with the FDC network when LTα−/− splenocytes were provided (Fig. 4). The data indicate that T cell-derived TNF is sufficient to assist TNF-deficient lymphocytes in the formation of FDC and GC. In contrast, LTα−/− mice reconstituted with BM cells from BCR−/− mice or with TNF-deficient T cells failed to form any FDC clusters (22) (Table I). Thus, our study demonstrates that LT from B cells, but not T cells, is essential for the formation of FDC, whereas TNF from either T cells or B cells can partially complement TNF-deficient splenocytes to form both FDC and GC.

To study whether T cell-derived TNF is required for GC reactions in the presence of intact FDC clusters, thymocytes (source of T cells) from TNF−/− mice were transferred into TCR−/− mice. Reconstituted mice were also immunized with SRBC. TCR−/− mice were chosen as recipients, because they have a well-established FDC network in the primary B cell follicle, but cannot form secondary B cell follicles in the absence of T cell help and SRBC (22). GCs were rapidly formed around the FDC network in TCR−/− mice reconstituted with T cells from either wt or TNF−/− mice (Fig. 5). Consistently, TNF-deficient lymphocytes were capable of forming GC in irradiated wt mice when the clustering of FDC remained (data not shown). It appears that TNF is required for the creation of the permissive environment to maintain GC in

**FIGURE 3.** Complementary effects of TNF and LT. Lethally irradiated mice were reconstituted with a mixture of BM cells from TNF−/− or LTα−/− mice. Six weeks after BMT the mice were immunized with 10^8 SRBC, and 10 days later the spleens and sera were collected. A, The spleen sections were stained with 8C12 (blue) to detect FDC, with PNA (blue) to detect GC, and with anti-B220 (brown). B, IgG to SRBC from wt, LTα−/− mice, or reconstituted LTα−/− mice was determined by ELISA. The complementary effects of TNF and LT from different cells can be demonstrated.
B cell follicles. However, once the FDC network is established, TNF may not be required for GC reactions.

Discussion

In optimal humoral immune responses the FDC/GC complex provides the microenvironment where Ag-specific B cells efficiently undergo clonal expansion and somatic hypermutation with the help of T cells and FDC, leading to the selection of B cells bearing higher affinity Ig and generation of memory B cells and plasma cells (1–4). Recent studies using knockout approaches have greatly facilitated our understanding of the factors required for the formation of FDC and GC (1, 2). For example, mice deficient in either TNF or LTα lack both FDC networks and PNA clusters inside B cell follicles (1, 9), but it is unclear whether the effects of LT and TNF can complement one another in the formation of FDC and GC. It has not been well defined which cellular sources of LT and TNF are essential for the formation of those structures. Here, we have clearly demonstrated that 1) either T or B cells are able to supply TNF needed for FDC and GC formation in the presence of B cell-associated LT; 2) TNF is not essential for GC formation if FDC is present; and 3) LTα−/− cells make enough TNF for the formation of FDC and GC. Due to the technical limitation of immunohistology, the expression of LT/TNF protein by either T or B cells in the FDC/GC area has not been demonstrated directly. We propose that putative FDC precursors depend on signals from both TNFR and LTβR delivered by various lymphocytes for the differentiation, migration, and maturation of FDC. Therefore, T and B cells coordinate their effort to form GC around FDC via the complementary efforts of TNF and LT.

Targeted disruption of genes that encode ligands and receptors in the TNF/LT family has clearly established the important roles of these molecules in the development and maintenance of secondary lymphoid tissue (1, 14, 15). However, it was proposed that compound defects in the production of both TNF and LT may occur in LTα−/− mice, and the resulting defect in TNF caused by the nearby gene targeting may impair the formation of FDC/GC (14). However, many other studies strongly support the idea that an interaction between membrane LT and LTβR is essential for the formation of the T/B zone, FDC, and GC, independently from TNF (8, 15–18). In fact, wt mice treated with LTβR-Ig, but not TNFR-Ig, are prevented from developing GC and FDC (8, 15). If defective TNF expression in LTα−/− mice is the cause of the altered lymphoid structure, then transferring cells from LTα−/− to TNF−/− mice should not restore the formation of FDC clusters and GC in TNF−/− mice. LT-deficient cells still make sufficient TNF to assist TNF-deficient cells to form FDC and GC in B cell follicles. Together with other evidence, it is likely that the lack of FDC/GC in LTα−/− mice is directly attributed to the absence of LT, independent of the reduced expression of TNF. The data also suggest that LTα and TNF signals could be delivered by different subsets of lymphocytes (Fig. 4).

In contrast to TNFR−/− mice, wt mice treated with TNFR-Ig fail to block the formation of FDC/GC (8, 15). Our study indicates that prolonged deficiency of TNF, but not short term deficiency, could lead to the loss of FDC and GC. It is possible that signaling via TNFR is important for the early development of the FDC network but is less important for the clustering of FDC and their formation of GC. Prolonged blockage of TNF may be required for the loss of established FDC due to the slow turnover rate of FDC and the presence of LT. In contrast, LT may be more important for the maintenance of FDC clustering. Blockage of LT will lead to the rapid loss of FDC clustering. It was recently proposed that the lack of a detectable FDC network in primary B cell follicles of TNF−/− mice may be due to the failure of FDC precursors to migrate through the disorganized marginal zone to the follicles (24). FDC-like cells are retained within the defective marginal zone of TNF−/− or TNFR−/− mice, but not in LTα−/− mice (24, 25). Therefore, TNF and LT may act preferentially on different stages of FDC differentiation.
Endres et al. recently found that the reconstitution of BCR<sup>−/−</sup> mice with the mixture of TNF<sup>−/−</sup> and BCR<sup>−/−</sup> BM led to few FDC clusters (18). No PNA clusters were located at B cell follicles, although some small clusters of PNA cells were ectopically located around central arterioles in T cell areas. They concluded that TNF-expressing B cells, but not T cells, are required for the development of a mature splenic FDC network and GC. In contrast, we have found that the reconstitution of TNF<sup>−/−</sup> mice with the mixture of TNF<sup>−/−</sup> and BCR<sup>−/−</sup> BM led to the restored FDC clusters and GC in B cell follicles (Fig. 2). The mechanisms for the apparent discrepancy are still under investigation. It is possible that the BMT protocol, the treatment of the recipient, the Ag used, and the housing conditions of the mice may contribute to the underlying discrepancy.

There may be several explanations why LT from B cells, but not T cells, is required for FDC clustering, whereas TNF from either T or B cells can regulate FDC clustering: 1) the expression of LT<sub>α</sub> may be more limited to B cell follicles than that of TNF; 2) the time course of the expression of TNF and LT<sub>α</sub> may be different; 3) the soluble form of TNF binds to TNFR, whereas only the membrane form of LT<sub>α</sub> binds to LT<sub>βR</sub>; or 4) FDC clusters are surrounded by B cells inside B cell follicles. Therefore, it may be difficult for cells other than B cells to deliver membrane LT signal to FDC, because cell-cell contact is required for the interaction between a membrane ligand and its receptor. In contrast, both activated T and B cells near FDC could deliver enough soluble TNF to FDC inside B cell follicles without cell-cell contact.

Interestingly, transfer of either TNF- or LT-deficient splenocytes to sublethally irradiated wt mice (750 rad) could generate GC when radioresistant FDC still remained (15, 21). Soluble TNFR-Ig administered in vivo failed to prevent GC (8, 15). Furthermore, we have found that TNF-deficient T cells can help B cells to form GC in TCR<sup>−/−</sup> recipients (Fig. 5). Therefore, it appears that TNF is not essential for the formation of GC inside B cell follicles once FDC is established, but TNF is necessary for creating the permissive environment for B cell migration and GC formation, probably by the development and/or maintenance of FDC.

Both TNF and LT may not always be required for FDC maturation or clustering. In some situations, a single signal from over-expression of TNF or LT alone may be sufficient for lymphoid neogenesis. Expression of TNF or LT by the transgenic approach can also lead to the generation of some FDC networks and ectopic GC (14, 26). The development of FDC and GC is closely associated with the development of a pathological process, such as chronic inflammation or autoimmunity. For example, patients with myasthenia gravis can manifest lymphoid follicles and GC within the thymus. Similar follicles and GCs are found with inflamed synovial tissues of patients with rheumatoid arthritis. Lymphoid follicles, including FDC clusters, are developed in the islets before the destruction of β-cells in autoimmune diabetic mice. We speculate that the formation of such follicles may greatly facilitate Ag presentation and maintenance of the local immune response. It will be of interest to study which cytokine(s) and which cells are involved in the development and maintenance of such a lymphoid microenvironment and their roles in chronic inflammation and autoimmune diseases.

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**References**


