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*J Immunol* 2004; 173:3524-3534; doi: 10.4049/jimmunol.173.5.3524

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Mechanism of Action of Transmembrane Activator and Calcium Modulator Ligand Interactor-Ig in Murine Systemic Lupus Erythematosus

Meera Ramanujam,*† Xiaobo Wang,*† Weiqing Huang,*† Lena Schiffer,*† Christine Grimaldi,† Alla Akkerman,*† Betty Diamond,*† Michael P. Madaio,‡ and Anne Davidson2*†

B cell-activating factor belonging to the TNF family (BAFF); also known as B lymphocyte stimulator; TNF-homolog that activates apoptosis, NF-kB, and JNK; and zTNF4), is an important survival factor for B cells. BAFF is produced predominantly by myeloid cells and binds to three distinct receptors on the B cell surface, transmembrane activator and calcium modulator ligand interactor (TACI), B cell maturation Ag (BCMA), and BAFF-R (1, 2). Engagement of BAFF-R by BAFF is an absolute requirement for B cell survival and maturation in secondary lymphoid organs. BAFF also mediates class switching to IgG (3) and promotes the survival of plasmablasts (2, 4, 5). A proliferation-inducing ligand (APRIL), another TNF family member, is highly homologous to BAFF, but binds to only two of the three BAFF receptors, TACI and BCMA (6, 7). Recombinant APRIL induces B cell proliferation and class switching (3), and APRIL transgenic mice have increased levels of serum IgM and increased IgM responses to T-independent Ags (8).

BAFF-deficient mice have small spleens with few B cells, and they mount an attenuated humoral immune response to T-dependent and T-independent Ags (9, 10). Conversely, BAFF transgenic mice develop B cell hyperplasia and an autoimmune lupus-like disease (2, 11, 12). Overexpression of BAFF has been observed in spontaneous murine systemic lupus erythematosus (SLE) models and in humans with autoimmune diseases (13, 14). These findings have suggested that antagonism of BAFF may be a useful therapeutic approach for autoimmune disease.

Antagonism of BAFF can be achieved by administration of a soluble form of any of the three BAFF receptors. Both TACI-Ig and BAFF-R-Ig have been reported to delay the onset of SLE in the murine NZB/W F1 model (2, 15). We have generated an adenovirus expressing a fully murine TACI-Ig fusion protein to study the mechanism for this effect and to determine whether antagonism of BAFF would be synergistic with an agent that blocks T cell costimulation. We show that administration of a single dose of adenovirus expressing TACI-Ig to prenephritic NZB/W F1 mice together with six doses of CTLA4-Ig results in profound depletion of splenic B cells and a delay in disease onset. The long delay in disease onset after a short course of TACI-Ig, with or without CTLA4-Ig, is associated with a decrease in the absolute number of activated lymphocytes of all lineages. TACI-Ig treatment also results in a long term survival disadvantage for IgM-producing plasma cells in the spleen, but does not appear to have a sustained effect on the survival of IgG-producing plasma cells. Finally, a short course of TACI-Ig and CTLA4-Ig can reverse nephritis and prolong survival even in older mice, suggesting that B cell depletion may be an effective therapeutic strategy for active SLE.

Materials and Methods

Generation of an adenovirus expressing fully murine TACI-Ig

The extracellular domain of murine TACI (aa 2–129) was cloned from NZB/W F1 spleen cDNA by RT-PCR and fused to a protease signal...
Mice were challenged with 50 μg/CTLA4-Ig or Ad-LacZ/CTLA4-Ig. Mice were bled at weekly intervals, mice were killed at 26 wk for ELISPOT and Flow cytometry experiments and to groups of Ad-TACI-Ig/CTLA4-Ig- and Ad-LacZ/CTLA4-Ig-treated mice, 10 mice). Comparisons were performed with 10 mice that received CTLA4-Ig alone and 20 untreated controls.

Mice were bled, and urine was tested for proteinuria by dipstick (Multistick; Fisher Scientific, Pittsburgh, PA) every 2 wk. Mice were followed until death. Separate groups of Ad-TACI-Ig/CTLA4-Ig- and Ad-LacZ/CTLA4-Ig-treated mice were killed at 26 wk for ELISPOT and flow cytometry experiments and at 36 wk for renal histology. Finally, a separate group of 10 mice was treated with Ad-TACI-Ig/CTLA4-Ig at the age of 30 wk. We elected to use the combination treatment for remission induction studies, because it appeared to be more efficacious at preventing the onset of proteinuria than Ad-TACI-Ig alone. These mice were compared with five mice treated at 30 wk with Ad-LacZ/CTLA4-Ig and 15 untreated controls.

Anti-dsDNA Abs

Anti-dsDNA ELISA was performed as previously described (17). For measurement of affinity for dsDNA, sera with maximal IgG anti-dsDNA titers were identified for each mouse, and inhibition assays were performed as previously described (18) by preincubating sera at the predetermined concentration that resulted in 80% maximal binding to the plate with increasing amounts of dsDNA from 2–200 μg/ml.

Oxazolone (Ox) immunization of NZB/W F1 mice

To evaluate the effect of TACI-Ig on the humoral immune response to a T cell-dependent Ag, groups of five NZB/W F1 mice were immunized with 750 μg of Ox by skin paint 8 wk after treatment with Ad-TACI-Ig/CTLA4-Ig or Ad-LacZ/CTLA4-Ig. Mice were bled at weekly intervals, and anti-Ox Abs were measured by ELISA as previously described (19). Mice were challenged with 50 μg of Ox by skin paint 4–6 wk later, and spleens were harvested for ELISPOT analysis as previously described (18) 3 days after boosting. To determine whether TACI-Ig interfered with established memory B cells in treated mice, 10 NZB/W F1 mice, aged 14 wk, were immunized with 750 μg of Ox. Four weeks later, Ad-TACI-Ig/CTLA4-Ig or Ad-LacZ/CTLA4-Ig was administered as described above to groups of five mice. Once serum TACI-Ig levels became undetectable (6 wk after virus administration), mice were challenged with 50 μg of Ox by skin paint. The mice were bled 7 days later for measurement of anti-Ox Abs, and bone marrow was harvested 14 days after challenge for analysis of anti-Ox responses by ELISPOT as described above.

Anti-dsDNA ELISPOT assay

Spleens were harvested at 35–37 wk of age by survival splenectomy. At this time control mice were beginning to develop proteinuria, but treated mice remained well. ELISPOT assays for total IgG-secreting cells and for anti-dsDNA-secreting B cells were performed as previously described (17).

Generation of hybridomas

Hybridomas were generated from spleen cells by standard techniques using NSO cells as the fusion partner. Hybridomas were screened for anti-Ox or anti-dsDNA activity, and positive hybridomas were isotyped as previously described (20).

Analysis of class switching and somatic mutation

Evidence of active class switching was sought by semi quantitative RT-PCR analysis of IgG2b I-C transcripts from spleens of five Ad-TACI-Ig/CTLA4-Ig-treated and five age-matched control mice as previously described (17). PCR of these transcripts yielded a single band. The amount of activation-induced cytidine deaminase (AID) was measured by semi quantitative RT-PCR using specific primers (5'-CCTCCGCTAATGACTGACATC and 3'-GTCTGTACCCGAGCAGA).

To determine whether somatic mutation was occurring in autoreactive B cells, we analyzed the autoreactive V<sub>λ</sub>BW-16 gene, a marker gene for the anti-dsDNA response (21). IgG CDNA libraries were constructed by RT-PCR from the spleens of seven Ad-TACI-Ig/CTLA4-Ig treated mice, four Ad-TACI-Ig treated mice, and eight age-matched controls and were screened for V<sub>λ</sub>BW-16 as we have previously described in detail (17). Sequences were compared with the germ line V<sub>λ</sub>BW-16 sequence using BLAST search (www.ncbi.nlm.nih.gov/blast/).

Flow cytometry and immunofluorescence analysis of spleens

Spleen and peripheral blood cells were analyzed for B and T cell markers using Abs to CD4 (Caltag Laboratories, Burlingame, CA), CD8 (Caltag Laboratories), and CD19. T cell subsets were identified using FITC-anti-CD4, PE-anti-CD69, CyChrome-anti-CD44, and PE-anti-CD62L. B cell subsets were identified using biotin-anti-CD23, FITC-anti-CD21, PE- or FITC-anti-IgM, PE-anti-IgD, allophycocyanin-anti-CD19, CyChrome-anti-CD5, PE-anti-B220, PE-anti-CD43, and PE-anti-CD69. Streptavidin-PerCP was used as a second stain for biotinylated Abs. Spleen and peripheral blood dendritic cells were identified using PE-anti-CD11b and FITC-anti-CD11c. Except where indicated, all Abs were purchased from BD Pharmingen (San Diego, CA).

For immunofluorescence staining, 5-μm cryosections were stained with FITC-anti-mouse IgM, PE-anti-mouse IgD, FITC-anti-CD11c, FITC-anti-CD4, FITC-peanut agglutinin, and PE-anti-mouse CD45R/B220 (BD Pharmingen) in 10% normal goat serum-FCS/PBS for 1 h at room temperature. Images were captured using a digital CCD-camera system (Diagnostic Instruments, Sterling Heights, MI) connected to a microscope (Nikon, Melville, NY).

Immunohistochemical analysis of kidneys

Examination and scoring of the kidneys were performed as previously described (22).

Statistical analysis

Proteinuria and survival data shown in Fig. 2, A and B, and Fig. 10A were analyzed using Kaplan-Meier curves and log-rank test. The comparisons shown in Figs. 2C, 3, 4, 6, 7, 10B, 11, and 12G and in Tables I and II were performed using Wilcoxon’s rank sum test. The comparisons shown in Fig. 10C were performed using paired t test. The comparisons in Table III were performed using χ² analysis or Fisher’s exact test. Only significant p values are shown.

Ethical approval

All animal experiments were approved by the institutional animal review board of Albert Einstein College of Medicine.

Results

Clinical effects of TACI-Ig in NZB/W F1 mice

TACI-Ig was only detected in the serum of NZB/W F1 mice for 7–10 days after Ad-TACI-Ig injection. For this reason and because Ad-TACI-Ig treatment alone had only a modest effect on the development of proteinuria, in some mice the adenovirus was administered together with six doses of CTLA4-Ig. This resulted in the expression of TACI-Ig protein for 4–5 wk (Fig. 1). No IgG1 or

FIGURE 1. Mean ± SD serum TACI-Ig levels over time in five NZB/W F1 mice injected on day 0 with 10⁸ PFU of Ad-TACI-Ig alone (left panel) or together with six doses of 100 μg of CTLA4-Ig (right panel).
IgG2b Abs to TACI-Ig were detected 6 wk after virus administration (not shown).

The life span of NZB/W F1 mice treated with either Ad-TACI-Ig alone or both Ad-TACI-Ig and six doses of CTLA4-Ig (Ad-TACI-Ig/CTLA4-Ig) was significantly prolonged; however, Ad-TACI-Ig-treated mice developed proteinuria significantly earlier than those treated with Ad-TACI-Ig/CTLA4-Ig (Fig. 2C).

NZB/W F1 mice have a polyclonal increase in serum levels of IgM that continues to increase with age. Both Ad-TACI-Ig and Ad-TACI-Ig/CTLA4-Ig treatment caused a drop in serum IgM levels that was sustained for up to 6 mo after treatment (Fig. 3A). Serum IgG levels decreased for a short time after Ad-TACI-Ig/CTLA4-Ig, but were unaffected by treatment with Ad-TACI-Ig or Ad-LacZ/CTLA4-Ig (Fig. 3B). Serum IgA levels followed a similar pattern as IgG (Fig. 3C). The drop in serum levels of IgG after Ad-TACI-Ig/CTLA4-Ig was due predominantly to a decrease in levels of IgG1 and, to a lesser extent, of IgG2a (Fig. 3C).

IgM anti-DNA Abs were undetectable in the serum of /H1102270% of the Ad-TACI-Ig-treated mice up to the time that proteinuria developed, even in mice that had high serum titers of IgG anti-DNA Abs (Fig. 4A). The appearance in serum of IgG anti-DNA Abs was delayed in mice that received Ad-TACI-Ig/CTLA4-Ig compared with untreated controls (p < 0.001), but not compared with mice that received Ad-LacZ and CTLA4-Ig (Ad-LacZ/CTLA4-Ig; p = 0.09), suggesting that this delay was partly due to CTLA4-Ig (Fig. 4B). The affinity of the Abs for dsDNA, measured by inhibition assay, was the same in treated and control mice (Fig. 4C).

Analysis of B cell phenotype

At 26 and 36 wk of age (8 and 18 wk after treatment), the spleens of TACI-Ig-treated mice were significantly smaller and contained fewer cells than the spleens of control 16-, 26-, and 36-wk-old NZB/W F1 mice (Tables I and II). Thirty-six-week-old control NZB/W F1 mice had more B cells in the spleen than 16-wk-old controls due to an increase in follicular B cells and CD19/IgM/IgD-class-switched B cells (Table II). The spleens of both 26- and 36-wk-old, Ad-TACI-Ig/CTLA4-Ig-treated mice consistently had a lower percentage and absolute number of CD19-positive B cells than the spleens of age-matched, Ad-LacZ/CTLA4-Ig-treated mice and untreated controls. A decrease in the percentage of B cells was also observed at 26 wk in the lymph nodes (not shown) and peripheral blood of Ad-TACI-Ig/CTLA4-Ig treated mice compared with controls (25.2 ± 4.7 vs 45.4 ± 4.3%; p < 0.001).

To determine whether certain B cell subsets were preferentially depleted in the treated mice, CD19-gated cells were examined for markers to distinguish T1 (CD23/CD21/IgMhigh), T2 (CD23/CD21high/IgMhigh), marginal zone (MZ; CD23/CD21high), and B1 (B220low/CD5+) subsets. Because down-regulation of CD21...
and CD23 has been reported in BAFF-deficient mice (23), immature (IgM<sup>high</sup>/IgD<sup>low</sup>) and mature (IgM<sup>low</sup>/IgD<sup>high</sup>) B cells were also distinguished using staining with anti-IgM and anti-IgD with similar results (Fig. 5). Twenty-six-week-old, Ad-TACI-Ig/CTLA4-Ig-treated mice had the same number of B1 and T1 cells as controls, but fewer T2, MZ, and follicular B cells, consistent with a TACI-Ig-induced block in B cell maturation. This abnormality persisted in mice examined at 36 wk of age. The number of peritoneal B1 cells was unaffected by Ad-TACI-Ig/CTLA4-Ig treatment (not shown). Splenic CD19<sup>+</sup> cells were also examined for class-switched IgM<sup>+</sup>/IgD<sup>−</sup> cells and for the CD69 activation marker. Although the frequency of these cells was unchanged in Ad-TACI-Ig/CTLA4-Ig-treated mice (Table I), their total number was diminished compared with that in age-matched controls (Table II). Of the four Ad-TACI-Ig spleens examined, two resembled Ad-TACI-Ig/CTLA4-Ig mice, and two resembled age-matched control mice, reflecting a similar, but more short-lived, treatment effect in the absence of CTLA4-Ig.

Analysis of B cell function

To enumerate the frequency of Ig-secreting cells, ELISPOT assay was performed on equal numbers of spleen cells from treated and control mice. At both 26 and 36 wk of age, the frequency of total Ig-secreting cells was 2- to 3-fold less in Ad-TACI-Ig/CTLA4-Ig-treated mice than in controls, but due to the spleen enlargement in older untreated mice, it was 10- to 20-fold less in 36-wk-old, Ad-TACI-Ig/CTLA4-Ig-treated mice than in age-matched controls (Fig. 6C). Ad/TACI-Ig-treated mice displayed a mixed picture, and the mean frequency of Ig-secreting cells was between those in young and age-matched controls.

To determine whether Ad-TACI-Ig- and Ad-TACI-Ig/CTLA4-Ig-treated mice could mount a humoral immune response to a foreign Ag during the period of delay in disease onset, mice were immunized with Ox at 30 wk of age. IgM anti-Ox Abs were undetectable in the serum of both Ad-TACI-Ig- and Ad-TACI-Ig/CTLA4-Ig-treated mice 7 days after primary immunization. The IgG serum response to Ox was diminished compared with that in NZB/W F<sub>1</sub> controls only in the Ad-TACI-Ig/CTLA4-Ig-treated mice, but was higher than that in immunized BALB/c mice (Fig. 7A). The frequency of IgM and IgG anti-Ox-secreting splenic B cells, as evaluated by ELISPOT, was decreased by 2- to 3-fold in Ad-TACI-Ig/CTLA4-Ig-treated mice compared with controls, but not in Ad-TACI-Ig-treated mice, reflecting the decrease in the total number of Ig-secreting cells in Ad-TACI-Ig/CTLA4-Ig mice (Fig. 6). Few hybridomas were recovered from the Ad-TACI-Ig/CTLA4-Ig-treated mice, but hybridomas were readily recovered from the spleens of two Ad-TACI-Ig-treated mice that had low serum IgM anti-Ox Ab levels; the frequency of both IgM and IgG anti-Ox hybridomas in these mice was the same as that in controls (percent Ox-positive hybridomas, 8.8% with an IgG:IgM ratio of

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Table 1. Frequency of spleen cell subsets<sup>a</sup>

| % of Cells | 16 wk NZB/W (n = 5) | 26 wk NZB/W (n = 5) | Ad-LacZ CTLA4-Ig, 26 wk (n = 5) | Ad-TACI-Ig/CTLA4-Ig, 26 wk (n = 5) | 36 wk NZB/W (n = 8) | Ad-TACI-Ig/CTLA4-Ig, 36 wk (n = 7) | Ad-TACI-Ig, 36 wk (n = 4) |
|------------|---------------------|---------------------|-------------------------------|-------------------------------|---------------------|-------------------------------|---------------------|
| CD19 (mean ± SD) | 38.2 ± 2.1 | 43.7 ± 12.6 | 46.8 ± 3.3 | 24.2 ± 7.8 | 401 ± 59 | 22.2 ± 3.7 | 36.7 ± 11.4 |
| CD19/CD69 | 3.7 ± 0.4 | 3.5 ± 1.1 | 3.7 ± 1.3 | 4.2 ± 1.4 | 16.5 ± 2.7 | 18.6 ± 5.2 | 24 ± 4.8 |
| Follicular | 65.1 ± 1.3 | 61.2 ± 5.3 | 65.5 ± 5.1 | 53.5 ± 4.2<sup>a</sup> | 69.3 ± 4.9 | 52.0 ± 1.3<sup>a</sup> | 61.6 ± 5.8 |
| T<sub>1</sub> | 8.2 ± 1.8 | 5.9 ± 3.2 | 4.2 ± 1.7 | 15.1 ± 10.4<sup>ab</sup> | 5.6 ± 1.1 | 16.1 ± 2.7<sup>b</sup> | 7.8 ± 3.5 |
| T<sub>2</sub> | 4.6 ± 0.9 | 4.4 ± 2.8 | 5.1 ± 1.9 | 3.7 ± 1.6 | 3.5 ± 1.1 | 3.1 ± 0.8 | ND |
| MZ | 11.8 ± 2.5 | 13.8 ± 6.1 | 14.5 ± 4.9 | 7.2 ± 2.0<sup>c</sup> | 4.3 ± 2.0 | 4.1 ± 1.8 | ND |
| B1 | ND | ND | 3.8 ± 1.4 | 12.0 ± 2.9<sup>d</sup> | ND | ND | ND |
| IgM<sup>a</sup>/IgD<sup>b</sup> (switched) | 5.3 ± 1.0 | ND | ND | ND | 13.2 ± 3.5 | 120.0 ± 1.8 | 146.0 ± 5.6 |
| CD4 | 25.3 ± 2.0 | 30.0 ± 9.0 | 26.3 ± 4.8 | 42.5 ± 5.3<sup>e</sup> | 29.5 ± 6.0 | 33.3 ± 10.6 | 25.9 ± 5.4 |
| CD4/CD69 | 7.4 ± 2.3 | 17.4 ± 4.2 | 16.1 ± 9.2 | 11.0 ± 4.1 | 30.7 ± 2.9 | 16.2 ± 1.4<sup>f</sup> | 36.8 ± 12.2 |
| CD4/CD4<sup>+</sup>/CD62L<sup>e</sup> (memory) | 28.9 ± 10.1 | 48.1 ± 13.7 | ND | ND | 83.7 ± 3.6 | 60.0 ± 9.0<sup>e</sup> | ND |
| CD4/CD4<sup>+</sup>/CD62L<sup>e</sup> (naive) | 42.8 ± 13.5 | 29.5 ± 13.6 | ND | ND | 5.7 ± 1.6 | 15.0 ± 3.3<sup>e</sup> | ND |
| CD8 | 8.8 ± 1.6 | 9.6 ± 6.7 | 9.0 ± 4.3 | 18.3 ± 3.2<sup>e</sup> | 5.2 ± 1.0 | 20.5 ± 3.3<sup>e</sup> | ND |

<sup>a</sup> The p values are compared with age-matched controls. Only significant p values are shown.
<sup>b</sup> p < 0.002.
<sup>c</sup> p < 0.05.
<sup>d</sup> p < 0.01.
<sup>e</sup> p < 0.02.
<sup>f</sup> p < 0.001.
2.03 in Ad-TACI-Ig-treated mice vs 5.7% with an IgG:IgM ratio of 2.2 in controls).

To determine the effect of treatment on somatic mutation, analysis of the V_{H}-Ox-1 gene derived from IgG cDNA libraries from spleen was performed in Ad-TACI-Ig/CTLA4-Ig-treated mice. Examination of the V_{H} region showed a 28% decrease in the frequency of somatic mutations (35 mutations in 17 sequences compared with 65 in 23 control sequences), but no difference in the R:S ratio in the CDR regions (14:5 vs 22:7; Table III). Furthermore, the affinity of anti-Ox Abs of the IgG1 and IgG2a isotypes, measured using different coupling frequencies of Ox-BSA to coat the plates, was no different in Ad-TACI-Ig/CTLA4-Ig- or Ad-TACI-Ig-treated mice than in untreated NZB/W F1 controls (not shown). This suggests that functional germinal centers were formed. The difference in IgG titers between Ad-TACI-Ig/CTLA4-Ig and control mice appears to reflect a decrease in the total number of B cells in the treated mice.

The results presented above indicate that despite the small spleen size and the decrease in B cell frequency observed in Ad-TACI-Ig/CTLA4-Ig-treated mice at 36 wk of age, their B cells were still able to respond to T cell signals, undergo class switching and somatic mutation, and generate IgG-producing plasma cells. To confirm this result, we performed semiquantitative RT-PCR on spleen cDNA for IgG2b class switch transcripts and AID. We found that IgG2b class switch transcripts and AID expression correlated with each other and were diminished by ~50% in the TACI-Ig-treated mice (ratio of IgG2b:actin, 0.27 ± 0.18 vs 0.63 ± 0.11; p < 0.005), consistent with the 50% decrease in B cell frequency.

Immunohistochemical studies of spleens from 36-wk-old, Ad-TACI-Ig/CTLA4-Ig-treated NZB/W F1 mice revealed that lymphoid organization was intact, but follicles were smaller and contained fewer B cells and more CD4+ T cells than those in controls (Fig. 8, A–D). However germinal centers were still present within the follicles (Fig. 8, E and F). Cells staining intensely for cytoplasmic IgM were abundant in the splenic red pulp of control mice, but were diminished in both number and staining intensity in the spleens of Ad-TACI-Ig/CTLA4-Ig-treated mice (Fig. 8, G–J).

It is still not known whether flares of SLE occur as a result of recruitment of naive B cells to the autoreactive response or whether autoreactive memory cells are reactivated. Because of the marked depletion of B cells in the spleen, we determined whether

**FIGURE 5.** Representative FACs plots of B and T cell subsets. Cells were gated on CD19 (panels 3–5), both CD19 and CD23 (panel 2) or CD4 (panels 6 and 7). Twenty-six-week-old controls are shown in A, panels 1–4, and B, panel 6. Thirty-six-week-old controls are shown in A, panels 5–7. Ad-TACI-Ig/CTLA4-Ig-treated mice are shown in B, panels 1–4 (26 wk) and panels 5 and 7 (36 wk). Panel 1, Loss of B cells and relative increase in T cells in treated mice. Panel 2, Only the CD23+ population is shown. Note the decrease in marginal zone B cells (upper box) and increase in T1 cells (lower box) in treated mice. Panel 3, The oval shows marginal zone B cells; panel 4, the square shows B1 B cells. Panel 5, Immature (IgM(IgD)+) and class-switched (IgM(IgD)−) B cells are shown. Note the increase in immature B cells in treated mice. Panel 6, Loss of naive CD44lo/CD62Lhigh and increase in memory CD44hi/CD62Llow CD4 cells are shown in 36-wk-old compared with 26-wk-old control NZB/W. Panel 7, The square shows activated T cells.
a short course of TACI-Ig treatment would result in decreased survival of memory B cells. No difference in the secondary IgG anti-Ox response was detected in mice that had been immunized with Ox 4 wk before Ad-TACI-Ig/CTLA4-Ig treatment and boosted 6 wk post-treatment by either serologic assay (Fig. 7o or ELISPOT (controls, 24.3/H11006 15.6; treated, 22.6/H11006 9.9/105 bone marrow cells vs 4 in naive controls), indicating that memory B cells survived Ad-TACI-Ig/CTLA4-Ig treatment and could be reactivated upon Ag boosting after TACI-Ig and CTLA4-Ig were no longer present in the serum.

**Effect of TACI-Ig treatment on autoreactive B cells**

The above data indicate that despite their low numbers, some B cells in the Ad-TACI-Ig/CTLA4-Ig-treated mice were activated, could undergo class switching, and could acquire autoreactivity. ELISPOT analysis revealed that the decrease in the frequency of anti-dsDNA-secreting B cells of the IgM and IgG isotypes in Ad-TACI-Ig/CTLA4-Ig-treated mice paralleled the decrease in total Ig-secreting cells, indicating that there was no specific deletion of autoreactive B cells (Fig. 6).

To determine whether somatic mutation of an autoreactive V gene could occur in the small spleen, we analyzed the autoreactive VH BW16 H chain gene. This gene is overexpressed among anti-DNA Abs in NZB/W F1 mice and is regulated in normal mice (21). The amount of IgG VH BW16 transcript was decreased in Ad-TACI-Ig/CTLA4-Ig-treated mice, reflecting the decreased number of mature B cells. Thirty-seven different IgG VH -BW-16 sequences were obtained from the Ad-TACI-Ig/CTLA4-Ig-treated mice, and their mutation frequency and pattern were compared with those of untreated mice (44 sequences) or mice treated with Ad-LacZ/CTLA4-Ig (14 sequences). The overall mutation frequency of VH BW-16 was decreased by 43% in the Ad-TACI-Ig/CTLA4-Ig-treated mice compared with untreated controls (p < 0.06), but the frequency of replacement mutations in the CDR

**FIGURE 6.** Mean ± SD frequency of Ab-secreting B cells of the IgM (A) and IgG (B) isotypes from spleens of control and treated NZB/W F1 mice (four or five per group). The frequency is per 105 cells for total Ig and per 106 cells for anti-dsDNA Ig and anti-Ox Ig. Ad-LacZ/CTLA4-Ig-treated mice were indistinguishable from untreated controls (not shown). Ad-TACI-Ig/CTLA4-Ig-treated mice had fewer IgM and IgM anti-dsDNA- and anti-Ox-secreting cells than age-matched controls at both times studied. Ad-TACI-Ig/CTLA4-Ig-treated mice had fewer IgG and IgG anti-dsDNA- and anti-Ox-secreting cells than controls at 36 wk. Only significant p values are shown. C. Shown are absolute numbers of spots per spleen for the 36-wk mice.

**FIGURE 7.** TACI-Ig had a prolonged effect on the primary IgM response to Ox in NZB/W F1 mice (p < 0.02; A). Only the IgG2a response to Ox in Ad-TACI-Ig/CTLA4-Ig-treated mice was significantly different from controls (p < 0.02). In contrast, if the mice were primed before administration of TACI-Ig and were boosted after TACI-Ig was no longer present in the serum, the memory response to Ox was intact (B). Naive mice in this experiment received the boost immunization only. Five mice were examined per group, and values are expressed as the mean ± SD. A titration assay is shown in C.
regions was not decreased, and the pattern of mutations, including the accumulation of positively charged residues in CDR2, was similar in all three groups of mice analyzed (Table III and data not shown), indicating little effect of treatment on selection of B cells expressing \( V_{\mu} \text{BW-16} \).

Finally, to determine whether pathogenic Abs were present in Ad-TACI-Ig/CTLA4-Ig-treated mice, kidneys from three mice killed at the age of 36 wk were examined and compared with kidneys from three Ad-LacZ/CTLA4-Ig-treated controls. Glomerular damage was present in all three Ad-LacZ/CTLA4-Ig-treated mice (glomerular score, 2–3/100), but none was evident in Ad-TACI-Ig/CTLA4-Ig-treated mice (glomerular score, 0–1/100). Deposition of IgG was present in the glomeruli of all three Ad-TACI-Ig/CTLA4-Ig-treated mice as well as in the controls, indicating that the autoantibodies generated in Ad-TACI-Ig/CTLA4-Ig-treated mice had pathogenic potential (Fig. 9).

### Table III. Analysis of mutations of the \( V_{\mu} \text{BW-16} \) and \( V_{\text{Ox-1}} \) genes

|           | CDR Replacement Mutations/Sequence | R/S Ratio | CDR FR | Mutations/Sequence | R/S Ratio | Total Mutations/Sequence | R/S Ratio | No. Sequences |
|-----------|-----------------------------------|-----------|--------|-------------------|-----------|--------------------------|-----------|--------------|
|           |                                   |           |        |                   |           |                          |           |              |
| \( V_{\text{Ox-1}} \) |                                   |           |        |                   |           |                          |           |              |
| Untreated |                                   |           |        |                   |           |                          |           |              |
| Ad-T/C    |                                   |           |        |                   |           |                          |           |              |
| \( V_{\mu} \text{BW-16} \) |                                   |           |        |                   |           |                          |           |              |
| Untreated |                                   |           |        |                   |           |                          |           |              |
| Ad-L/C    |                                   |           |        |                   |           |                          |           |              |
| Ad-T/C    |                                   |           |        |                   |           |                          |           |              |

*FR, Framework; R, replacement; S, silent.

### Phenotypic analysis of spleen T cells and dendritic cells

Changes in the phenotype of T cells induced by TACI-Ig were less dramatic than those observed with B cells and were consistent with the state of B cell deficiency in the treated mice. Thirty-six-week-old control NZB/W F1 mice had a 2-fold increase in the number of splenic CD4\(^+\) T cells compared with 16-wk-old NZB/W F1 controls, but a 7- to 10-fold increase in the number of activated and memory CD4 T cells. Ad-TACI-Ig/CTLA4-Ig-treated mice had a 20–30% decrease in the number of CD4 T cells compared with pretreatment controls. The frequency of activated and memory CD4 T cells in 36-wk-old, Ad-TACI-Ig/CTLA4-Ig-treated mice was intermediate between young and age-matched untreated controls. Because of the small spleen size, however, the absolute numbers of activated CD4\(^+\) T cells were significantly decreased in Ad-TACI-Ig/CTLA4-Ig- and Ad-TACI-Ig-treated mice compared

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Immunohistochemistry of spleens from Ad-TACI-Ig/CTLA4-Ig-treated mice (A, B, F, I, J, and L) and age-matched control NZB/W F1 mice (C–E, G, H, and K) stained with anti-B220 (red; A–F, K, and L), anti-CD4 (green; A–D), peanut agglutinin-FITC (green; E and F), anti-IgM (green; G–J), anti-IgD (red; G–J), and anti-CD11c (green; K and L). B cell follicles in treated mice contain fewer B cells, more CD4 T cells (A–D), and a similar number of CD11c\(^+\) cells as control mice (K and L). Germinai centers are present in both treated mice and controls (E and F). IgM-containing plasma cells are abundant in the red pulp of control mice, but are smaller and less frequent in treated mice (G–I, representative of five mice).
pretreatment levels for C, IgG anti-dsDNA levels rebounded promptly after TACI-Ig was no longer present in the serum, but IgM anti-dsDNA levels remained lower than D and both treated (B) and control (E) mice. Fluorescence staining shows IgG deposits in the glomeruli of mice. immunostaining shows no glomerular damage or cellular infiltrates in the treated mice.

with age-matched controls (Tables I and II). These data indicate a significant inhibitory effect of TACI-Ig treatment on CD4 T cell activation and proliferation.

No difference in the percentage or distribution of CD11c-positive cells was observed between treated and control mice in either spleen (Fig. 8, K and L) or peripheral blood, but again, because of the small spleen size, the absolute number of these cells in the spleen was reduced in TACI-Ig-treated mice (data not shown).

**Induction of remission by TACI-Ig and CTLA4-Ig**

To determine whether Ad-TACI-Ig/CTLA4-Ig therapy would be as effective in treating established disease in NZB/W F1 mice as it was in delaying disease onset, we studied its effects at 30 wk when high titer IgG anti-dsDNA Abs were present and ~50% of the mice had developed proteinuria. The combination of TACI-Ig and CTLA4-Ig was found to reverse or delay the onset of proteinuria and prolong the life span in these mice (Fig. 10 A). The two treated mice that died shortly after treatment had established >300 mg/dl proteinuria at the time of treatment and expressed low levels of TACI-Ig in the serum after treatment, probably because of renal excretion of TACI-Ig. Even at this late age, Ad-TACI-Ig/CTLA4-Ig treatment resulted in a prompt decrease in serum levels of IgM and IgG and a decrease in IgM and IgG anti-DNA Abs (Fig. 10B). Similar to our observations with Ad-TACI-Ig/CTLA4-Ig treatment in younger mice, the decrease in IgG anti-DNA Abs was temporary, lasting only as long as TACI-Ig was expressed in the serum, whereas the decrease in IgM anti-DNA Abs was sustained for up to 3 mo (Fig. 10C).

Analysis of the spleens of surviving mice, performed at 40-44 wk of age, 10-14 wk after treatment, revealed that they were smaller (11.7 ± 8.1 vs 21.2 ± 6.7 × 10⁷ cells; p = 0.09) and contained fewer B cells than control, 40- to 42-wk-old spleens (32.8 ± 5.3 vs 48.8 ± 3.1%; p < 0.02), but had no change in the absolute number of CD4⁺ and CD8⁺ T cells. The only other statistically significant difference from controls was an increase in the frequency of naive CD4⁺ T cells (23.7 ± 14.0 vs 8.3 ± 4.0%; p < 0.02) and a decrease in the frequency of memory CD4⁺ T cells (60.1 ± 9.7 vs 75.9 ± 3.0%; p < 0.01), suggesting that the transition from the naive to the memory phenotype is still occurring at 30 wk and can be delayed either directly or as a result of B cell depletion, by TACI-Ig. Consistent with the flow cytometry data,

No difference in the percentage or distribution of CD11c-positive cells was observed between treated and control mice in either spleen (Fig. 8, K and L) or peripheral blood, but again, because of the small spleen size, the absolute number of these cells in the spleen was reduced in TACI-Ig-treated mice (data not shown).

**Induction of remission by TACI-Ig and CTLA4-Ig**

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the frequency of IgM- and IgG-secreting B cells as measured by ELISPOT was 2-fold lower in spleens from treated mice compared with controls. In contrast, there was no difference in the frequency of IgG-secreting cells or IgG anti-DNA-secreting cells in the bone marrow of treated and control mice (Fig. 11).

Immunofluorescence studies of the kidneys of treated mice 10–14 wk after treatment revealed IgG deposits in the glomeruli of treated mice. However, compared with controls, the Ad-TACI-Ig/CTLA4-Ig-treated mice had less glomerular damage and less interstitial lymphocytic infiltrate (Fig. 12).

Discussion

The studies presented in this report have confirmed that a short course of the murine BAFF and APRIL inhibitor TACI-Ig, given as a single dose of adenovirus, has a profound effect on disease onset in NZB/W F1 mice. We also showed that the addition of a 2-wk course of CTLA4-Ig prolonged the duration of TACI-Ig expression and the nephritis-free survival of the mice; this combination also had beneficial effects in older mice with established disease.

Our studies addressed the question of how TACI-Ig modulates the immune system to induce these clinical outcomes. As has been observed in nonautoimmune mice (10), TACI-Ig induced a profound depletion of MZ, T2, and mature B2 B cells that persisted for many months after treatment, with sparing of T1 and B1 B cells. The B cells that remained in the spleen after TACI-Ig treatment, however, were able to form germinal centers and respond to T cell help. Once TACI-Ig was no longer present in the serum, however, were able to form germinal centers and respond to TACI-Ig treated mice showed that IgM-producing plasma cells were present in the splenic red pulp of treated mice.

that the selection, migration, and survival of high affinity IgG-producing plasma cells in bone marrow could be supported.

In contrast to the temporary effect of TACI-Ig treatment on serum levels of IgG, TACI-Ig with or without CTLA4-Ig induced a profound decrease in serum levels of IgM and IgM anti-DNA Abs that was sustained for many months after cessation of therapy. TACI-Ig-treated mice were also unable to mount a serum IgM response to a T-dependent Ag, Ox, despite the presence of anti-Ox IgM-secreting B cells in the spleen that could be detected by ELISPOT and rescued as hybridomas. This result might be directly related to the loss of MZ B cells and/or B2 B cells. However, administration of the selective BAFF antagonist BAFF-R-Ig had an even more profound effect on the number of MZ and B2 B cells, but had no effect on serum levels of IgM or the IgM response to Ox (M. Ramanujam and A. Davidson, manuscript in preparation).

Serum IgM levels reflect both the frequency of IgM-secreting cells and the amount of Ig secreted per cell. Our data suggest that the low serum IgM level in treated mice is due to the absence of a few B cells that produce large amounts of Ig. TACI-Ig has previously been shown to prevent the IgM response to a T-independent Ag in nonautoimmune mice (5) due to decreased survival of plasmablasts. Immunohistochemical staining of the spleens from treated mice showed that IgM-producing plasma cells were present in the splenic red pulp of untreated NZB/W F1 mice, and that both the frequency and the size of these plasma cells in the red pulp of the spleen were significantly decreased in treated mice compared

FIGURE 11. Frequency of Ab-secreting B cells of the IgM and IgG isotypes from spleens and bone marrows of control and treated NZB/W F1 mice (seven or eight mice per group) after late treatment with Ad-TACI-Ig/CTLA4-Ig, shown as the mean ± SD. The frequency is per 10⁷ cells for total Ig and per 10⁸ cells for anti-dsDNA Ig. There are significantly fewer total Ig-secreting cells (p < 0.01 for IgM and IgG) and anti-dsDNA-secreting cells (p < 0.03 for IgM and IgG) in the spleen, but not in the bone marrow, of treated mice.

FIGURE 12. Effect of Ad-TACI-Ig/CTLA4-Ig treatment at 30 wk on renal histology in NZB/W F1 mice. All mice still alive at 44 wk were killed for analysis. Control mice, shown in A, C, and E, had evidence of proliferative glomerulonephritis, tubular casts (A), and vasculitis (C). Ad-TACI-Ig/CTLA4-Ig-treated mice are shown in B, D, and F. These mice had much less glomerular damage (B) and interstitial disease (D), but deposition of IgG in the glomeruli (F) was no different from that in controls (E). Mean glomerular and interstitial disease/vasculitis score are shown in G for the six surviving control mice and for six Ad-TACI-Ig/CTLA4-Ig-treated mice that remained free of proteinuria. A score of 5 indicates a mouse that died within 15 wk of treatment with >300 mg/dl proteinuria. The p values for treated vs control mice were calculated for the whole group, with scores of 3 attributed to dead mice: glomerular score, p < 0.01; tubular score, p = 0.002.
with controls. We therefore believe that TACI-Ig induces an environment in which these extrafollicular plasma cells, elicited by either T-dependent or T-independent Ags, do not survive. The reduction of serum IgM levels by TACI-Ig, but not BAFF-R-Ig, is consistent with the finding that TACI-deficient mice have low serum IgM levels (25), whereas IgM levels are normal in BAFF-R-deficient mice (26). These data suggest that the survival of short-lived IgM plasma cells in the spleen is dependent on the interaction of BAFF and APRIL with TACI. However, the recent identification of BCMA as an important BAFF receptor on human and murine bone marrow plasma cells (27–29) suggests that this point needs further investigation.

It is unlikely that the decrease in serum levels of IgM autoantibodies was responsible for the long delay in onset of renal disease, because mice treated with BAFF-R-Ig also had prolonged delay in disease onset (15) (M. Ramanujam and A. Davidson, manuscript in preparation). The beneficial effect of TACI-Ig could not be explained by a decrease in the titer or affinity of IgG autoantibodies or their ability to deposit in the kidney. It has previously been reported that T cells are important mediators of renal damage in mice with SLE even in the absence of circulating Ig (30). As NZB/W F1 mice age, they have increased numbers of CD4+ cells, increased expression of T cell activation markers, and loss of the naive T cell marker CD62L (31). We have shown that kidneys of diseased NZB/W F1 mice contain large aggregates of lymphoid cells, including B cells, T cells, and dendritic cells, resulting in local secretion of inflammatory mediators and cytokines (22). We therefore asked whether there was an alteration in T cell activation in treated mice. In TACI-Ig-treated mice, the proliferation of CD4+ T cells that accompanies aging and disease onset did not occur, and the frequency of activated and memory T cells was intermediate between those in young and aged controls. Of note, despite previous reports of a costimulatory function for TACI on T cells that accompanies aging and disease onset did not occur, and the frequency of activated and memory T cells was intermediate between those in young and aged controls. Of note, despite previous reports of a costimulatory function for TACI on T cells (32, 33), we were unable to demonstrate any effect of TACI-Ig on delayed-type hypersensitivity in BALB/c mice (not shown). Finally, although the frequency of dendritic cells in spleen and peripheral blood was not changed, their absolute numbers were diminished as a result of the small spleen size.

Our findings suggest that the profound effect of TACI-Ig on disease onset in NZB/W F1 mice is secondary to B cell depletion. B cells produce soluble mediators involved in the organization of lymphoid tissues, and their absence results in shrinkage of the spleen and lymph nodes (34, 35). B cells also function as APCs that can participate in cycles of autoantigen presentation to autoreactive T cells (36). Because of the small spleen size and the decreased number of activated B cells in TACI-Ig-treated mice (36), the total number of activated T cells was decreased 4–5-fold. In addition, the small spleen size resulted in a decrease in the total number of dendritic cells. Dendritic cells that migrate to the kidneys of NZB/W F1 mice secrete chemokines that attract inflammatory cells, including B and T cells, into the kidney (22). The suppression of T cell activation and the decrease in the total number of dendritic cells probably contributed to the delay in onset of proteinuria despite the absence of Abs in renal glomeruli.

A striking observation in this study was the ability of Ad-TACI-Ig/CTLA4-Ig to induce improvement of established disease in NZB/W F1 mice with prolongation of life span and in some cases even reversal of proteinuria. As in the younger NZB/W F1 mice, the major effect of Ad-TACI-Ig/CTLA4-Ig was a decrease in the frequency of B cells accompanied by a sustained decrease in IgM and a temporary decrease in IgG anti-DNA Abs. Thus, even in older autoimmune mice, B cell survival and maintenance of high circulating autoantibody levels are BAFF and APRIL dependent. The requirement for CTLA4-Ig to mediate the observed effect on IgG anti-DNA Abs needs to be examined further, but it is possible that both BAFF/APRIL and T cell cytokines contribute to the survival of plasmablasts (28).

The findings reported in this study with TACI-Ig treatment of NZB/W F1 mice are in contrast to previous studies using other costimulatory blocking reagents. In preventive studies neither CTLA4-Ig, anti-CD40L, nor the combination of the two agents induced either a change in serum levels of Ig or a decrease in titers of IgM anti-DNA Abs, even in young mice (17, 20, 31). In contrast, these treatments inhibited class switching and somatic mutation and appeared to alter selection of autoreactive V region genes, consistent with their effect on delivery of T cell help to B cells (37). In remission induction studies, a 2-wk course of combination anti-CD40L and CTLA4-Ig did not induce remission in nephritic NZB/W F1 mice (22). The combination of CTLA4-Ig and cyclophosphamide induced remission in nephritic mice, but it did not alter serum Ig or autoantibody levels (22). Anti-CD40L treatment of 26-wk-old mice did result in a decrease in the titer of IgG anti-DNA Abs (31), but this took much longer to occur than the 1 wk effect with TACI-Ig treatment, consistent with an effect of anti-CD40L on an earlier autoreactive precursor rather than on the effector B cell (31). Similar findings were reported in human patients treated with anti-CD40L Ab (38). Taken together, our findings are consistent with the hypothesis that a large amount of the abnormally high circulating autoantibody levels, even in older NZB/W F1 mice, derives from plasma cells that are at least in part dependent for survival on signals from BAFF and/or APRIL. TACI-Ig does not, however, appear to affect memory B cells, and the rapid rebound of IgG anti-dsDNA to pre-treatment levels once TACI-Ig has cleared from the serum might reflect the generation of new plasma cells from the memory compartment, which appears functionally resistant to BAFF blockade.

As large numbers of early plasmablasts are found in the peripheral blood of patients with active SLE (39), blockade of BAFF and APRIL may be an effective means of rapidly depleting these cells during an acute flare. Once TACI-Ig is no longer present in the serum, treated mice are capable of mounting an effective response to a T cell-dependent Ag, and established memory cells can be readily reactivated. However, the profound and prolonged effect of TACI-Ig on spleen size, B cell number, and serum levels of IgM raises concerns about its immunosuppressive potential. In view of these considerations, additional experiments are needed to determine the optimal balance of BAFF blockade and to define potential toxicities so that this strategy for treatment can be safely used in humans with autoimmune diseases.

Acknowledgments

We thank Dr. H. Keiser for critical reading of the manuscript.

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