Activation and Tolerance in CD4$^+$ T Cells Reactive to an Immunoglobulin Variable Region

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Abstract

Antibody diversity creates an immunoregulatory challenge for T cells that must cooperate with B cells, yet discriminate between self and nonself. To examine the consequences of T cell reactions to the B cell receptor (BCR), we generated a transgenic (Tg) line of mice expressing a T cell receptor (TCR) specific for a κ variable region peptide in monoclonal antibody (mAb) 36-71. The κ epitope was originally generated by a pair of somatic mutations that arose naturally during an immune response. By crossing this TCR Tg mouse with mice expressing the κ chain of mAb 36-71, we found that κ-specific T cells were centrally deleted in thymi of progeny that inherited the κTg. Maternally derived κTg antibody also induced central deletion. In marked contrast, adoptive transfer of TCR Tg T cells into κTg recipients resulted in T and B cell activation, lymphadenopathy, splenomegaly, and the production of IgG antichromatin antibodies by day 14. In most recipients, autoantibody levels increased with time, Tg T cells persisted for months, and a state of lupus nephritis developed. Despite this, Tg T cells appeared to be tolerant as assessed by severely diminished proliferative responses to the Vκ peptide. These results reveal the importance of attaining central and peripheral T cell tolerance to BCR V regions. They suggest that nondeletional forms of T tolerance in BCR-reactive T cells may be insufficient to preclude helper activity for chromatin-reactive B cells.

Key words: lymphocytes • SLE • antinuclear antibody • self-tolerance • glomerulonephritis

Introduction

The sequence diversity within Ab V regions presents a challenge to a CD4$^+$ T cell repertoire that provides help to B cells during humoral immunity, yet seems to avoid helping B cells with self-reactive receptors under physiological circumstances. Although several studies have provided evidence that the T cell repertoire attains a state of tolerance to germline-encoded Ab sequences (1, 2), it is unknown whether tolerance results from T cell interactions with self-presenting B cells or with soluble Ab products presented by professional APCs and whether tolerance occurs primarily in the thymus or the periphery. Moreover, almost nothing is known regarding T cell tolerance to somatically generated V region sequences.

Various experimental models have revealed that failure to develop or maintain tolerance to Ig-derived peptides results in either B cell death, or survival of B cells that no longer present antigenic T cell epitopes (e.g., through isotype switching; references 3–7). However, in models where tolerance is not attained, T cells specific for self-antigens that are ubiquitously expressed by B cells can induce B cell activation and autoantibody production (8–11). Given that B cells can self-present clonally unique B cell receptor (BCR)–derived peptides (1, 12–16), we and others have proposed that such peptides might provide an unregulated avenue of help for B cells in spontaneous systemic autoimmunity. Several studies in spontaneously lupus-prone mice have provided support for this idea (17–23).

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Abbreviations used in this paper: BCR, B cell receptor; CFSE, carboxyfluorescein diacetate succinimidyl ester; FR1, framework-1; GVH, graft-versus-host; H&E, hematoxylin and eosin; RT, room temperature; Tg, transgenic.

To investigate the consequences of a T cell encounter with a BCR-derived peptide, we generated a complementary pair of transgenic (Tg) mice. Our κTg mice express the κ chain of mAb 36-71, in which a class II–restricted T cell epitope is located within Vκ framework-1 (FR1). This epitope was originally created by two somatic mutations in codons 7 and 8 (1, 2). CA30 Tg mice express corresponding αβ TCR transgenes encoding a receptor specific for the Vκ 36-71 epitope. We found that central tolerance was tight in double Tg mice. However, adoptive transfer of mature CA30 T cells into κTg recipients resulted in multiclonal B cell activation, antinuclear antibody production, and lupus nephritis. This was associated with a persistence of CA30 T cells that, paradoxically, appeared to be refractive in proliferation assays. Although our results underscore the importance of maintaining specific T cell tolerance to Ig V regions, they suggest that nondeletional forms of tolerance may be insufficient to protect against the development of antinuclear Ab.

Materials and Methods

Tg Mice. κTg and Ars/A1 mice were described previously (1, 24). The κTg and μδ Tg mice were crossed to an A/J genetic background (8 and 10 backcrosses, respectively). To produce CA30 Tg mice, α and β TCR genes of the hybridoma A30.2.11 were amplified by PCR from cDNA using DyNAzyme (Finnzyme Oy; MJ Research Inc.) and primers 1 and 2 (α) or 3 and 4 (β). All primers are listed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20031234/DC1). The resulting PCR products were cloned independently into the EcoRI site of the VA-CD2 vector, provided by D. Kioussis (Medical Research Council, London, England, UK; references 25–27). The V gene sequences were verified, using the sequencing primers 5–8. TCR Vα1 and Vβ8.2 sequences were identical to those published (National Center for Biotechnology Information accession no. XM 290046; reference 28). CDR3 sequences are listed in Fig. S1 A. After removal of plasmid sequences, the TCR α– and β–containing constructs were mixed together in equal proportions, and Tg mice were produced using standard techniques.

s*MHC Tetramer Construction.* Using an overlapping primer strategy, an oligonucleotide encoding the Vκ 36-71 FR1 peptide (DIQMTQIPSSLSA) was linked to the 5′ end of the gene encoding the β chain of I-A^k. To this end, the vector pBACp10ph (provided by J. Kappler, National Jewish Medical and Research Center, Denver, CO; reference 29), containing an I-A^k construct, was used in two rounds of PCR as a template for primers 11 and 12, and 11 and 13. Primers 12 and 13 contained DNA encoding the Vκ 36-71 peptide sequence. The resulting DNA consisting of the 5′ end of the I-A^k β construct connected to DNA encoding the Vκ 36-71 peptide was cloned into pBACp10ph (29) using the EcoRI and SpeI sites, and the integrity of the construct was verified by sequencing.

To increase protein stability, a leucine zipper was introduced to the carboxy terminal ends of the α and β chains. A plasmid encoding the zipper templates was provided by J. Bill (University of Colorado School of Medicine, Denver, CO). The basic zipper chain was linked to an I-A^k α chain, and the acidic zipper chain was linked to the I-A^k β chain. The acidic zipper template was amplified with primers 14 and 15 and cloned into the I-A^k β gene using the NheI and SphI restriction sites. The basic zipper template was amplified with primers 17 and 18, introduced into the I-A^k α gene via amplification with primers 16 and 17 and cloned into the pBACp10ph vector (29) using XhoI and KpnI sites. This strategy destroyed the thrombin cleavage sites. Sequence verification revealed zipper sequences that were otherwise identical to those published previously (30).

Soluble I-A^k-peptide tetramers were produced as described previously (29, 31, 32). Soluble I-A^k protein was detected with mAb 10.3.6 (33) and biotinylated mAb 11.5.2 (33) and affinity purified with sepharose conjugate of anti–I-A^k mAb 10.3.6. Bound protein was washed in sodium bicarbonate (50 mM, pH 8) and eluted with sodium carbonate (50 mM, pH 11). Staining with the tetrameric reagent was performed at 37°C for 3 h before the addition of secondary antibodies for FACS® analysis.

Cell Purification. Single cell suspensions were obtained from thymi, LNs, or spleens as described previously (1). Thymocytes and LN cells were used without further manipulation unless otherwise indicated. Erythrocytes were removed from splenocyte preparations by lysis with ammonium chloride. In some cases, splenocyte APCs were fractionated on the basis of their specific gravity with Percoll as described previously (1). For most proliferation assays and intracellular cytokine detection, T cells were isolated by negative selection following the StemSep™ magnetic separation protocol (StemCell Technologies Inc.). Typically, this protocol yielded T cells that were 85–95% pure as judged by FACS® analysis.

Adoptive Transfers, Immunizations, and Injections. CA30 LN cells were isolated as aforementioned, washed once in PBS, and injected into a lateral tail vein of A/J or κTg recipient mice (not irradiated). Some recipient mice were immunized with 30 μg of Vκ 36-71 peptide emulsified in CFA and injected either intraperitoneally or subcutaneously. Spleens or draining LNs were harvested for proliferation and cytokine assays.

Flow Cytometry. Conjugated mAbs were purchased from BD Biosciences, and cells were stained using standard staining procedures. Biotinylated antibodies were visualized with streptavidin-PE (Biosource International) in most cases. Cells were analyzed on FACsCalibur™ or FACscan™ flow cytometers using CELL Quest™ software (Becton Dickinson).

Proliferation Assays. Various numbers of purified T cells were cultured with a fixed number (2×10^5) of lightly irradiated (1,100 rad) splenocytes as a source of APCs from an unmanipulated mouse. Stimulator peptide was added at the indicated concentrations, and the culture was incubated for 4–5 d at 37°C in 5% CO_2. [^{3}H]Thymidine (1 μCi/well) was added during the final 20 h of culture, and incorporation was detected with a Wallac Microbeta, using Beta-Scint scintillation fluid (both obtained from Wallac).

Analysis of Serum Antibody. Chromatin-specific serum IgG was detected on plates coated with either 10 μg/ml of mouse chromatin or 10 μg/ml BSA. Total IgG was detected on plates coated with 5 μg/ml Fc-specific goat anti–mouse IgG (Sigma-Aldrich). All assay plates (Corning) were coated overnight at 4°C and incubated with blocking buffer (2% BSA, 1% gelatin in PBS containing 0.05% Tween 20) for 1–2 h at 37°C or room temperature (RT). Mouse serum was incubated at the indicated dilutions for 1 h at 37°C or RT. IgG antibodies were detected with biotinylated goat anti–mouse IgG (Southern Biotechnology Associates, Inc.) followed by europium-labeled streptavidin (Wallac). Europium fluorescence at 615 nm was measured as described previously (1). Chromatin-specific Ig isotypes were detected with horseradish peroxidase–coupled goat anti–mouse isotype-specific antibodies (Southern Biotechnology Associates, Inc.) followed by colorimetric detection of 2,2′-azino-bis(3ethylbenz-thiazoline-6-sulfonic acid) at 405 nm (Victor 2; Wallac).

Analysis of Antinuclear Antibodies. Sera from individual mice were incubated for 30 min in a humidified chamber on slides containing NOVA-Lite HEP-2 cells (Inova Diagnostics). Antinuclear antibodies were detected with a FITC-labeled $F(ab)_2$ fragment of goat anti–mouse IgG (Fc specific; Sigma-Aldrich).

Analysis of Kidney Pathology. For tests of Ig deposition, kidneys were quick frozen in Tissue-Tek OCT (Sakura Finetek Europe B.V.) and stored at −80°C. Serial sections of 6–8 μm were transferred to positively charged microscope slides (ProbeonPlus; Fisher Scientific), fixed in acetone, and stored at −80°C. Sections were thawed at RT and blocked with 5% FCS in PBS for 10 min at RT. Subsequently, the sections were incubated with FITC-coupled goat anti–mouse isotype antibodies for 30 min at RT. Sections were mounted using Gel/Mount (Biomeda). Images were taken on a Marianas workstation using Slidebook 4.0.6.4 (both obtained from Intelligent Imaging Innovations, Inc.). Hematoxylin and eosin (H&E) staining was performed on kidneys fixed in 1% formalin by the Histology Core Laboratory at the National Jewish Medical and Research Center.

Online Supplemental Material. Table S1 lists the primers used in the project. Table S2 gives weights and numbers of cells in LNs and spleens of κTg recipients on day 10. Table S3 illustrates activation markers on B cells at day 10 in κTg mice that received different numbers of CA30 cells. Table S4 shows the Ig isotypes of antichromatin antibodies. Fig. S1 illustrates normal expression levels and allelic exclusion of the CA30 transgene-encoded TCR. Fig. S2 shows intracellular IL-2 in stimulated CA30 cells. Fig. S3 illustrates poor allelic exclusion in peripheral CD4 cells of double Tg mice. Fig. S4 shows the total IgG and antichromatin titers in sera of recipients of 10^7 CA30 cells. Kidney pathology in recipients of CA30 cells is compared at days 28 and 130 in Fig. S5. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20031234/DC1.

Results

Phenotype of CA30 Tg T Cells. To study CD4 T cell reactions to antibody V region diversity, we developed two complementary lines of Tg mice. One line expresses the κ chain of mAb 36-71, which contains a well-characterized T cell epitope, created by a pair of somatic mutations at codons 7 and 8 within FR1 (1, 2). Expression of endogenous Ig heavy chains in these mice creates a relatively diverse B cell repertoire with normal numbers of resting B cells (1). The second line of Tg mice (CA30) expresses an αβ TCR from hybridoma A30.2.11, which is specific for the Vκ 36-71 peptide presented in the context of I-A^k. In CA30 Tg mice, >90% of $CD3^+$ LN cells were $CD4^+$ and $V\beta8^+$, and the level CD3 expression was nearly identical among $CD4^+$ T cells of Tg and nonTg littermates (Fig. 1 A and see Fig. 2 D, and Fig. S1 B, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1).

To identify Tg T cells, we also developed a covalent Vκ 36-71–I-A^k tetrameric staining reagent. The tetramer stained Vκ 36-71–specific T cell hybridomas and ~95% of peripheral CD4 T cells in CA30 Tg mice (Fig. 1 A and see Fig. 2 D). Allelic exclusion appears to be tight in Tg mice, as <1% of the peripheral CD4 T cells expressed an alternative endogenous α chain (Vα2) in both young and adult mice (Fig. 1 B and Fig. S1 C). In addition, CD44 expression was reduced in frequency among T cells of naive CA30 Tg mice relative to nonTg littermates, indicating that few T cells had encountered cognate Ag in CA30 mice (unpublished data).

Specificity and Function of CA30 Tg T Cells. To assess the biological activity of CA30 T cells, we performed a series of in vitro and in vivo tests. Initially, LN cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with various concentrations of Vκ 36-71 peptide. 2 d later, the cultures were analyzed by FACS® for tetramer staining and CFSE fluorescence. At high peptide concentrations, virtually all of the $tetramer^+$ cells had undergone at least one round, and most cells had undergone two rounds of division (Fig. 1 C). Furthermore, nearly all of these cells produced IL-2 as measured by intracellular cytokine staining (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1).

CA30 T cells were also directly stimulated by κTg splenocytes. As predicted from our previous studies (1) and shown in Fig. 1 D, CA30 Tg T cells proliferated vigorously to low density splenocytes ($\rho < 1.079$) from κTg mice. Similarly, CA30 T cells were strongly stimulated by spleno-

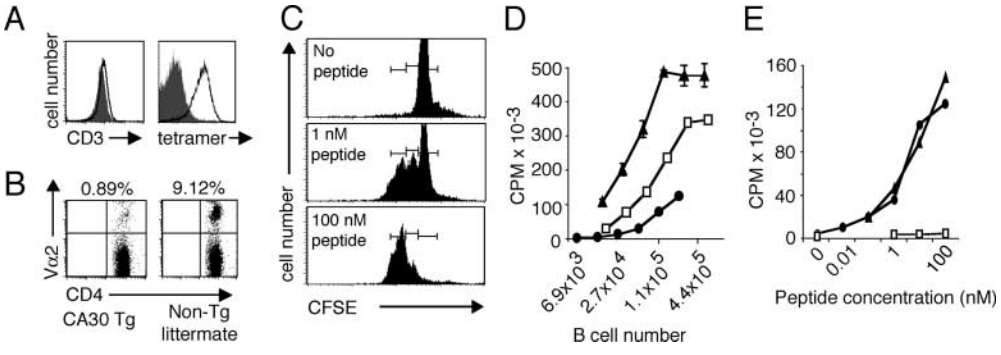


Figure 1. Tg expression and allelic exclusion in CA30 TCR Tg mice. (A) Comparison of CD3 levels and tetramer staining (Vκ 36-71 FR1-I-A^k) in $CD4^+$ LN cells from CA30 (dark line) and nonTg (shaded histogram) mice. (B) Allelic exclusion in $CD4^+$, CA30 cells. Vα2 is the only A/J α V region for which a specific antibody is available. (C) Proliferation of $tetramer^+$ cells in vitro as assessed by loss of CFSE intensity after 2 d in culture with indicated concentrations of Vκ 36-71 peptide. (D) Proliferation of CA30 T cells as assessed by [^{3}H]thymidine incorporation after 2 d in culture with various numbers of splenocytes from anergic Ars/A1 mice (closed triangles) or κTg mice (closed circles, high density, $\rho > 1.079$; open squares, low density, $\rho < 1.079$). The number of B cells per well was calculated based on the frequency B220 positive cells. (E) In vivo stimulation of CA30 T cells. LNs were harvested from nonTg A/J mice that received 2×10^6 CA30 Tg LN cells and were either immunized subcutaneously (closed symbols) or not (open symbols) with 30 μg of Vκ 36-71 peptide in CFA. T cells were purified and proliferation was measured by [^{3}H]thymidine incorporation.

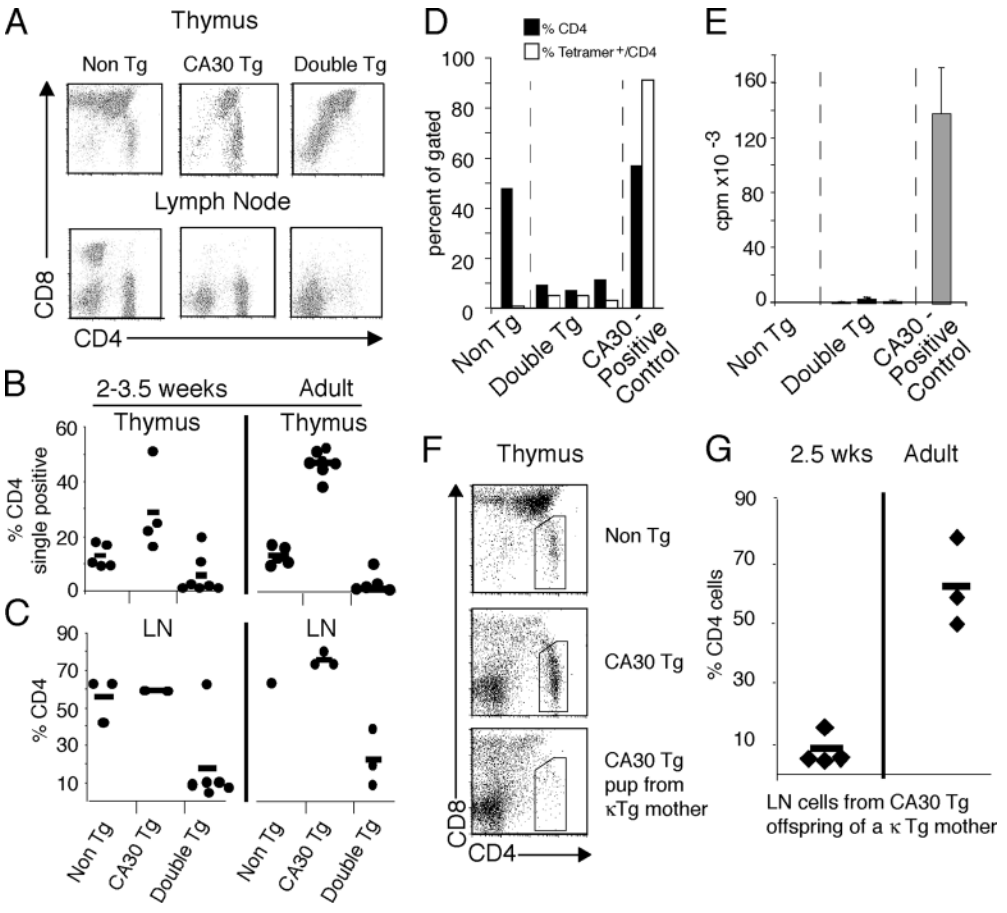


Figure 2. Central deletion of CA30 CD4$^+$ T cells in mice that produce the κTg Ab or that have maternally derived κTg Ab. (A) Representative FACS® plots of thymic and LN cells from double Tg, CA30 single Tg (derived from TCR-only breeders), and κTg adult mice stained with anti-CD4 and CD8. (B and C) Frequencies of single positive CD4$^+$ thymic and LN T cells from multiple mice represented graphically. (D) Percentages of CD4 T cells among total LN cells and of Vκ 36-71–I-A^k tetramer$^+$ cells among CD4 T cells. (E) Proliferation of LN T cells purified and stimulated with 1 μM Vκ 36-71 peptide for 5 d. (F) Representative FACS® plots of thymocytes from 2.5-wk-old offspring of a male CA30 mouse bred with a female κTg mouse. (G) Frequencies of CD4$^+$ cells among CD4$^+$ LN cells from CA30 single Tg progeny of κTg mothers.

cytes containing anergic Ars/A1 Tg B cells, which express the mAb 36-71 κ chain in conjunction with a μδ Tg (24). In contrast, proliferation induced by high density resting splenocytes was weak in agreement with our previous findings (1). For in vivo tests of biological activity, 2×10^6 CA30 Tg LN cells were transferred into nonTg recipients. Immediately after transfer, the recipient mice were immunized subcutaneously with the Vκ 36-71 peptide emulsified in CFA. T cells recovered from peripheral LNs of recipient mice at day 10 responded strongly to the Vκ 36-71 peptide in vitro, both by proliferation and intracellular IL-2 production (Fig. 1 E and not depicted).

Thymic Deletion of CA30 T Cells in Mice Expressing Vκ 36-71. In a test for central tolerance, CA30 Tg mice were bred to Tg mice expressing the κ chain of mAb 36-71. Fig. 2 A shows representative FACS® analyses of thymic and LN cells from double Tg mice and age-matched controls. Compiled data from several mice are shown in Fig. 2 (B and C). In addition to the percent decrease in CD4 cells shown in Fig. 2 (A–C), a total reduction of 30–40-fold in CD4$^+$ cell numbers was seen in thymi and LNs of double Tg mice (not depicted). This large reduction in cell numbers is consistent with central thymic deletion at the CD4$^+$/CD8$^+$ double-positive stage.

A closer analysis of peripheral CD4 T cells in double Tg mice indicated that very few of these cells expressed the Tg receptor, as shown by MHC tetramer staining (Fig. 2 D). Relative to CA30 Tg mice, double Tg mice had a higher percentage cells expressing Vα2 among CD4$^+$ T cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1), although this percentage was similar to that in age-matched nonTg littermates. These appear to be cells that escaped thymic deletion due to incomplete enforcement of allelic exclusion by the TCR Tg mice. Consistent with the low proportion of tetramer staining cells in double Tg mice, purified LN T cells from such mice failed to proliferate above background levels to the Vκ 36-71 peptide (Fig. 2 E). This result further reveals the extent of CD4$^+$ T cell tolerance to the Vκ epitope.

Central Tolerance Induced by Maternally Derived Vκ 36-71. To test whether APCs that process and present exogenous antibody could mediate thymocyte deletion, we analyzed offspring of κTg mothers that had inherited the TCR Tg but not the κ transgene. These mice should only be exposed to maternally derived κ Ig transmitted either transplacentally or through milk. In very young mice (2–3.5 wk of age), we observed profound deletion of Tg T cells in both the thymus and the periphery (Fig. 2, F and G). However, the CD4 T cell populations began recovering shortly after weaning, and by 7 wk of age, the percentages of CD4, Vβ8, and tetramer-staining populations were nearly normal (Fig. 2 G and not depicted). Moreover,

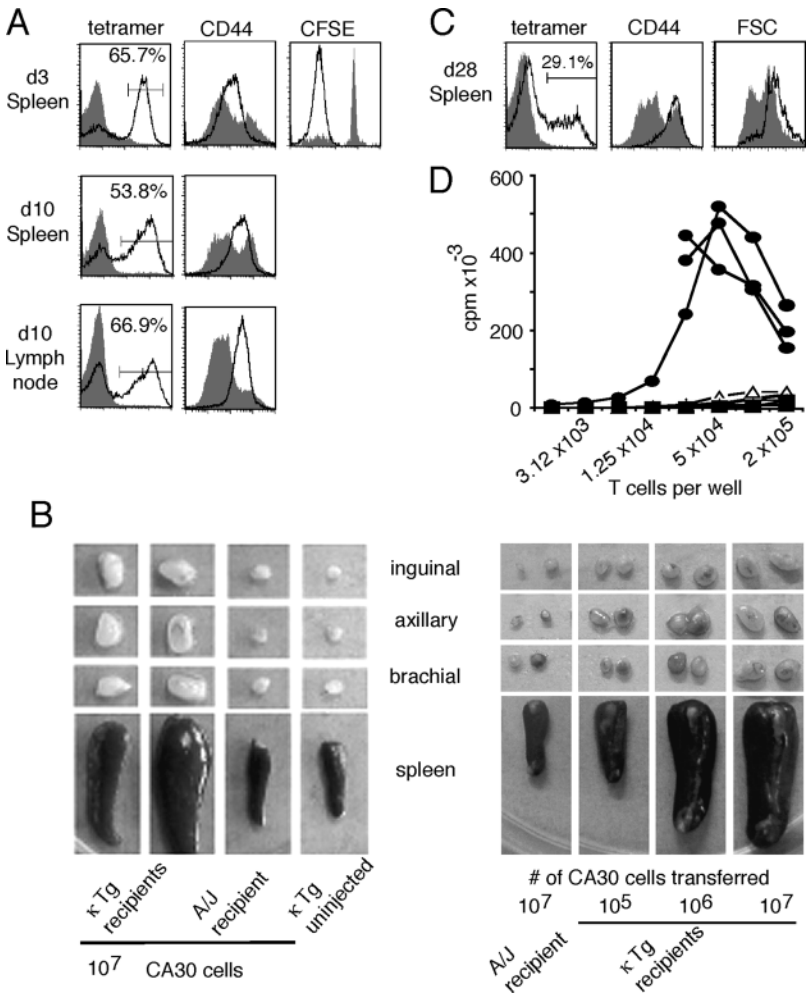


Figure 3. Activation and proliferation of B and T cells in κTg recipients of CA30 cells. (A) Representative FACS® plots of tetramer$^+$ T cells at 3 and 10 d after injection of 10^7 CA30 LN cells into κTg recipients (dark line) or control A/J recipients (shaded histogram). Day 3 tetramer staining is shown for CD4-gated cells, and day 10 tetramer staining is shown for CD4$^+$, CD16/CD32$^-$ cells. CFSE profile is shown for tetramer$^+$, CD4$^+$ cells. CD44 profile is shown for tetramer-gated cells (dark line) and tetramer$^-$ cells (shaded histogram). (B) LNs and spleens harvested from mice 10 d after CA30 cell transfer. Spleen images are not proportional to lymph node images (left). (C) Representative FACS® plots of tetramer$^+$ T cells 28 d after the injection of 10^7 CA30 LN cells into a κTg recipient. Tetramer staining is shown for CD4$^+$, CD16/CD32$^-$ splenocytes. CD44 staining and forward scatter (FSC) analyses are shown for tetramer$^+$ cells (dark lines) and tetramer$^-$ T cells (shaded histograms) from the same mouse. (D) Proliferation of purified T cells from spleens or LNs of κTg recipients 10 d (closed circles) after transfer or from spleens of κTg recipients (open triangles) and A/J recipients (closed triangles) 28 d after transfer. The purified T cells were stimulated for 5 d with 1 μM of Vκ 36-71 peptide. Immunized κTg recipients (closed squares) were injected i.p. on day 24 and isolated on day 28 for proliferation tests.

Vα2-expressing T cells were more abundant in young than adult CA30 Tg offspring of κTg mothers (unpublished data). These results demonstrate that Vκ-specific T cells are subject to deletion by maternally derived antibody, and that the repertoire recovers with time presumably due to antibody clearance.

T Cell Activation in κTg Adoptive Recipients of CA30 Cells. Although our results implicated central tolerance as a mechanism to regulate Ig V region–specific CD4 T cells, they did not address possible redundant regulation by peripheral tolerance mechanisms. To test for peripheral tolerance, we transferred 10^7 CA30 LN cells into κTg recipients or control A/J recipients. By 3 d after transfer, the Tg T cells had expanded dramatically in κTg recipients, as assessed by tetramer staining and loss of CFSE fluorescence in cells that were labeled before transfer. At day 10, tetramer-binding T cells uniformly expressed elevated levels of CD44 and still comprised a large percentage of splenic and LN T cells (Fig. 3 A). Furthermore, κTg recipients of CA30 LN cells that were killed 10 d after transfer displayed enlarged LNs and spleens, suggesting massive proliferation (Fig. 3 B, left, and Table S2, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1). Even 28 d after transfer, the spleens of κTg mice receiving 10^7 CA30 cells were still dramatically enlarged, and tetramer-binding T cells comprised ~30% of the splenic CD4 T cell population (Fig. 3 C). Although splenic architecture was generally intact, the white pulp was significantly expanded, particularly follicles and germinal centers. Similar but less dramatic results were obtained upon transfer of fewer CA30 cells into κTg recipients (Fig. 3 B, right). Transfer of 10^6 CA30 cells induced less severe lymphadenopathy and splenomegaly at day 10, in which 7–11% of LN and splenic CD4$^+$ cells were tetramer$^+$. 10^5 CA30 T cells induced lymphadenopathy without splenomegaly, in which tetramer staining cells comprised ~3% of the peripheral CD4$^+$ population.

T Cells Become Refractive with Time after Transfer. T cells were recovered from κTg recipients and tested for proliferative responses to the Vκ 36-71 peptide in vitro. Interestingly, T cells recovered 10 d after transfer responded strongly, whereas those recovered 28 d after transfer did not proliferate above background levels (Fig. 3 D). Moreover, immunization with Vκ 36-71 peptide emulsified in CFA did not restore the proliferative capacity of the T cells recovered at day 28 (Fig. 3 D). As assayed by proliferation,

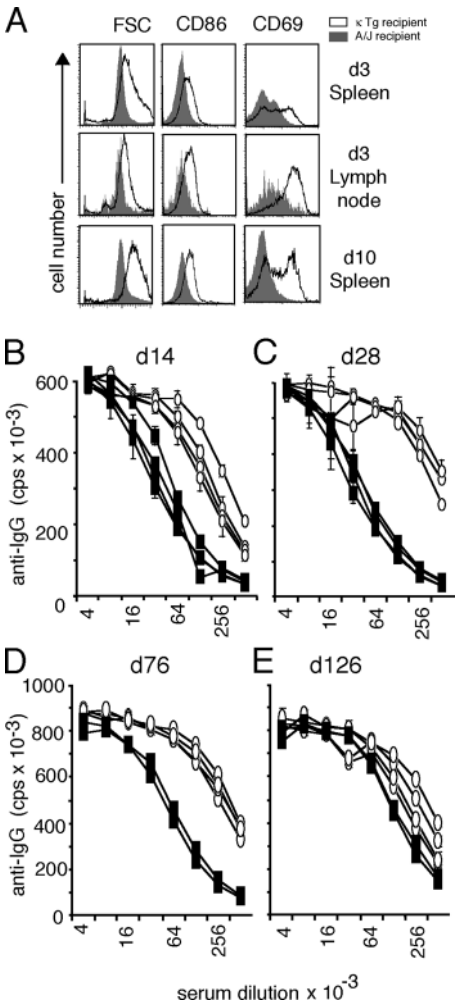


Figure 4. Activation of κTg B cells and induction of IgG antibody in κTg recipients of CA30 cells. (A) Representative FACS® plots showing forward scatter (FSC), CD69, and CD86 for B220-gated cells at 3 and 10 d after CA30 cell transfer. (B–E) IgG antibody detected in sera of κTg (open symbols) and A/J (closed symbols) recipients at the indicated days after CA30 cell transfer. Binding assays for all sera were conducted on the same day.

these results indicate that the transferred T cells were functional early, but rendered tolerant with time.

B Cell Activation in κTg Adoptive Recipients of CA30 Cells. We also investigated the effect of transferred CA30 T cells on B cells. As shown in Fig. 4 A, κTg B cells in recipients of 10^7 CA30 cells expressed increased levels of activation markers. On day 3 after transfer, CD86 levels were increased on most B cells in LNs and spleen, whereas high levels of CD69 were expressed on most B cells in LNs and ~35% of B cells in the spleen. By day 10, CD86 expression remained high on a substantial fraction of splenic B cells, whereas CD69 was elevated on ~50% of splenic B cells. In contrast, B cells of A/J mice that received CA30 cells had staining profiles identical to those of control uninjected κTg mice (unpublished data). Qualitatively similar results were seen in κTg mice that received fewer CA30 cells. However, in these recipients, frequencies of B cells expressing CD86 and CD69 were reduced relative to those of mice that received 10^7 CA30 cells (Table S3, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1).

We quantified serum Ig levels in several of the κTg recipients and observed significant increases in total IgG levels by day 14, with further increases at day 28 (Fig. 4, B and C). By day 76, IgG titers were still high, although reduced somewhat relative to those of day 28, and further reductions were evident at day 126, when titers approached baseline levels seen in control A/J recipients (Fig. 4, D and E). The same trend was observed in a second experiment involving five κTg recipients (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1). This observation suggests that the κTg recipients of CA30 cells were able to recover from a multiclonal B cell response that occurred at early time points.

Development of Antichromatin Ab in κTg Adoptive Recipients of CA30 Cells. Significant titers of antichromatin IgG antibodies were observed in sera of all κTg recipients tested at days 14 and 28 after adoptive transfer ($n = 9$; Fig. 5, A and B, and Fig. S4 B). The presence of antinuclear antibodies was verified by staining of HEP-2 cells (Fig. 5, C and F). However, only a small fraction of total serum IgG bound chromatin at these early time points. On day 14, the concentration of antichromatin IgG in κTg recipients was ~1–2 μg/ml, whereas total IgG concentration was several milligrams per milliliter. Moreover, from days 14 to 28, chromatin-specific IgG increased only proportionally to the total increase in IgG. These data indicate that the transferred T CA30 cells initially activated not only chromatin-specific B cells but also B cells with other antigenic specificities.

However, with time, chromatin-specific antibodies increased in both absolute and relative terms, with some variability among individual mice. In an initial experiment, titers of anti-chromatin antibodies were substantially increased in two out of four κTg recipients at day 76 relative to titers measured on day 28. One of these mice maintained elevated levels through day 126 (Fig. 5 E). In a second experiment, five out of five recipients demonstrated elevated antichromatin titers that were retained for at least 4 mo after transfer (Fig. S4 B). Using the high-affinity 3H9/Vκ4 as a quantitative standard (34, 35), we determined that the concentrations of antichromatin Ab ranged from 7–70 μg/ml at day 130. Collectively, the serological analyses indicated that, in most κTg recipients, chromatin-specific B cells were preferentially stimulated to produce Ab as time progressed after CA30 cell transfer.

Persistent CA30 T Cells in Most κTg Recipients. We speculated that failed tolerance in CA30 T cells might account for a prolonged production of autoantibodies in most of the recipients. To test this idea, we first assayed for tetramer-binding cells in blood samples from the four κTg recipients of the initial experiment. Tetramer$^+$ cells were only observed in the two recipients (nos. 3 and 4) that contained high titers of antichromatin Ab at day 76 (unpublished data). In a more rigorous test for Tg T cells, we immunized the two mice with lower autoantibody levels

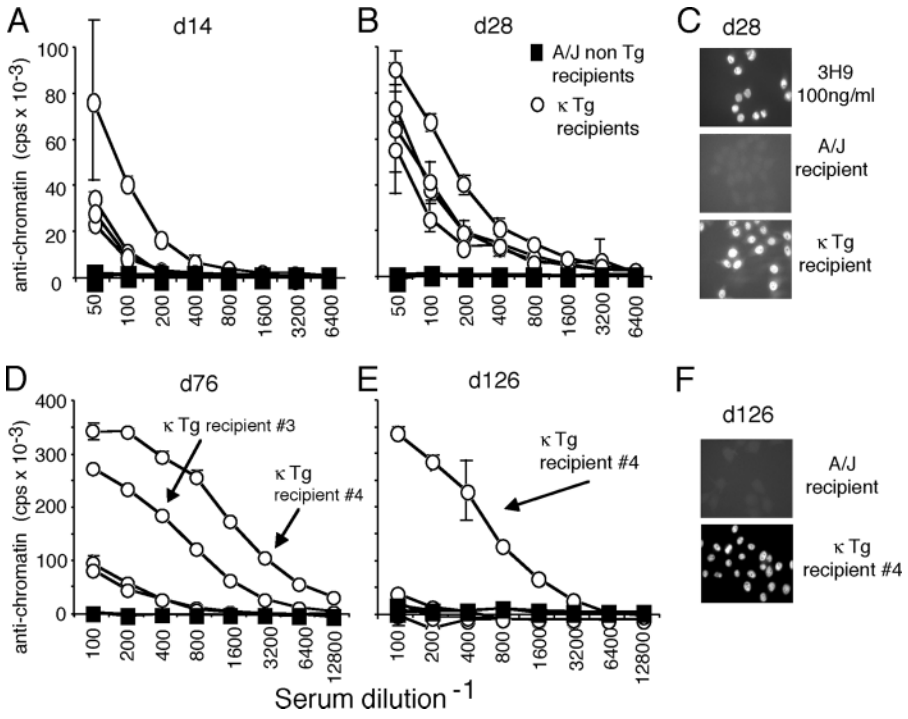


Figure 5. Induction of antichromatin IgG in κTg recipients of CA30 cells. (A, B, D, and E) Chromatin-specific IgG antibody in the sera of recipient mice. Counts detected on BSA-coated plates were subtracted from those detected on chromatin-coated plates at each dilution point. Binding assays for different sera were conducted on the same day. (C and F) Antinuclear staining of HEP-2 cells (sera diluted 1:100). 3H9 (Vκ4) is a prototypic antinuclear mAb.

(nos. 1 and 2) and stained splenocytes 3 d later (day 126) for tetramer-binding cells. Despite immunization, the frequencies of tetramer$^+$ cells were not significantly greater than those seen in control A/J recipients (Fig. 6 A). In contrast, clearly defined tetramer$^+$ populations were evident in the recipients with high antichromatin titers.

To test for Tg T cell function, we conducted in vitro proliferation assays using splenocytes from the κTg recipients of both experiments. In all cases, purified T cells proliferated poorly to the Vκ 36-71 peptide. Fig. 6 B illustrates results for the five κTg recipients (second experiment), which all had persistent antichromatin titers. In these mice, an average of 2.3% of CD4$^+$ splenocytes were tetramer$^+$. Nevertheless, their responses on a per cell basis were less than one-tenth of that by tetramer$^+$ cells from an immunized A/J recipient. Similar weak responses were seen for tetramer$^+$ T cells from κTg recipients of the first experiment (unpublished data). Thus, chronic antichromatin responses were accompanied by CA30 T cells in κTg recipients at late time points, but these cells demonstrated severely diminished proliferative responses to antigen in vitro.

Evidence of Inflammation and Kidney Involvement. To test for antibody deposition in glomeruli, we stained frozen kid-

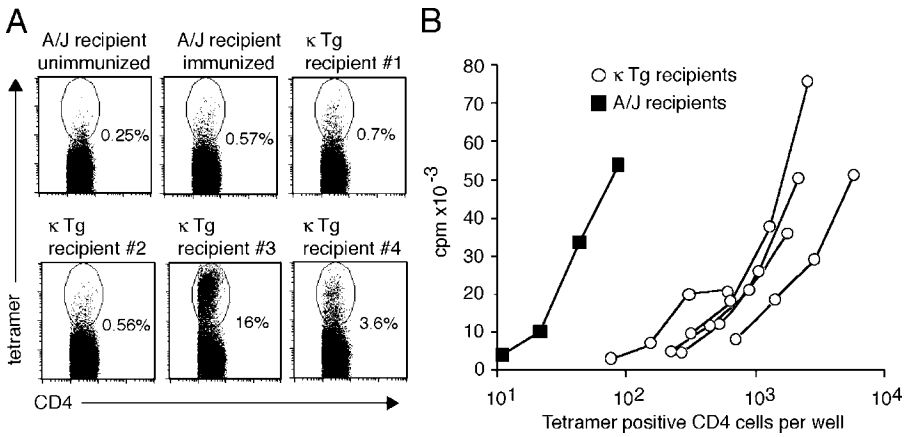
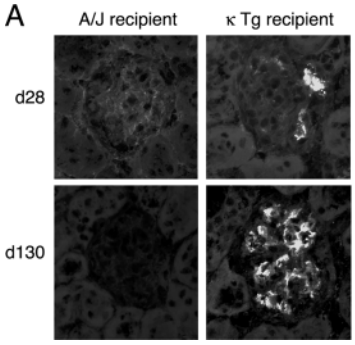


Figure 6. Persistent but refractive CA30 T cells in some κTg recipients. (A) Tetramer staining of CD4$^+$ CD16$^-$/CD32$^-$ splenocytes of κTg and A/J recipients killed 126 d after CA30 cell transfer. κTg mice nos. 1 and 2 and an A/J recipient mouse were immunized 3 d before the stain (Vκ 36-71 peptide, 30 μg of i.p. CFA). These two mice produced a much weaker antichromatin Ab response at day 76 than mice nos. 3 and 4, which were left unimmunized. (B) Proliferative responses of splenocytes from long-term κTg recipients of CA30 cells (second experiment). [^{3}H]Thymidine incorporation, normalized to frequencies of tetramer$^+$ cells, was measured at day 5 in splenocyte cultures containing 10 ng of Vκ 36-71 peptide. A/J recipients (closed squares) immunized on day 0. Five κTg recipients (open circles) of 10^7 CA30 cells killed on day 130. These are the same mice analyzed for antichromatin Ab and kidney pathology in Figs. S4 and S5, and in Table S4.



B

Recipient mouse	Day	Kidney Ig IgM	IgG1	IgG2a	IgG2b	IgG3	H&E
κ Tg	28	++	(+)	(+)	-	+	normal
		++	(+)	(+)	-	(+)	normal
	130	+++	+++	+++	-	++	EGCI
		+++	+++	+++	-	++	EG
		+++	+++	+++	-	++	EG
		+++	+++	+++	-	++	EGCI
		++	++	++	-	+	normal
A/J	28	+	-	(+)	-	(+)	normal
	130	+	-	-	-	(+)	normal

Figure 7. Kidney pathology in κTg recipients of CA30 cells. (A) Immunofluorescent detection of IgG2a in kidneys of recipients of CA30 cells. (bottom right) Data are representative of all five κTg recipients at day 130. (top right) Data show segmental staining seen at day 28 in 3 out of 10 mice that received CA30 cells. Two of these mice received 10^7 CA30 cells, and the third received 10^6 cells. Magnification, 40. For color images, see Fig. S5. (B) Isotype profile of IgG deposits and H&E results for various kidney sections. The two κTg recipients at day 28 (top) showed segmental staining pattern. EG, enlarged glomeruli; EGCI, enlarged glomeruli with cellular infiltration.

ney sections from κTg recipients of CA30 cells with a panel of antiisotype antibodies. Little or no staining was observed in kidneys of mice killed at day 10 after transfer. However, by day 28, some recipients of higher numbers of CA30 cells displayed infrequent segmental staining in a few glomeruli (3/10 mice). However, no evidence of disease was observed upon H&E staining. At day 130, in contrast, kidneys of all five κTg recipients of 10^7 CA30 cells demonstrated global deposition of antibody in virtually all glomeruli (Fig. 7 and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1). Moreover, H&E staining revealed enlarged glomeruli in four out of five mice, with cellular infiltrates in two of these four animals (unpublished data). In all five mice, antibodies of multiple classes, including IgM, IgG1, IgG2a, and IgG3 were deposited in glomeruli, with IgG1 and IgG2a predominating. Of note, these are the same two classes that were found to predominate in kidney deposits within spontaneously autoimmune (NZB × NZW)F1 mice (36). Because these were the same five mice aforementioned that contained persistent antichromatin Ab in sera, we were able to define their isotypes as well. In the case of sera, the predominant serum antichromatin IgG was IgG2a, followed by IgG3 and IgG2b (Table S4, available at http://www.jem.org/cgi/content/full/jem.20031234/DC1). The IgG2a and IgG3 subclasses are associated with the inflammatory cytokine, IFN-γ, which is known to induce class switch recombination to γ2a and γ3 constant region genes. IgG2a is also the predominant serum isotype of antinuclear Ab in two widely studied murine models of spontaneous lupus: the (NZB × SWR)F1 mouse and the MRL *lpr/lpr* mouse (37). Collectively, these data reveal manifestations of chronic lupuslike disease in recipients of T cells that react to a BCR-derived peptide.

Discussion

To examine activation and self-tolerance in helper T cells that are specific for BCR V regions, we generated a line of αβ TCR Tg mice with $CD4^+$ T cells reactive to a peptide derived from the somatically mutated κ V region of mAb 36-71. Central tolerance was examined by breeding these CA30 Tg mice to mice expressing a 36-71 κTg, and peripheral tolerance was assessed by transferring CA30 Tg T cells into mice expressing the κTg. Our results revealed clear evidence of central tolerance by thymic deletion in CA30 mice that either inherited the κTg or that acquired maternal antibody derived from it. However, results of peripheral tolerance studies were more complex. Transfer of CA30 Tg T cells into κTg recipients led to an initial widespread activation of B cells; massive proliferation of CA30 T cells; and a graft-versus-host (GVH)–like disease manifested by lymphadenopathy, splenomegaly, hypergammaglobulinemia, and low levels of serum antichromatin Ab. However, with time after transfer, hypergammaglobulinemia gave way to a stronger and more specific antichromatin response with kidney pathology in most recipients, and this was associated with a persistence of CA30 T cells. This antichromatin response progressed through day 76 in seven out of nine recipients, despite evidence of T cell tolerance by day 28 as assessed by proliferative responses to the Vκ 36-71 peptide. To our knowledge, this is the first clear example of chronic autoimmunity induced by a defined $CD4^+$ T cell reactive with an Ig V region sequence. It lends support to the hypothesis that BCR-derived peptides may provide an avenue of T cell help to autoreactive B cells in systemic autoimmune disease (17–23).

Evidence for central $CD4^+$ T cell tolerance to Ig sequences has been reported by several groups (38–42). However, two Tg systems previously developed to address this question yielded contrasting results. Bogen et al. (40) found that T cells specific for a V region peptide from the λ light chain of MOPC-315 underwent thymic deletion in mice that either expressed the cognate Ig or that were injected with soluble antibody at a high concentration. These results are consistent with others suggesting that wild-type $IgG2a^b$-specific T cells attain tolerance in the thymus (41). In contrast, Granucci et al. (7) found that $IgG2a^b$-specific T cells from a TCR Tg mouse failed to undergo negative selection in mice endogenously expressing $IgG2a^b$. In agree-

ment with the MOPC-315 result, we observed a near complete deletion of the Tg T cells. Although indirect evidence has been published suggesting that maternally derived Ig can induce central tolerance (3, 42–44), our results provide the first direct evidence of thymocyte deletion resulting from interactions with maternally derived Ig. Nevertheless, although it is clear from our data that high levels of serum Ig can induce central tolerance in Ig V region–reactive $CD4^+$ T cells, it is still unclear whether this tolerance can be induced centrally by physiologically low levels of IgM bearing a particular V region.

The consequences of a cognate T cell–B cell interaction mediated by a BCR-derived V region peptide are largely unknown. Two different outcomes might be predicted from clues in the literature. Some evidence suggests that B cells might be suppressed (3–5, 45). This outcome is predicted from studies using Ig allotype-specific T cells or clones that induce allotype suppression. Results from other models suggest instead that an Ig–peptide-directed interaction between a $CD4^+$ T cell and a B cell will result in B cell activation. Singh et al. (22) demonstrated that immunization of BALB/c mice with a V region peptide derived from a lupus-associated Ab, resulted in transient autoantibody production. The striking implication of this result is that chromatin-specific B cells received T cell help directed to BCR V region–derived peptides that were self-presented by B cells in the context of class II MHC. The temporary nature of autoantibody production observed by Singh et al. is similar to what was seen in two of our κTg recipients of CA30 T cells. Our results are also reminiscent of those from several models in which T cell help was directed to peptides derived from natural or Tg-encoded membrane antigens expressed by B cells (8–11). The early hypergammaglobulinemia, splenomegaly, and lymphadenopathy seen in our transfer model are particularly reminiscent of $CD4^+$ T cell–induced GVH disease with lupuslike manifestations (10, 11). Although it is clear that initial B cell activation is multiclonal, more interesting is the time-dependent specific increase in antichromatin IgG, which is accompanied by a significant reduction of total serum IgG. This indicates that chromatin-reactive B cells are selectively stimulated with time after CA30 cell transfer. A common feature of spontaneous murine lupus is a progressive, oligoclonal antichromatin response (17, 46, 47).

Although the outcomes of our transfer model and the previously described models are similar in some respects, our model is distinct in that recipient mice contained high levels of serum Ig bearing the cognate T cell epitope. In this sense, it is striking that CA30 Tg T cells were not immediately rendered tolerant due to high levels of 36-71 κ chain in recipient mice. There is considerable evidence that IgG can be tolerogenic for $CD4^+$ T cells, and our recipient mice contained milligram per milliliter quantities of serum IgGκ (48–53). However, studies from one group have provided evidence that IgM can be immunogenic (53–55). Thus, it is possible that APCs presenting Vκ 36-71 peptide derived from IgM stimulated the transferred CA30 T cells at early time points before tolerance could be induced by APCs presenting Vκ 36-71 peptide derived from IgG. The subsequent rise in total IgG levels could explain an observed delay in the acquisition of T cell tolerance; CA30 Tg T cells recovered 10 d after transfer proliferated strongly in vitro in response to the Vκ 36-71 peptide, whereas T cells recovered 28 d after transfer were much less responsive to antigenic stimulation.

Although all κTg recipients of CA30 cells consistently developed early manifestations of GVH-like disease, there was considerable variation in the duration and level of the specific antichromatin IgG response. Seven out of nine mice had high serum Ab titers at day 76, and in six of these mice, the titers were maintained or further increased to varying levels at the final, 4-mo time point. The two recipients with only low titers of antichromatin IgG at day 76 had no clearly detectable CA30 cells at the end point of the experiment. In all others, defined populations were evident upon tetramer staining. At face value, these observations suggest that a persistent CA30 population is required for long-term production of antichromatin Ab. Nevertheless, the recovered CA30 cells responded only poorly to the Vκ 36-71 peptide in proliferation assays.

In a well-defined model of peripheral tolerance, transfer of Tg $CD4^+$ T cells into a host ubiquitously expressing cognate antigen led to a state of "adaptive tolerance" that was manifested by poor proliferative and cytokine responses to antigen (56). An interesting feature of adaptive tolerance is its apparent reversibility, which can occur if T cells are removed from the antigenic environment that provides constant stimulation (57, 58). In our transfer model, it is intriguing that an antichromatin IgG response persisted in the face of antigen-refractive CA30 T cells. This apparent discrepancy could be explained by the adaptive tolerance idea if chromatin-specific B cells are more effective stimulators of T cells than are other APCs. CA30 T cells that are refractive to the Vκ 36-71 peptide presented by professional APCs may be able to respond to the same peptide that is self-presented by chromatin-specific B cells. This might occur if the B cells present a higher concentration of the Vκ peptide due to BCR internalization after chromatin engagement. Alternatively, chromatin-activated B cells may provide enhanced or unique costimulatory signals for T helper cells. CpG motifs in chromatin apparently enhance B cell activation by triggering Toll-like receptor 9 (59, 60). It is conceivable that this pathway might enhance T cell costimulation as well. Further studies will be required to address this hypothesis.

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