Self-limiting systemic autoimmune disease during reconstitution of T cell-deficient mice with syngeneic T cells: support for a multifaceted role of T cells in the maintenance of peripheral B cell tolerance

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Abstract

The T cell compartment can be partially reconstituted in mice with targeted inactivation of the TCR Cβ and Cδ genes by injection of mature, syngeneic T cells. Surprisingly, during this reconstitution high titers of IgG anti-nuclear antibodies and symptoms of systemic autoimmune disease develop. However, this autoimmune response is transient and aged, reconstituted mice show no overt signs of disease. The autoantibody response appears to be derived from a pre-existing population of host self-reactive B cells and requires CD40 ligand-mediated co-stimulation from donor cells. Diminution of this response is coincident with a vigorous germinal center reaction and the disappearance of a subpopulation of activated B cells that expresses elevated levels of Fas. Collectively, our data support the idea that T cells play a multifaceted role in the maintenance of peripheral B cell tolerance that includes mediating the activation-induced death of autospecific B cells.

Introduction

Autoreactive cells are eliminated from the murine B cell compartment at several developmental stages. During primary differentiation in the adult bone marrow, many autoreactive B cells are either deleted via apoptosis or undergo revision of their antigen receptors due to secondary V(D)J rearrangements (1–3). However, B cells with certain autoreactivities are not eliminated at this stage and enter the periphery (4). It has been argued that the inclusion of ‘multireactive’ B cells whose BCR specificities have low to moderate affinity for self-antigens is required to allow the B cell compartment to encompass recognition of any of the multitude of epitopes present in the foreign antigen universe (5). Nonetheless, such peripheral autoreactive B cells may be subjected to tolerance pathways that result in either clonal deletion or anergy (1). In addition, autoreactive B cells that arise as a result of V region somatic hypermutation during germinal center (GC) responses may be regulated by analogous mechanisms (6–9).

There is a growing body of evidence suggesting that T cells play an active role in these peripheral B cell tolerance pathways (10–14). For example, the use of Ig transgenic systems has shown that certain autoreactive B cells, in the presence of cognate self-antigens, are eliminated after migrating to the T–B interface of splenic follicles, thereby implicating T cells as a regulatory factor, possibly through Fas–Fas ligand-mediated programmed cell death (15–19). Moreover, it has recently been observed that B cells expressing autoreactive, transgene-encoded BCR display distinct phenotypes and behaviors in T cell-deficient mice, as compared to when they are present in a T cell sufficient environment (20,21).

In this report we show that autospecific B cells are present in T cell-deficient TCRβδ−/− mice and that these mice mount a vigorous serum autoantibody response and develop symptoms of autoimmune disease shortly after they are injected with mature, syngeneic splenic T cells. However, this
autoimmune response is transient, suggesting that, on the one hand, reconstitution of the T cell compartment allows activation of quiescent autoreactive B cells and, on the other, that reconstituting T cells ultimately participate in the restoration of peripheral B cell tolerance mechanisms. This T cell reconstitution system provides a means to evaluate the role of various T cell subsets and T cell regulatory factors in peripheral B cell tolerance, and has advantages over transgenic systems since it examines the activity of natural autoreactive B cells.

**Methods**

**Mice and adoptive transfers**

C57BL/6J, C57BL/6J-Tnfsf4em1tmx (CD40 ligandf/f) (23), C57BL/6J-Igh-a-Thy1.1 (Thy1.1), C57BL/6J-Tcrbem1Mom Tcrbem1Mom (TCRβδ) (24-26) and C57BL/6-Igh-6em1Cgn (μMT) mice (27) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our facilities. Lymphocyte suspensions were prepared as described previously (28). Briefly, spleens were ground between microscope slides and red blood cells were lysed using Tris-buffered ammonium chloride (29). Cells were then used for adoptive transfer or flow cytometry. Prior to transfer, splenocytes were depleted of B cells using antibodies to I-A (25-9-3S) (ATCC, Rockville, MD) and heat stable antigen (J11d) (a gift of Dr Michael Cancro) or T cells by using antibodies to Thy1.2 (J1j) (also from MD) and heat stable antigen (J11d) (a gift of Dr Michael Cancro) or mouse anti-rat IgG (Jackson ImmunoResearch) or peroxidase-labeled donkey anti-mouse IgM (Jackson ImmunoResearch). T cells were identified using anti-CD3–FITC (Southern Biotechnology Associates), anti-CD28 clone 37.51, FITC (Southern Biotechnology Associates), anti-CD44 (Pgp1) clone IM7, PE or–anti-CD45R B220 clone RA3-6B2, anti-CD45R B220 (very early activation antigen) clone H1.2F3, FITC–anti-CD86 (B7-2) clone GL1, FITC–anti-CD90.1 (Thy1.1) clone HIS51, anti-CD3–FITC, anti-CD45R B220 clone RA3-6B2, anti-CD44 (Pgp1) clone IM7, PE or–anti-CD45R B220 clone RA3-6B2, biotin–anti-CD95 (Fas) clone Jo2, biotin–anti-CD138 (syndecan-1) clone 281-2, PE–anti-CD154 (CD40 ligand) clone MR1, anti-Fas ligand clone MFL3, biotin–anti-NK1.1 (NK1.1) clone PK136 and PE–anti-DX5 (Pan Nk cells) clone DX5 were all purchased from PharMingen (San Diego, CA). Biotin–rat anti-mouse IgD was purchased from Southern Biotechnology Associates (Birmingham, AL) and FITC–donkey anti-mouse IgM from Jackson ImmunoResearch (West Grove, PA). Streptavidin–Red670 was purchased from Gibco/BRL (Grand Island, NY). PL9-6 was the kind gift of Marc Monestier (30).

**Immunohistochemistry**

Immunohistochemical studies were performed as described previously (31,32). Briefly, spleens were quick frozen in OCT (Elkhart, Indianapolis, IN) and 6 μm sections were prepared. Sections were fixed in acetone and air dried. The slides were incubated in 0.3% H2O2 to destroy endogenous peroxidase activity. The slides were blocked with TBS + 5% BSA. The sections were incubated with horseradish peroxidase (HRP)- and biotin-labeled antibodies followed with streptavidin–alkaline phosphatase (Southern Biotechnology Associates). HRP-labeled antibodies were revealed with 3-amino-9-ethyl-carbazole and alkaline phosphatase-labeled antibodies were revealed with Fast BB Blue Base (Sigma, St Louis MO).

B cells were identified using a rat anti-B220 (6B2) ascites and mouse anti-rat IgG–alkaline phosphatase (Jackson ImmunoResearch) or peroxidase-labeled donkey anti-mouse IgM (Jackson ImmunoResearch). T cells were identified using anti-CD3–biotin and streptavidin–alkaline phosphatase (Dako, Glostrup, Denmark). Germinal centers were identified using peanut agglutinin (PNA) linked to horseradish peroxidase (Sigma).

**Serology**

Mice were bled at various intervals pre- and post-transfer via the retro-orbital sinus and the levels of anti-chromatin, anti-single-stranded DNA and anti-double-stranded DNA, and various antibody isotypes were determined by previously described ELISA assays (7,31,33). Chromatin was prepared as described elsewhere (34).

**Anti-nuclear antibody (ANA) assays**

ANA staining activity was analyzed using human epitheloid HEP-2 cells on prepared slides (Antibodies Inc., Davis, CA). HEP-2 cells were stained with a 1:50 dilution of sera from time points corresponding to the peak of autoantibody production as analyzed by ELISA. The slides were washed with PBS and the presence of mouse antibodies was revealed using a goat anti-mouse IgG–FITC (Southern Biotechnology Associates).

**Immune complex deposition**

Kidneys from mice at days 0 and 28 post-T cell transfer were frozen in OCT and 6 μm sections were prepared. Sections were stained with a FITC-labeled anti-mouse κ mAb, clone EM-34.1 (Sigma), to reveal immune complex (IC) deposition.

**Mitogen stimulation of B cells in vitro**

Spleen cells were depleted of T cells via treatment with anti-Thy1 (mAb J1) and complement, and small, resting B cells...
purified on Percoll gradients. Cells were plated at 10^6 cells/ml in RPMI media containing 10% FCS, 50 µg/ml lipopolysaccharide (LPS, Difco, Detroit, MI) and 5 µg/ml dextran sulfate (Amersham Pharmacia, Piscataway, NJ). After 5 days, supernatants were harvested and assayed for levels of antibodies with various specificities by ELISA.

Results

The T cell compartment can be partially reconstituted in TCRβδ^-- mice by injection of mature C57BL/6 T cells

As judged by immunohistochemistry of spleen sections and flow cytometric analysis of spleen cells using anti-CD3, mice with previously described targeted inactivations of the TCR Cβ and Cδ genes (TCRβδ^-- mice) (24–26) are completely devoid of mature T cells (Fig. 1A and B, top panels). To attempt to reconstitute the T cell compartment of TCRβδ^-- mice (congenic on a C57BL/6 background), 1 × 10^7 β-depleted C57BL/6 splenocytes were transferred i.v. to each TCRβδ^-- mouse. The recipient mice were not irradiated prior to T cell transfer. Mice were sacrificed at various times after injection and spleens taken. Approximately one-third of each spleen was used for flow cytometric analysis and the other two-thirds was frozen for histological analysis.

Frozen spleen sections were stained with reagents to visualize B cells, reconstituting T cells and PNA binding B cell follicular areas surrounded by marginal zones were observed in spleen sections at all times examined, including in pre-transfer TCRβδ^-- mice (red staining in left and top right two panels of Fig. 1A). Numerous T cells were seen at day 7 post-transfer and these T cells were scattered throughout the red pulp as well as the follicles (blue staining middle left panel of Fig. 1A). By day 14 post-transfer the T cells began to aggregate around central arterioles inside of follicular areas, although a decrease in spleen size was observed as early as three days post-T cell transfer. The weight of day 7 post-transfer spleens was approximately twice that seen in pre-transfer mice. Pre-transfer spleens weighed an average of 130 mg, while day 7 spleens increased to an average of 220 mg. Spleen weights were observed to decrease at later time points. At day 35 the weight of spleens decreased to 125 mg and by day 49 they began to approximate that seen in C57BL/6 mice which weighed an average of 114 mg.

Concomitantly with the increase in spleen size was an increase in various splenocyte subsets (Fig. 1C). Control C57BL/6 mice had ~5 × 10^7 splenic B cells and ~3 × 10^7 splenic T cells. Pre-transfer TCRβδ^-- mice had increased numbers of splenic B cells compared to C57BL/6 mice with ~9 × 10^7 B cells (Fig. 1C). Following transfer there was an average of 1.6 × 10^8 B cells in the spleens of transfer mice at day 7. These numbers decreased over time to ~5 × 10^7 cells by days 35–49. Flow cytometry also revealed a transient increase in a population of non-B/non-T cells during reconstitution (data not shown). Preliminary phenotypic analysis of this subpopulation revealed that it included NK cells.

During T cell reconstitution TCRβδ^-- mice produce a vigorous but transient serum antibody response to self-antigens

As expected, TCRβδ^-- pre-transfer mice had lower total serum Ig concentrations, especially of switched isotypes G1, G2a and G2b, compared to C57BL/6 mice. However, IgM and IgG3, isotypes associated with T-independent responses, were elevated. After T cell reconstitution, mice displayed an increase in total serum antibody. Individual isotypic classes of the antibodies varied in their degree of increase. The most pronounced elevation was seen in IgG2a and there was no increase in IgG3 (data not shown).

During the course of T cell reconstitution of TCRβδ^-- mice we observed temporary symptoms consistent with systemic disease, including reduced skin elasticity, tufted fur and lethargy. These symptoms peaked 2–3 weeks after injection of T cells and subsequently disappeared. Mice followed up to 1 year after T cell transfer showed no recurrence of disease symptoms. For this reason, sera obtained from TCRβδ^-- mice at various times after T cell transfer were assayed for levels of autoantibodies with specificities associated with systemic autoimmune disease. Figure 2(A) shows that light chain-bearing serum antibodies capable of binding single-stranded DNA, double-stranded DNA and chromatin appeared at high levels shortly after T cell transfer. At 2–3 weeks post-transfer the titers of these autoantibodies approached 50% of those characteristic of aged autoimmune MRL/Ipr/lpr mice. However,
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This autoantibody response was transient. An increase in autoantibody titers was seen as early as day 7 and reached a peak in the day 14–21 time frame, and then declined to levels characteristic of normal mice. Heavy chain isotype analysis of serum anti-chromatin (Fig. 2B) and anti-DNA antibodies (data not shown) revealed high levels of IgG2a, particularly 28 days after T cell transfer, consistent with the overall increase in total serum IgG2a observed after T cell

Fig. 1. Reconstitution of the T cell compartment in TCRβδ−/− mice. Individual TCRβδ−/− mice were injected in the lateral tail vein with 1 × 10^7 B-depleted splenocytes from C57BL/6 mice. Spleens were taken at the indicated time points, and one-third of each spleen was used for flow cytometric analysis and the remainder was frozen for immunohistochemical analysis. (A) Spleen sections were stained with anti-IgM (red) and anti-CD3 (blue) as described in Methods. To elaborate GC a day 28 section was stained with anti-B220 (blue) and PNA (red). The original magnification of all images was ×100. The data are representative of two experiments using three recipient mice per time point. (B) Splenocytes were prepared as described in Methods, and stained with anti-CD3 and anti-B220. Stained cells were analyzed with a Coulter Epics Elite cytometer using a live lymphocyte forward and side scatter gate. The data were representative of those obtained from two experiments using at least three mice per time point. (C) Numbers of total splenocytes were determined by Trypan blue cell counts. The number of T and B cells was determined by multiplying the number of total splenocytes by the proportion of CD3+ and B220+ cells obtained from the experiments illustrated in (B). The data were obtained from at least three mice per time point.
Fig. 1. Legend on facing page.
Fig. 2. Serum autoantibody responses of TCRβδ−/− mice after transfer of T cells. Mice were bled at the indicated time points. (A) Titers of antigen-specific κ light chain-bearing antibodies were determined by ELISA. Levels of autoantibodies are presented as µg/ml equivalents of the PL9-6 anti-chromatin antibody (IgG2b, that was cross-reactive with our anti-IgG2a anti-sera) which also binds single-stranded DNA and double-stranded DNA. (B) Data from a separate experiment using three additional TCRβδ−/− mice not used for the studies summarized in (A) showing IgG2a anti-chromatin responses after T cell transfer. Results obtained from the analysis of the pooled sera of aged-matched C57BL/6 mice and old MRL lpr/lpr mice are shown for comparison.
injection. Interestingly, there was substantial mouse to mouse variation seen in the kinetics of expression of autoantibodies with particular specificities.

As expected from the high titers of anti-double-stranded DNA and anti-chromatin antibodies revealed by ELISA assays, sera from many post-transfer mice contained IgG ANA as determined by ANA analysis (Fig. 3A). Such antibodies were not present at detectable levels in pre-transfer or C57BL/6 mice. To investigate whether these serum autoantibodies might in part be responsible for the disease symptoms observed, kidneys were taken from three mice each at 21 and 28 days after T cell transfer. Frozen kidney sections were then stained for IC deposition. All of the kidneys revealed extensive IC deposition in glomeruli. Figure 3(B) illustrates this point.

The autoantibody response in reconstituting TCRβδ+/– mice appears to depend on cell contact-dependent co-stimulatory interactions between pre-existing recipient autoreactive B cells and donor T cells

The increases in B cell numbers and the induction of autoantibodies observed in C57BL/6→TCRβδ+/– mice could have been a consequence of activation of donor T cells during purification or transfer, resulting in the indirect activation of B cells in the recipients. To test this idea, reciprocal transfers were performed. TCRβδ+/– B cells (3×10^7 per mouse) were injected i.v. into congenic C57BL/6 µMT (B cell-deficient) mice and levels of serum anti-single-stranded DNA, anti-double-stranded DNA and anti-chromatin antibodies were analyzed at various times thereafter. These mice accumulated serum autoantibodies (Fig. 4A), albeit at levels lower than observed in TCRβδ+/– mice reconstituted with C57BL/6 T cells. Transfer of C57BL/6 B cells into µMT mice resulted in only very transient, low-level autoantibody production. Therefore, TCRβδ+/– B cells can produce a sustained autoantibody response in the presence of unmanipulated T cells.

To further evaluate whether TCRβδ+/– mice contained an increased level of autoreactive B cells as compared to normal mice, splenic B cells were purified and equivalent numbers were stimulated with LPS plus dextran sulfate in vitro. After 5 days, anti-single-stranded DNA, anti-double-stranded DNA and anti-chromatin antibody levels in culture supernatants were measured. TCRβδ+/– B cells produced 2–3 times more autoantibodies under these conditions as compared to C57BL/6 B cells (Fig. 4B).

To investigate whether activation of autoantibody producing B cells in the TCRβδ+/– reconstituted mice required direct T cell co-stimulation, congenic C57BL/6 CD40 ligand-deficient mice were used as a source of T cells for transfer. Figure 5 illustrates that transfer of splenic T cells from these mice into TCRβδ+/– mice resulted in long-term partial T cell reconstitution equivalent to when C57BL/6 T cells were used (Fig. 5B) and modest increases in total serum antibody at day 28 post-T cell transfer (IgM and IgG, Fig. 5A). However, post-transfer sera obtained from these mice at this time lacked detectable levels of anti-chromatin (Fig. 5A) and anti-double-stranded DNA antibodies (data not shown). The mice were also bled a multiple time points before and after day 28, up to the time when they were sacrificed to evaluate T cell reconstitution. At no time were anti-chromatin or anti-DNA antibodies levels above background controls detected (data not shown). This indicated that the autoantibody response in TCRβδ+/– mice receiving normal T cells is not a T-independent response stimulated by antigens expressed by T cells and that conventional T cell–B cell co-stimulatory interactions are required for autoantibody production. However, since T cell reconstitution by CD40 ligand-deficient T cells was only evaluated at a late time point, we cannot exclude the formal possibility that the kinetics of reconstitution differed in this situation as compared to when CD40 ligand-sufficient T cells were used and that this difference influenced the magnitude of the autoimmune response in the recipients.

Activated splenic B cells appear during reconstitution of the T cell compartment of TCRβδ+/– mice

To determine whether the generation of autoantibodies in TCRβδ+/– reconstituted mice was the result of polyclonal activation of B cells, we examined expression of markers associated with B cell activation at various times after T cell transfer (37–39). Splenic B cells through day 49 post-transfer showed no detectable increase in CD23 (FcRRII) CD69 (very early activation marker), CD86 (B7-2) or CD138 (syndecan). However, we did observe increases in CD95 (Fas), CD35 and CD43 (Fig. 6). All three of these proteins were up-regulated on a small percentage of B cells present from days 14 through 35 post-transfer. However, by day 49 these cells had disappeared.

We also analyzed the B cell compartment of reconstituting mice for expression levels of surface IgM and IgD (Fig. 7). Controls for this experiment revealed that prior to T cell transfer many TCRβδ+/– B cells expressed reduced levels of slgD as compared to C57BL/6 B cells. However, at day 7 post-transfer, these slgD levels appeared normal. Beginning at day 21 there was a substantial increase in the number of B cells that lacked expression of both slgM and IgD. This was consistent with our serum antibody results in suggesting that a subpopulation of B cells had undergone isotype class switching by this stage of T cell reconstitution.

Discussion

The data presented here show that TCRβδ+/– mice, which are completely devoid of CD3+ T cells, mount a vigorous serum autoantibody response during reconstitution with syngeneic mature T cells. This response includes antibodies analogous to those present in mice with progressive systemic autoimmune disease, i.e. IgG that are reactive with purified single-stranded DNA, double-stranded DNA and chromatin, and that stain nuclear structures in ANA assays. The production of these autoantibodies coincides with a robust splenic GC response, abundant deposition of IC in kidney glomeruli and the presence of a variety of pathological symptoms. However, this autoimmune response is transient, as serum autoantibody titers begin to wane 3–4 weeks after reconstitution and aged reconstituted mice show no overt signs of autoimmune disease. Concurrent with autoantibody production and the GC response, an increase in Fas (CD95) expression as well as several other markers associated with activation-induced cell death is observed on a small percent-
Fig. 3. Legend on facing page.
age of splenic B cells. As the serum autoantibody response subsides, the Fas\textsuperscript{hi} subpopulation of B cells disappears. We considered that a by-product of T cell reconstitution was a transient and perhaps non-specific polyclonal activation of the B cell compartment. Our data strongly argue against this possibility. Transfer of syngeneic CD40 ligand\textsuperscript{−/−} T cells into TCR\textsuperscript{βδ}\textsuperscript{−/−} mice did not result in a serum autoantibody response, demonstrating that conventional B cell co-stimulation by T cells is required for this response. The unique ANA staining patterns obtained from the sera of individual C57BL/6 T → TCRαβ\textsuperscript{−/−} reconstituting mice and large mouse to mouse variation in expression levels of various autospecificities are indicative of a restricted number of stochastically selected B cell clones giving rise to the majority of autoantibody in each

Fig. 4. Autoantibody production by B cells from TCR\textsuperscript{βδ}\textsuperscript{−/−} mice after injection into B cell-deficient mice and after mitogen stimulation in vitro. (A) T cell-depleted spleen cells from either TCR\textsuperscript{βδ}\textsuperscript{−/−} mice (diamonds) or C57BL/6 mice (squares) were transferred to B cell-deficient µMT mice as described in Methods. The levels of anti-single-stranded DNA, anti-double-stranded DNA and anti-chromatin antibodies in the sera of recipient mice just before (day 0) or at various times thereafter are shown (as µg/ml equivalents of the PL9-6 mAb). The data were obtained from four mice that received TCR\textsuperscript{βδ}\textsuperscript{−/−} cells and five that received C57BL/6 cells. The results shown are representative of those obtained in three separate experiments. (B) Small resting splenic B cells were purified via T cell depletion and Percoll gradient centrifugation from C57BL/6 and TCR\textsuperscript{βδ}\textsuperscript{−/−} mice, and cultured for 5 days either in media or in media containing LPS and dextran sulfate as described in Methods. Supernatants were then assayed for levels of anti-chromatin, and anti-double-stranded DNA and anti-single-stranded DNA by ELISA. Squares and diamonds indicate data obtained from control and mitogen-stimulated C57BL/6 B cells respectively. Circles and triangles indicate analogous data obtained from TCR\textsuperscript{βδ}\textsuperscript{−/−} B cells.

Fig. 3. Serum ANA and kidney IC deposition in TCR\textsuperscript{βδ}\textsuperscript{−/−} mice reconstituted with T cells. (A) ANA analysis of sera from TCR\textsuperscript{βδ}\textsuperscript{−/−} mice after T cell transfer (see Methods for details). Staining by the PL9-6 anti-chromatin mAb and pooled sera from aged MRL lpr/lpr mice were used as positive controls. The data shown are representative of those obtained in two separate experiments from a total of nine mice that received T cells. Of the sera obtained from these animals, six gave rise to strong nuclear staining only, one gave rise to strong perinuclear staining and two gave rise to strong cytoplasmic staining only. Original magnification of all images was ×128. (B) An example of IC deposition in the kidneys of TCR\textsuperscript{βδ}\textsuperscript{−/−} at day 28 post-T cell transfer. Immune complexes were revealed using a FITC-labeled goat anti-mouse κ light chain mAb. Kidneys taken from aged-matched mice just prior to transfer (day 0) were used as controls. The data are representative of those obtained from six mice. Original magnification was ×128.
Most convincingly, flow cytometric analysis of the B cell compartment in C57BL/6 T → TCRβδ+− reconstituting mice revealed that only a subpopulation of B cells in this compartment transiently expressed cell-surface markers characteristic of activation.

Nonetheless, transfer of mature T cells may have other, more global effects on the B cell population of TCRβδ+− mice. This was suggested by our observation that sIgD levels were low on the majority of splenic B cells in these mice, but returned to normal shortly after injection of T cells. A role for T cells in the primary development of B cells has been previously suggested (21,40,41). For example, Wortis and colleagues have shown that mice bearing both the nude mutation and the mutation in Btk characteristic of CBA/N mice lack both mature B cells and T cells, whereas CBA/N mice display an absence of only certain mature B cell subpopulations. The influence of T cells on the development of B cells in this system appears to be manifested during pre-B cell development. While further experiments on the influence of mature T cells on primary B cell development in TCRβδ+− mice is clearly warranted, we think it unlikely that such an effect could alone account for the transient autoimmune response we have described, for reasons detailed above.

Autoantibody production has not been detected in previous experiments in which mature splenic T cells were transferred into T cell-sufficient mice (42,43). However, temporary autoantibody responses to single-stranded DNA and cardiolipin have been observed after repeated immunization of non-autoimmune mice with apoptotic thymocytes (42). Indeed, there is mounting evidence that perturbation of apoptotic pathways or of the removal of apoptotic cells is a contributory factor to the development of systemic autoimmune disease (44,45). It is possible that transfer of T cells to TCRβδ+− mice results in increased levels of apoptotic cells in the secondary lymphoid organs. This might result from death of a major fraction of the transferred cells or from perturbation of homeostatic regulatory pathways leading to death of large numbers of host cells. Further studies will be required to stringently test these ideas. However, we did not observe a reduction in T cell numbers following transfer, and B and NK cell populations were observed to expand. Moreover, immunization with apoptotic cells does not give rise to antibodies characteristic of frank systemic autoimmune disease (e.g. ANA−, anti-double-stranded DNA) (42), whereas we observed high titers of such antibodies in C57BL/6 T → TCRβδ+− mice.

The autoantibody response in C57BL/6 T → TCRβδ+− mice appears to be derived from a pre-existing subpopulation of autoreactive cells in TCRβδ+− mice that is absent in T cell-sufficient mice. Transfer of TCRβδ+− B cells into syngeneic, B cell-deficient μMT mice resulted in increased and sustained serum levels of anti-DNA and anti-chromatin autoantibodies as compared to B cells from control mice. The level of autoantibody production in the TCRβδ+− B → μMT mice was lower than in the C57BL/6 T → TCRβδ+− mice, but this could have resulted from a poor reconstitution of the B cell compartment in the non-irradiated hosts, as previously observed in experiments using SCID mice (35,36). In addition, polyclonal activation of purified TCRβδ+− B cells in vitro resulted in secretion of 2- to 3-fold higher levels of anti-

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**Fig. 5.** T cell reconstitution in the absence of autoantibody production in TCRβδ+− mice injected with CD40 ligand-deficient splenic T cells. (A) Splenic T cells (1×10⁶) purified from CD40 ligand-deficient mice were injected into TCRβδ+− mice, and the mice were bled 28 days later and sera assayed for levels of total IgM and IgG antibodies (closed symbols) and κ-bearing anti-chromatin autoantibodies (open symbols) by ELISA. In the total Ig assay, closed squares connected by a dotted line indicate pre-T cell transfer serum levels (values and standard errors obtained from five mice), closed circles indicate Ig levels in pooled C57BL6 sera and closed triangles indicate Ig levels in pooled sera obtained from aged MRL lpr mice. In the anti-chromatin assay, open squares indicate data obtained from five post-transfer mice, and open circles and open triangles indicate data obtained from pooled C57BL6 sera and pooled sera from aged MRL lpr mice respectively. (B) Flow cytometric evaluation of T cell reconstitution of TCRβδ+− mice by CD40 ligand-deficient splenic T cells. The data were obtained as described in Fig. 1(B) from a mouse 116 days post-transfer and are representative of results obtained from a total of four animals at this time point.
Fig. 6. Analysis of surface activation markers on splenic B cells in TCRβδ−/− mice after T cell transfer. Cells were prepared as described in Methods, and simultaneously stained with anti-B220 and mAb specific for the indicated activation markers. Stained cells were analyzed with a Coulter Epics Elite cytometer using live lymphocyte forward and side scatter and B220− gates. Each panel shows data in the form of histograms from three mice per time point (indicated with lines of different colors) except day 0 and C57BL/6 controls, which show data obtained from two mice.
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Fig. 7. Levels of surface IgM and IgD expression on B cells in TCRβδ−/− mice before and at various times after T cell transfer. Spleen cells were simultaneously stained with FITC-labeled anti-IgM, PE-labeled anti-B220 and biotin-labeled anti-IgD plus streptavidin-Red670. Stained cells were analyzed with a Coulter Epics Elite cytometer using a live lymphocyte forward and side scatter gate. Data from B220+/H11001 cells are shown in the form of two-dimensional density plots. The results are representative of the data obtained from three mice per time point and two separate experiments. The R3 gate highlights cells that appear to lack expression of both surface IgM and IgD, and the percentages of total cells in this gate at each time point are indicated. This gate was set using the data obtained from C57BL/6 cells.

Peripheral B cell tolerance is effected at several stages of differentiation (46,47). The first stage seems to target a population of 'transitional' B cells that are recent emigrants from the adult bone marrow. Studies exploiting mice expressing transgene-encoded, autoreactive BCR have shown that engagement of autoantigen by recent B cell bone marrow emigrants inhibits their developmental maturation and results in a state of 'anergy'. In several transgenic systems, this state of 'anergy' is associated with partial activation, alterations in homing and a reduced life span (10–12,48). In these systems, autoreactive B cells accumulate in the T cell rich PALS of the spleen, close to the follicular boundary. Studies by Goodnow and colleagues have also suggested that in this microenvironment, the death of anergic B cells is facilitated by interaction with CD4 T cells via the Fas–Fas ligand pathway (17).

Given the results of these previous studies, and the data presented here, we suggest that the deficiency in T cells in TCRαβ−/− mice leads to perturbations in this first step of peripheral B cell tolerance induction. In such mice, T cells and the T cell-rich microenvironments that prevent autoreactive transitional/anergic B cells from entering the follicle are absent. Once such B cells have gained access to the follicle in TCRβδ−/− mice, we suggest they often undergo further maturation and their life span is increased, despite the autoreactivity of their BCR. Upon introduction of T cells into TCRβδ−/− recipients, those B cells whose BCR are being cross-linked by multivalent antigens would be receptive to T cell help while other B cells would not. These B cells would then mount a vigorous GC and antibody response. These conclusions are consistent with our findings that TCRβδ−/− mice have elevated levels of peripheral B cells, many of which express reduced levels of sIgD, and that a subset of B cells in these mice can produce autoantibodies after stimulation with LPS in vitro or after transfer to B cell-deficient mice.

However, since the T cells transferred in our experiments are self-tolerant, how could they provide cognate help to follicular autoreactive B cells? Although a general, yet transient breakdown of T cell tolerance during reconstitution cannot be ruled out, we suspect that this is due to the presence of T cells, many of which are previously primed and have specificities for commonly encountered environmental...
antigens. In TCRβδ−/− mice, all B cells, including the pre-existing autoreactive subset, would be processing and presenting such antigens at low levels, irrespective of the specificity of their BCR (49,50). In addition, a large fraction of B cells in normal mice have been shown to express ‘multireactive’ BCR, capable of binding both self and foreign antigens (51–56). The subset of the latter type of B cells specific for environmental antigens would be particularly receptive to the help provided by incoming T cells specific for these same antigens, due to their ability to efficiently endocytose and shunt this antigen into the MHC class II antigen processing and presentation pathway via the BCR.

Recent studies have shown that subsequent to transfer of mature T cells into syngeneic, irradiated, T-depleted or congenitally T cell-deficient mice donor T cell proliferation is induced. This proliferation does not take place in hosts with intact T cell compartments and seems to require the recognition of the same MHC-peptide ligands by the donor cells that positively selected their precursors in the thymus (57,58). In the experiments described here, such homeostatic and self-ligand-induced proliferation might have promoted both the reconstitution of the T cell compartment, as well as the activation of T cells specific for environmental antigens, that could then provide help for ‘multireactive’ B cells. Further studies will be required to investigate these ideas.

Given the scenario for the development of the autoimmune response in C57BL/6 T cell → TCRβδ−/− mice discussed above, why would this response be transient? The establishment of a functional T cell compartment would be expected to reduce the further entry of autoreactive ‘transitional/anergic’ B cells into the follicular microenvironment. The production and maintenance of serum levels of antibodies to environmental antigens would result in the clearance of these antibodies, inhibiting the further recruitment of naive T cells into this response. These processes would inhibit the continued induction of a primary immune responses to autoantigens, as well as the differentiation of autoreactive pre-plasma cells that would require high levels of T cell help for further differentiation (59,60).

It seems unlikely that the activity of previously primed autoreactive and ‘multireactive’ B cells participating in the GC reaction would be substantially influenced by reduction in circulating environmental antigen levels, however. In fact, the stimulatory form of antigen in the GC is most probably IC. In the experiments described here, such homeostatic and self-ligand-induced proliferation might have promoted both the reconstitution of the T cell compartment, as well as the activation of T cells specific for environmental antigens, that could then provide help for ‘multireactive’ B cells. Further studies will be required to investigate these ideas.

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