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CD4⁺CD25⁺ T Cell-Dependent Inhibition of Autoimmunity in Transgenic Mice Overexpressing Human Bcl-2 in B Lymphocytes

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Regulation of lymphocyte survival is essential for the maintenance of lymphoid homeostasis preventing the development of autoimmune diseases. Recently, we described a systemic lupus erythematosus associated with an IgA nephropathy in autoimmune-prone (NZW × C57BL/6)F₁ overexpressing human Bcl-2 (hBcl-2) in B cells (transgenic (Tg) 1). In the present study, we analyze in detail a second line of hBcl-2 Tg mice overexpressing the transgene in all B cells and in a fraction of CD4⁺ and CD8⁺ T cells (Tg2). We demonstrate here that the overexpression of hBcl-2 in T cells observed in Tg2 mice is associated with a resistance to the development of lupus disease and collagen type II-induced arthritis in both (NZW × C57BL/6)F₁ and (DBA/1 × C57BL/6)F₁ Tg2 mice, respectively. The disease-protective effect observed in autoimmune-prone Tg2 mice is accompanied by an increase of peripheral CD4⁺CD25⁺ hBcl-2⁺ regulatory T cells (Treg), expressing glucocorticoid-induced TNFR, CTLA-4, and FoxP3. Furthermore, the in vivo depletion of CD4⁺CD25⁺ Treg in (DBA/1 × C57BL/6)F₁ Tg2 mice promotes the development of a severe collagen type II-induced arthritis. Taken together, our results indicate that the overexpression of hBcl-2 in CD4⁺ T cells alters the homeostatic mechanisms controlling the number of CD4⁺CD25⁺ Treg resulting in the inhibition of autoimmune diseases. The Journal of Immunology, 2007, 178: 2778–2786.

The control of autoreactive lymphocytes is one of the most important functions of the immune system. This is accomplished by a sophisticated network of cellular interactions that occur during lymphocyte ontogeny in the primary lymphoid organs as well as in the periphery. Central-induced immune tolerance mainly involves the elimination by apoptosis of developing autoreactive T and B lymphocytes (1, 2). The deletion of autoreactive clones occurs at specific stages of lymphocyte maturation that are characterized by an increased susceptibility to cell death (1–3). Cell death by apoptosis is also a crucial process shaping the lymphoid repertoire in the periphery and accounts for the demise of long-term activated lymphocytes and of autoreactive B cells generated in the germinal centers after somatic mutation in their rearranged H and L chain Ig genes (3–5). The importance of this apoptotic mechanism is manifested by the fact that alterations in the genetic program that regulates lymphocyte survival facilitate the development of autoimmune diseases. Thus, gene mutations in fas, fasL, or several caspases result in the development of a lymphoproliferative autoimmune syndrome both in humans and mice (6, 7). In addition, mice deficient in bim or that overexpress bcl-2 in B lymphocytes develop an systemic lupus erythematosus (SLE)-like disease (8–11).

Despite its unquestionable importance, the deletion of autoreactive T and B cell clones is not sufficient to ensure a state of complete self-tolerance. Autoreactive T and B cells are normal components of the peripheral mature lymphoid repertoire although in normal conditions, these cells are maintained silent (12–14). Several mechanisms operate in the periphery to guarantee an efficient immunological tolerance. During the activation of mature T and B cells, the absence of costimulatory signals leads to a state of functional unresponsiveness, i.e., anergy (15). In addition to the induction of anergy, several cell populations (T, NK, and dendritic cells) with a suppressive or regulatory activity have been recently characterized (16, 17). Among them, naturally occurring CD4⁺CD25⁺ regulatory T cells, which are characterized by high expression of CD25 and FoxP3, have emerged as central regulatory elements of peripheral tolerance. The role of CD4⁺CD25⁺ T cells in the control of autoimmunity has been well established in rodent models of experimental autoimmune encephalomyelitis (EAE) and experimental allergic allograft rejection (18–20). However, the mechanisms by which CD4⁺CD25⁺ T cells act to inhibit autoimmune diseases are still not completely understood. In this regard, multiple mechanisms have been proposed, including secretion of cytokines and chemokines, expression of inhibitory receptors, and induction of peripheral tolerance (21–23). Although the precise mechanism by which CD4⁺CD25⁺ T cells inhibit autoimmunity remains unclear, several lines of evidence indicate that Fas ligation downregulates the immunological response initiated by CD4⁺ autoreactive T cells (24–27).

Abbreviations used in this paper: SLE, systemic lupus erythematosus; Treg, regulatory T cell; GITR, glucocorticoid-induced TNFR; hBcl-2, human Bcl-2; CIA, collagen type II-induced arthritis; Tg, transgenic; col-II, bovine collagen type II; PT, pertussis toxin; TU, titration unit; IgAN, IgA nephropathy; IC, immune complex; EAE, experimental autoimmune encephalomyelitis.

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T cells (T<sub>reg</sub>) attract special attention because their involvement in the maintenance of immunological tolerance. These cells are produced in the thymus as a distinct population and their elimination in neonates (after neonatal thymectomy) leads to the development of autoimmune diseases (20). CD4<sup>+</sup> T<sub>reg</sub> cells represent a variable proportion of CD4<sup>+</sup> T helper (Th) cells within the peripheral blood lymphocytes (PBL) with a wide spectrum of functions and regulatory capacities. Th2 cells inhibit the development of autoimmune diseases (20). CD4<sup>+</sup> T<sub>reg</sub> cells control allospecific immune responses (16, 17). However, little is known about the mechanisms that regulate the number and/or activity of these cells in the periphery.

Although there are several controversial results, it has been recently reported that CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> have an increased susceptibility to different cell death stimuli (26–30). Nevertheless, the relevance of this phenomenon in terms of CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> activity has not been yet established. In the present study, we demonstrate that the overexpression of human Bcl-2 (hBcl-2) in T lymphocytes renders susceptible mice resistant to the development of secondary lymphoid organs and is abrogated by in vivo depletion of CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub>.

### Materials and Methods

#### Mice and treatments

C57BL/6 (B6) and B6-hbcl-2-22 Tg mice (from now referred as B6-Tg1 mice) were purchased from The Jackson Laboratory or provided by Dr. S. J. Korsmeyer (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Tg2 mice were provided by Harlan Ibérica. The F<sub>1</sub> female hybrids used in this study were obtained in our animal facilities. The presence of the hbcl-2 Tg in F<sub>1</sub> mice was assessed by flow cytometry using a specific mAb against hBcl-2 (clone 6C8; BD Pharmingen), as described previously (11).

The in vivo depletion of CD8<sup>+</sup> T cells was performed from birth up to 12 mo of age in the experiments with the SLE model, or from day 3 before immunization with bovine collagen type II (col-II) emulsified in CFA until the 12th week after immunization in the experiments with the CIA model, using an anti-CD8 mAb (H35-17.2: rat IgG2b) as described previously (32). Cells were treated i.p. (three times per week) with anti-CD8 mAb. The dose of mAb per week was dependent on the age of animals: 0.5 mg/week in mice from birth to 1 mo of age and 1.5 mg/week from 1 mo of age up to the end of the experiment. Control mice were treated with PBS.

The in vivo depletion of CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> in non-Tg and Tg-2 (DBA/1 x B6)<F<sub>1</sub> hybrid mice was performed by flow cytometry. Single-cell suspensions from bone marrow, thymus, spleen, lymph nodes, or peripheral blood were stained with different combinations of conjugated mAbs specific of surface or intracellular cell markers. Similarly, the quantification and phenotypic characterization of lymph node CD4<sup>+</sup> CD25<sup>+</sup> T cells overexpressing or not hBcl-2 was performed by flow cytometry. The following reagents were used: FITC-anti-CD4 (clone S7), FITC-anti-IgD (clone 11-26c.2a) FITC-anti-IgM (clone R6-60.2), FITC-anti-hbcl-2 (clone 6C8), PE or PerCP-anti-hbcl-2 (clone RM4-5), PE-anti-CD8 (clone 53-6.7), PE-anti-B220 (clone RA3-6B2), PE-anti-CD25 (clone 7D4), PE-anti-CD11c (clone 2C12; clone 2B8), PE-anti-CTL4-A (clone F10-11) and allophycocyanin-anti-25 (clone PC61), obtained from BD Pharmingen, PE-anti-FoxP3 (clone FJK-16s; eBioscience) and biotin-anti-GITR (clone DTA-1; supplied by Dr. S. Sakaguchi, Kyoto University, Kyoto, Japan). For biotinylated mAbs, PerCP-streptavidin (BD Pharmingen) was used as a second stage reagent. A total of 5 × 10<sup>4</sup> viable cells were analyzed in a FACS Calibur flow cytometer using CellQuest Pro software (BD Biosciences).

#### Cell cultures

Purified B cells (>99% purity in all cases) from different mice were obtained by cell sorting on a FACSaria cell sorter (BD Biosciences). The effects of hBcl-2 overexpression on B cell survival in the different lines of hBcl-2 Tg mice and non-Tg controls was evaluated in vitro using purified cell populations as described previously (34).

#### Serological studies

Serum levels of IgG anti-DNA and anti-nucleosome autoantibodies and of gp70-anti-gp70 immune complexes were determined in sera by ELISA, and the results were expressed in titration units (TU) in reference to a standard curve obtained from a serum pool from 6- to 8-month-old MRL- Fas<sup>+/–</sup> mice, as described previously (35).

#### Histopathology

Samples of all major organs were obtained at autopsy. For kidney, the organs were processed, stained, and scored as described previously (11). For the study of CIA, all mice were killed 10–12 wk after immunization and the hind limbs were fixed in 10% phosphate-buffered formaldehyde solution, decalcified in Parey’s decalcification solution overnight. The tissue was then embedded in paraffin. Sections (5 μm) were stained with H&E, examined in a light-phase microscope and scored according to a 0–3

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**Induction and assessment of arthritis**

Col-II (provided by Dr. M. Griffiths, University of Utah, Salt Lake City, UT) was dissolved at a concentration of 2 mg/ml in 0.05 M acetic acid and emulsified with CFA containing 4 mg/ml *Mycobacterium tuberculosis* (Chondrex). For the induction of CIA, 8- to 10-wk-old female non-Tg, Tg1, and Tg2 (DBA x B6F<sub>1</sub>) hybrid mice were immunized once at the base of the tail with 150 μg of Ag in a final volume of 150 μl. A clinical evaluation of arthritis severity was performed as described (33).

For radiological studies, mice were previously anesthetized by i.p. injection of a mixture containing: 50 mg/kg ketamine (Ketolar; Parke-Davis), 200 μg/kg atropine sulfate (B. Braun Medical), and 4 mg/kg diazepam (Valium; Roche). Rx pictures were obtained using a CCX Rx ray source of 70 Kev with an exposition of 90 ms (Trophy Irix X-Ray System; Kodak Spain). The radiological signal was digitalized with a Trophy RVG Digital Imaging system and analyzed using the Trophy Windows software. The severity of CIA was quantified radiologically with a graded scale according to the presence of five different radiological lesions (soft tissue swelling, subchondral osteopenia, and alterations in bony density, joint space narrowing or disappearance, marginal erosions, and periosteal new bone formation). The extension of every individual lesion (local: affecting one digit or one joint in the carpus; diffuse: affecting two or more digits and/or two or more joints in the carpus) was graded from 0 to 1 as follow: 0: absence; (1/2): local; 1: diffuse. To clearly establish the radiological score in each paw, plain radiographs were assessed using a magnifying glass. Then, each paw was graded from 0 to 5, giving a maximum possible score of 20 for each mouse.

#### Flow cytometry studies

The expression of hBcl-2 in B cell subpopulations, in T lymphocytes and in dendritic cells in the different hBcl-2 Tg and non-Tg mice was explored by flow cytometry. Single-cell suspensions from bone marrow, thymus, spleen, lymph nodes, or peripheral blood were stained with different combinations of conjugated mAbs specific of surface or intracellular cell markers. Similarly, the quantification and phenotypic characterization of lymph node CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> cells were performed by flow cytometry. The following reagents were used: FITC-anti-CD4 (clone S7), FITC-anti-IgD (clone 11-26c.2a) FITC-anti-IgM (clone R6-60.2), FITC-anti-hbcl-2 (clone 6C8), PE or PerCP-anti-hbcl-2 (clone RM4-5), PE-anti-CD8 (clone 53-6.7), PE-anti-B220 (clone RA3-6B2), PE-anti-CD25 (clone 7D4), PE-anti-CD11c (clone 2C12; clone 2B8), PE-anti-CTL4-A (clone F10-11) and allophycocyanin-anti-25 (clone PC61), obtained from BD Pharmingen, PE-anti-FoxP3 (clone FJK-16s; eBioscience) and biotin-anti-GITR (clone DTA-1; supplied by Dr. S. Sakaguchi, Kyoto University, Kyoto, Japan). For biotinylated mAbs, PerCP-streptavidin (BD Pharmingen) was used as a second stage reagent. A total of 5 × 10<sup>4</sup> viable cells were analyzed in a FACS Calibur flow cytometer using CellQuest Pro software (BD Biosciences).

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scale as described previously (36). All histological preparations were analyzed in a blinded fashion by a pathologist.

**Statistical analysis**

Statistical analysis of differences between groups of mice was performed using the Mann-Whitney U test and Student’s t test. Probability values <0.05 were considered significant.

**Results**

**Induction of SLE in (NZW × B6-Tg1)F1, but not in (NZW × B6-T2)F1, mice**

Several lines of Tg mice overexpressing hBcl-2 within the B cell compartment have been produced and studied (10, 31, 37). Recently, we had reported the development an IgA nephropathy (IgAN) associated with SLE in F1 animals between NZW and B cell B6-Tg1 mice, in which hBcl-2 expression is under the control of the SV40 early region promoter and the Ig H chain enhancer (10, 11). To further explore the pathogenic mechanisms involved in the induction of systemic autoimmunity in hBcl-2 Tg animals, the development of IgAN and SLE was analyzed in F1 hybrids of NZW mice with a second line of B cell hBcl-2 Tg mice, B6-Tg2 mice, in which the Tg expression is driven by the Ig H chain promoter and enhancer (31). Strikingly, unlike the already described (NZW × B6-Tg1)F1 mice, (NZW × B6-Tg2)F1 female mice failed to show signs of active SLE as evidenced by the absence of IgG anti-DNA autoantibodies (autoantibody) production at 8 mo of age (Fig. 1A). Other IgG as well as IgA autoantibodies associated with murine SLE, such as anti-nucleosome autoantibodies and gp70-anti-gp70 immune complexes, were also undetectable in the sera of (NZW × B6-Tg2)F1 mice (data not shown).

In addition, no evidences of glomerular abnormalities were observed in these mice (mean histological grades of glomerular lesions at 8–10 mo of age in Tg1 F1 mice: 3.8 ± 0.5, n = 8; in Tg2 F1 mice: 0.6 ± 0.5; n = 9, p < 0.005; Fig. 1B), and their life span was essentially identical to that of non-Tg controls (Fig. 1C).

**Protection of CIA in (DBA/1 × B6-Tg2)F1, but not in (DBA/1 × B6-Tg1)F1, mice**

The absence of IgAN associated to SLE observed in (NZW × B6-Tg2)F1 mice could be the result of a protective effect of the Tg2 strain of mice on autoimmune disease development. However, since (NZW × B6F1) non-Tg mice also failed to develop lupus, it could be also possible that (NZW × B6-Tg1)F1 mice had a particular susceptibility to undergo SLE. To explore these two possibilities, we used a model of induced autoimmune disease, the experimental model of CIA in (DBA/1 × B6)F1 mice, in which immunized non-Tg mice developed disease. Thus, we compared the development of CIA between (DBA/1 × B6)F1 non-Tg, (DBA/1 × B6-Tg1)F1, and (DBA/1 × B6-Tg2)F1, female mice after immunization with col-II. When analyzed for clinical signs of disease, both (DBA/1 × B6)F1 non-Tg and (DBA/1 × B6-Tg1)F1 mice developed an aggressive arthritis in the paws that evolved to severe inflammation and/or ankylosis around the 12th week after col-II immunization (Fig. 2A). Except at the 4 and 6 wk of CIA evolution, no differences in the severity of CIA were observed between these two F1 hybrid mice. In contrast, (DBA/1 × B6-Tg2)F1 mice exhibited less severe signs of paw inflammation during the whole period of CIA development (p < 0.05, Fig. 2A). Notably, 12 wk postimmunization with col-II, (DBA/1 × B6)F1 non-Tg, and (DBA/1 × B6-Tg1)F1 mice showed similar radiological signs of severe arthritis such as intense soft tissue swelling, hyperostosis, periosteal new bone formation, juxta-articular osteopenia, narrowing, or disappearance of the interosseous spaces and in some instances, marginal articular erosions reflecting cartilage loss (radiological scores: non-Tg mice, 17.6 ± 1.2; Tg1 mice, 16.9 ± 1.7; p > 0.1; Fig. 2B). However, none of the above-mentioned radiological signs of arthritis were observed in the paws of immunized (DBA/1 × B6-Tg2)F1 mice, except for a mild or moderate swelling of soft tissue that may explain the observed clinical score (radiological scores: 3.1 ± 0.5; p < 0.001; Fig. 2B). In accordance with the radiological findings, very limited histological abnormalities, such as a slight thickening of synovial cell layer, were detectable in the joints of some col-II-immunized (DBA/1 × B6-Tg2)F1 mice, which markedly contrasted with the severe joint destruction observed in the majority of col-II-immunized (DBA/1 × B6)F1 non-Tg and (DBA/1 × B6-Tg1)F1 mice analyzed (histological scores: non-Tg mice, 2.6 ± 0.2; Tg1 mice, 2.7 ± 0.1; and Tg2 mice, 0.5 ± 0.4; p < 0.001; Fig. 2C).

**Differential expression of the hBcl-2 Tg between Tg1 and Tg2 mice**

To elucidate the cellular basis for the absence of autoimmunity in Tg2 mice, the pattern of Tg expression was compared by flow cytometry between both lines of Tg mice in either the (NZW × B6)F1 and (DBA/1 × B6)F1 hybrid mice. The expression of hBcl-2 in peripheral B220+ B cells was comparable between (NZW × B6-Tg1)F1, (NZW × B6-Tg2)F1, and (DBA/1 × B6-Tg2)F1 mice (Fig. 3) and similar to that observed in (DBA/1 × B6-Tg1)F1 and Tg1 and Tg2 parental B6 mice (data not shown). Accordingly, purified B220+ peripheral B cells from B6-Tg1 and B6-Tg2 mice showed an analogous prolonged in vitro survival in comparison to B cells from non-Tg controls (data not shown).
addition, similar levels of hBcl-2 were also observed in bone marrow pro-B, pre-B, and immature B cell subpopulations and in splenic plasma B cells from Tg1 and Tg2 mice of both (NZW × B6)F1 and (DBA/1 × B6-Tg1)F1, (DBA/1 × B6-Tg2)F1 mouse strains (data not shown). CD4+ and CD8+ T cells from (NZW × B6-Tg1)F1 (Fig. 3) and (DBA/1 × B6-Tg1)F1 (data not shown) mice were negative for hBcl-2. As previously demonstrated (10), an increase in the number of B cells, but not T cells, in the spleen of these F1-Tg1 mice was observed (data not shown). In contrast, in (NZW × B6-Tg2)F1 and (DBA/1 × B6-Tg2)F1 mice, a significant proportion of peripheral mature spleen CD4+ (12.4 ± 0.8% and 13.9 ± 1.4%, respectively) and CD8+ (35.7 ± 2.6% and 38.8 ± 3.4%, respectively) T cells expressed high levels of hBcl-2 (Fig. 3). The overexpression of hBcl-2 in B and T cells of (NZW × B6-Tg2)F1 and (DBA/1 × B6-Tg2)F1 mice promoted an accumulation of mature spleen B cells, as previously reported (data not shown; Ref. 31), and a slight increase in the number of spleen CD8+ T cells (number of CD8+ T cells in the spleen of (NZW × B6)F1 non-Tg mice: 24.4 ± 4.8 × 10^6; in (DBA/1 × B6-Tg1)F1, (DBA/1 × B6-Tg2)F1, (NZW × B6-Tg2)F1 mice depleted from birth of CD8+ T cells by in vivo administration of a cytolytic anti-CD8 mAb, in comparison to PBS-treated (NZW × B6)F1 mice: 23.1 ± 3.4 × 10^6; in (DBA/1 × B6-Tg2)F1 mice: 32.7 ± 2.5 × 10^6; in (DBA/1 × B6-Tg2)F1 mice: 31.8 ± 5.1 × 10^6), but not CD4+ T cells (data not shown). The expression of hBcl-2 was also observed in developing and mature T cells within the thymus (in (NZW × B6-Tg2)F1 mice; percent of hBcl-2+ cells in CD4+ CD8− thymocytes: 5.6 ± 2.1; in CD4+ CD8− thymocytes: 15.2 ± 1.8 and in CD4− CD8− thymocytes: 40.5 ± 3.9). Other cell populations in the spleen of both (NZW × B6)F1 and (DBA/1 × B6)F1 Tg1 and Tg2 mice such as CD11c+ dendritic cells or NK cells were negative for hBcl-2 (Fig. 3 and data not shown). Similar results were obtained in the parental B6-Tg1 and B6-Tg2 mice (data not shown).

Lack of involvement of CD8+ hBcl-2+ T cells in the absence of autoimmunity in Tg2 mice

In view of the expression of hBcl-2 in T cells of Tg2 mice, we hypothesized that the absence of autoimmune diseases observed in these mice was a consequence of the overexpression of hBcl-2 in T cell subsets that could be associated with an increased Treg activity. Because the percentage of T cells overexpressing hBcl-2 in Tg2 mice was higher in the CD8+ T cell population than in CD4+ T cells, we assessed the possible involvement of CD8+ T cells in the autoimmune suppressive effect observed in such animals. To this end, the development of SLE was determined in (NZW × B6-Tg2)F1 mice depleted from birth of CD8+ T cells by in vivo administration of a cytolytic anti-CD8 mAb, in comparison to PBS-treated (NZW × B6-Tg1)F1 and (NZW × B6-Tg2)F1 mice. As additional controls, anti-CD8 and PBS-treated (NZW × B6)F1 non-Tg mice were used. This treatment caused a complete depletion of CD8+ T cells during the whole length of the study (12 mo; data not shown). However, the depletion of CD8+ T cells failed to promote the production of IgG anti-DNA autoantibodies in (NZW × B6-Tg2)F1 and non-Tg (NZW × B6)F1 mice; their
circulating anti-DNA titers were not significantly different from those of their respective PBS-treated controls ($p > 0.1$), but were lower than those in PBS-treated (NZW × B6-Tg1)F1 mice ($p < 0.005$; Fig. 4A). Accordingly, anti-CD8-treated (NZW × B6-Tg2)F1 mice did not develop glomerulonephritis and showed a similar life span than PBS-treated (NZW × B6-Tg2)F1 or (NZW × B6)F1 non-Tg mice (data not shown).

The lack of involvement of CD8$^+$ T cells in the autoimmune-protective effect observed in Tg2 mice was further explored using the experimental model of CIA in (DBA/1 × B6-Tg1)F1 and non-Tg mice. Again, the elimination of CD8$^+$ T cells in (DBA/1 × B6-Tg2)F1 mice failed to promote the development of a severe CIA after col-II immunization, as judged by radiological (radiological scores: anti-CD8-treated mice: 3.2 ± 0.4; PBS-treated mice: 2.9 ± 0.6; $p > 0.1$) and histopathological criteria (histological scores: anti-CD8-treated mice: 0.3 ± 0.2; PBS-treated mice: 0.6 ± 0.1; $p > 0.1$) (Fig. 4B).

**Expansion of CD4$^+$CD25$^+$hBcl-2$^+$ Tregs in Tg2 mice and induction of severe CIA in CD4$^+$CD25$^+$ Treg-depleted Tg2 mice**

We next assessed the possible contribution of CD4$^+$CD25$^+$ Tregs in the absence of SLE and CIA in CD4$^+$CD25$^+$ Treg-depleted Tg2 mice. To this end, we determined the percentages of CD4$^+$CD25$^+$ Tregs in the two CD4$^+$ T cell populations, the major CD4$^+$hBcl-2$^+$ population and the minor CD4$^+$ non-hBcl-2$^+$ population, present in both F1-Tg2 mice and in parental B6-Tg2, and compared with the percentages of CD4$^+$CD25$^+$ Tregs in both F1 and B6 non-Tg and Tg1 mice. As illustrated in Table I, a 2- to 3-fold expansion of CD4$^+$CD25$^+$ Tregs was observed in the CD4$^+$hBcl-2$^+$ population of the different Tg2

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<th>Mice</th>
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<th>Peripheral Blood</th>
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<td>B6 non-Tg</td>
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</tr>
<tr>
<td>(DBA/1 × B6)F1 non-Tg</td>
<td>CD4$^+$hBcl-2$^+$</td>
<td>9.8 ± 0.7</td>
<td>12.7 ± 1.2</td>
<td>9.7 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>(DBA/1 × B6-Tg1)F1</td>
<td>CD4$^+$hBcl-2$^+$</td>
<td>13.2 ± 1.2</td>
<td>14.4 ± 1.1</td>
<td>10.9 ± 1.2</td>
<td>ND</td>
</tr>
<tr>
<td>(DBA/1 × B6-Tg2)F1</td>
<td>CD4$^+$hBcl-2$^+$</td>
<td>14.4 ± 1.3</td>
<td>16.1 ± 1.1</td>
<td>7.8 ± 0.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cell suspensions from the mentioned primary and secondary lymphoid organs were obtained from 2- to 4-mo-old non-Tg, Tg1, and Tg2 mice, stained with fluorochrome-conjugated anti-CD4, anti-CD25, and anti-hBcl-2 mAbs and analyzed by flow cytometry. Results are expressed as the mean ± SD of CD4$^+$CD25$^+$ Treg as a percentage of CD4$^+$ T cells (five to seven mice per group). $*, p < 0.01.$
strain of mice, compared with CD4+ hBcl-2- cells, in every peripheral lymphoid organs analyzed. In both F1-Tg2 mice, CD4+ CD25+hBcl-2+ Tregs, as well as CD4+ CD25+hBcl-2- Tregs, expressed FoxP3, GITR, and CTLA-4 (Fig. 5A), markers associated to naturally arising Tregs (16, 17). The overexpression of hBcl-2 in (NZW × B6-Tg2)F1 mice promoted an increased resistance to dexamethasone-induced cell death in peripheral CD4+ hBcl-2+ cells of (NZW × B6-Tg2)F1 mice (B). A, Spleen cells from 2-mo-old (NZW × B6-Tg2)F1 and (DBA/1 × B6-Tg2)F1 mice were stained with different combinations of conjugated mAbs and analyzed by flow cytometry. The expression of different cell markers associated with CD4+ CD25+ Tregs, such as FoxP3, GITR, and CTLA-4, were studied by flow cytometry in the gated CD4+ hBcl-2- and CD4+ hBcl-2+ splenic T cell populations of (NZW × B6-Tg2)F1 (left panels) and (DBA/1 × B6-Tg2)F1 mice (left panels). Results are representative of three independent experiments. B, Two-month-old (NZW × B6)F1 non-Tg and Tg2 mice were treated i.p. with 1 mg of dexamethasone (Dex) or PBS as controls. The elimination of CD4+ CD25- and CD4+ CD25+ cells within the hBcl-2- and hBcl-2+ lymphoid populations in the spleen were analyzed 72 h later by flow cytometry. Results of two independent experiments are expressed in dexamethasone-treated mice (considered as 100%). *, Statistically significant differences (p < 0.05 in all cases).

To directly explore the contribution of the increased population of CD4+ CD25+ Tregs to the absence of autoimmune diseases in Tg2 mice, we performed in vivo depletion experiments using a cytolytic anti-CD25 mAb. To avoid the elimination of autoimmune effector CD4+ T cells, that can express CD25 after activation, and due to the CD4 T cell dependency of SLE and CIA, in our experimental approach, we performed a single short-term treatment with the anti-CD25 mAb before the initiation of any potential autoimmune response. For this reason, we decided to choose the experimental model of CIA that apart from being induced in otherwise healthy animals, it also had the advantage of its smaller evolution period (10 wk instead of 12 mo in the SLE model). To this end, Tg2 and non-Tg (DBA/1 × B6)F1 mice were treated with anti-CD25 mAb during the 4 days before the immunization with col-II. This treatment promoted the complete depletion of CD4+ CD25+ Tregs in both F1 hybrid mice (data not shown). Depletion of CD4+ CD25+ Tregs did not significantly modified the time course of CIA in (DBA/1 × B6)F1 non-Tg mice (radiological scores 10 wk postimmunization in PBS-treated mice: 17.4 ± 2.7, and in anti-CD25-treated mice: 18.1 ± 1.9; p > 0.1; histological scores 10 wk postimmunization in PBS-treated mice: 2.5 ± 0.3, and in...
B6-Tg2)F1 Tg mice. The protective effect conferred by hBcl-2 overexpression in these Tg2 mice is likely mediated by CD4+ CD25+ Tregs that are augmented in the periphery. These results show for the first time that new strategies addressed to interfere with the apoptotic program of CD4+ CD25+ Tregs can be useful for the treatment of autoimmune diseases.

Programmed cell death in the immune system takes place during lymphocyte differentiation and activation in both the primary and secondary lymphoid organs. It accounts for the demise of autoreactive T and B cell clones as well as for the elimination of long-term activated lymphocytes (3, 4). Using hBcl-2 Tg mice as an experimental system of inhibition of lymphoid cell death, we and others have demonstrated previously that the overexpression of hBcl-2 in B cells, in the context of an appropriate autoimmune predisposing genetic background, causes the development of an SLE-like syndrome (10, 11). These results are in agreement with the potential of Bcl-2 to interfere with the negative selection of developing B and T lymphocytes, particularly those self-reactive clones with low affinity for autoantigens (38–40). However, we clearly show here that the deregulated expression of a hBcl-2 Tg in CD4+ T cells, even in a minor fraction of such cells and independently whether the Tg is also expressed in B cells, not only fails to promote or enhance autoimmune manifestations but confers protection against the development of autoimmune diseases. In contrast, humans and mice with abnormalities in the lymphoid cell death cascade, secondary to natural or targeted mutations in several other apoptotic regulators, such as Bim, Fas, Fasl, or different caspases, show an accumulation of T and B cells in the secondary lymphoid organs in association with the development of severe autoimmune diseases (6–9). These differences can be explained by the particular role that each of the mentioned molecules plays in the complicated molecular network that regulates the death or survival of autoreactive and regulatory lymphocyte subpopulations. Thus, in the present study, we demonstrate that the deregulation of Bcl-2 expression in Tg2 mice causes an increase in the number of CD4+CD25+ Tregs within the CD4+ T cells that overexpress the Tg, altering in that way the CD4+CD25+ Treg/CD4+CD25− T effector cell ratio. In fact, in vivo elimination of these cells in (DBA/1 × B6-Tg2)F1 promotes the development of a CIA similar to that of non-Tg F1 controls. Our results are indicative of a potential role of Bcl-2 in the control of CD4+ CD25+ Treg homeostasis. In this regard, Chen et al. (30) have demonstrated that human CD4+CD25+ Treg express lower levels of endogenous Bcl-2 than CD4+CD25− cells, in correlation with an increased susceptibility to cell death stimuli in vitro. In contrast, Bim participates in the induction of cell death of immature precursors associated with the negative selection of B and T cells as well as in the elimination of activated lymphocytes after cytokine deprivation (8, 9, 41) and the Fas/Fasl cell death pathway plays a central role in the elimination of long-term activated lymphocytes in a process called activation-induced cell death (3, 4). Whether deficiencies in Bim or in the Fasl pathway also affect the number and/or activity of CD4+ CD25+ Tregs is at present unknown, but this hypothetical function apparently cannot compensate the described proautoimmune effects, as observed in mice bearing mutations in these proapoptotic regulators.

In agreement with our present study, it has been described recently that the constitutive Tg expression of Bcl-xL in T cells attenuates the development of CIA (42). Although the authors of this study do not clarify the mechanism directly involved in such protective effect, due to the similarities between Bcl-2 and Bcl-xL, in the regulation of programmed cell death, and more particularly in the regulation of lymphocyte survival (3, 43), it is likely that analogous mechanisms account for the protection against CIA in both anti-CD25-treated mice: 2.6 ± 0.1; p > 0.1; Fig. 6). However, in marked contrast to PBS-treated (DBA/1 × B6-Tg2)F1 mice, which developed only mild signs of CIA (radiological scores 10 wk postimmunization: 3.1 ± 0.6; histological scores 10 wk postimmunization: 0.4 ± 0.2), anti-CD25 treatment in these Tg2 F1 mice led to the development of an aggressive CIA (radiological scores 10 wk postimmunization: 16.5 ± 2.9; histological scores 10 wk postimmunization: 2.7 ± 0.5; Fig. 6), the severity of which was comparable to that observed in PBS-treated non-Tg F1 mice (p > 0.1).

**Discussion**

In recent years, much attention has been focused in describing the involvement of CD4+ CD25+ Tregs in the control of autoreactive or alloreactive immune responses. However, until now, the mechanisms controlling the number and/or activity of these cells in the periphery remain largely unknown. Using two different lines of hBcl-2 Tg mice in T and/or B lymphocytes, we demonstrate here that the overexpression of hBcl-2 in a minor population of T cells makes Tg2 mice refractory to the development of two autoimmune diseases: SLE in (NZW × B6-Tg2)F1 mice and CIA in (DBA/1 × 2784 T CELL OVEREXPRESSION OF Bcl-2 SUPPRESS AUTOIMMUNITY

**FIGURE 6.** Induction of severe CIA in (DBA/1 × B6-Tg2)F1 mice after in vivo depletion of CD4+ CD25+ Tregs. A, Clinical scores of CIA in 8- to 10-wk-old CD4+ CD25+ Treg-depleted (open symbols) or PBS-treated (solid symbols) (DBA/1 × B6)F1 non-Tg (□) and (DBA/1 × B6-Tg2)F1 (△) female mice immunized with 150 μg of col-II. Results of one representative experiment (from a total of three independent experiments) are expressed as the mean value (±1 SD) of 7–10 mice/group at the indicated weeks of CIA evolution. *p < 0.05 in all cases. B, Representative radiological images of the front paws of CD4+ CD25+ Treg-depleted or PBS-treated (DBA/1 × B6)F1 non-Tg and (DBA/1 × B6-Tg2)F1 mice, 10 wk after immunization with col-II. C, Representative histological appearance of the joints (×10) of CD4+ CD25+ Treg-depleted or PBS-treated (DBA/1 × B6)F1 non-Tg and (DBA/1 × B6-Tg2)F1 mice, 10 wk after immunization with col-II.
Bcl-x<sub>L</sub> T cell Tg and Bcl-2 Tg2 mice. However, Issazadeh et al. (44) have reported that Bcl-x<sub>L</sub> T cell Tg mice develop an accelerated and more chronic autoimmune encephalomyelitis (EAE) after immunization with myelin oligodendrocyte glycoprotein. These discrepancies can be explained by the recent discovery of the effects of the pertussis toxin (PT) on the survival of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> (45). Coadministration of PT is required for the induction of EAE after immunization with encephalitogenic Ags emulsified in CFA (44, 45). Interestingly, PT causes an intense depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> in vivo that may explain why Bcl-x<sub>L</sub> T cell Tg mice develop an intense EAE, resembling the effects of the treatment with anti-CD25 mAb in the development of CIA in Bcl-2 Tg2 Tg mice.

In Tg2 mice, the expression of the hBcl-2 Tg is driven by the Ig H chain promoter and enhancer and as expected, hBcl-2 is overexpressed in all B cell subpopulations (31). However, there is a leakage and a significant proportion of CD8<sup>+</sup> and CD4<sup>+</sup> T cells also express at high levels the hBcl-2 Tg. Based on this characteristic, these Tg mice constitute an excellent experimental model to directly compare in the same environment the outcome of particular T cell subpopulation expressing either physiological or elevated levels of Bcl-2. Thus, we have observed an increase of CD4<sup>+</sup>CD25<sup>+</sup>hBcl-2<sup>+</sup> T<sub>reg</sub> in the periphery of these Tg2 mice. In an elegant study, Jordan et al. (18) have demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> arise from precursors with a relatively high avidity for self-Ags. In addition to the interaction between TCR and self-peptides-MHC, other interactions such as those between CD28 and its B<sub>7</sub> ligands seem to be necessary for the appropriate thymic development of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> (46, 47). Different antiapoptotic factors such as Bcl-x<sub>L</sub> and A1 can be induced in activated T cells through CD28 costimulation (48, 49), suggesting that the development of high-avidity self-reactive CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> might require an enhanced resistance to cell death stimuli. In addition, it has been shown that the positive selection of developing T cells is increased in Tg mice overexpressing hBcl-2 in T cells (50). In this scenario, the increase in number of CD4<sup>+</sup>CD25<sup>+</sup>hBcl-2<sup>+</sup> T<sub>reg</sub> in the secondary lymphoid organs of Tg2 mice can be the result of an augmented selection of these cells within the thymus. However, the percentages of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> in the thymus of B6-Tg2 mice are similar between the CD4<sup>+</sup>hBcl-2<sup>+</sup> and CD4<sup>+</sup>hBcl-2<sup>+</sup> populations, indicating that other mechanisms could be responsible for the expansion of these regulatory cells in the periphery. A more reasonable possibility is that CD4<sup>+</sup>CD25<sup>+</sup> hBcl-2<sup>+</sup> T<sub>reg</sub> accumulate in the peripheral lymphoid organs as a result of the deregulated expression of hBcl-2 that prolongs their survival. Our results clearly show that the ectopic expression of hBcl-2 in CD4<sup>+</sup> T cells of Tg2 mice (both CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup>) augmented their resistance to apoptotic stimuli such as dexamethasone. However, the fact that the CD4<sup>+</sup>CD25<sup>+</sup>:CD4<sup>+</sup>CD25<sup>-</sup> ratio is higher within the CD4<sup>+</sup>hBcl-2<sup>+</sup> population than in CD4<sup>+</sup>hBcl-2<sup>+</sup> T cells, despite the enhanced resistance of CD4<sup>+</sup>CD25<sup>+</sup> hBcl-2<sup>+</sup> cells to proapoptotic stimuli, indicate that CD4<sup>+</sup>CD25<sup>-</sup> T cells constitute a cell population with a particular susceptibility to undergo homeostatic alterations after changes in its survival capacity. Accordingly, different studies indicate that these T<sub>reg</sub> are more sensitive to different cell death stimuli than CD4<sup>+</sup>CD25<sup>+</sup> T cells (26–28). Experiments are in progress in our laboratory to address this issue.

Finally, our present study constitutes the first description of a new approach that increases the suppressive capacity of the CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> population by interfering with its genetic cell death program and that results in the inhibition of autoimmune responses. This strategy can be then envisioned for the future treatment of autoimmune diseases.


