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Suppression of Disease in New Zealand Black/New Zealand White Lupus-Prone Mice by Adoptive Transfer of Ex Vivo Expanded Regulatory T Cells

Kenneth J. Scalapino,* Qizhi Tang,† Jeffrey A. Bluestone,† Mark L. Bonyhadi,‡ and David I. Daikh2*†

An increasing number of studies indicate that a subset of CD4⁺ T cells with regulatory capacity (regulatory T cells; Tregs) can function to control organ-specific autoimmune disease. To determine whether abnormalities of thymic-derived Tregs play a role in systemic lupus erythematosus, we evaluated Treg prevalence and function in (New Zealand Black × New Zealand White)F₁ (B/W) lupus-prone mice. To explore the potential of Tregs to suppress disease, we evaluated the effect of adoptive transfer of purified, ex vivo expanded thymic-derived Tregs on the progression of renal disease. We found that although the prevalence of Tregs is reduced in regional lymph nodes and spleen of prediseased B/W mice compared with age-matched non-autoimmune mice, these cells increase in number in older diseased mice. In addition, the ability of these cells to proliferate in vitro was comparable to those purified from non-autoimmune control animals. Purified CD4⁺ CD25⁺ CD62Lhigh B/W Tregs were expanded ex vivo 80-fold, resulting in cells with a stable suppressor phenotype. Adoptive transfer of these exogenously expanded cells reduced the rate at which mice developed renal disease; a second transfer after treated animals had developed proteinuria further slowed the progression of renal disease and significantly improved survival. These studies indicate that thymic-derived Tregs may have a significant role in the control of autoimmunity in lupus-prone B/W mice, and augmentation of these cells may constitute a novel therapeutic approach for systemic lupus erythematosus. The Journal of Immunology, 2006, 177: 1451–1459.

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by a marked loss of tolerance to self-Ags followed by immune-mediated injury to multiple organ systems. The etiology of SLE remains unknown, but activation and expansion of autoreactive lymphocytes is a feature of lupus that is shared by other autoimmune diseases. CD4⁺ CD25⁺ regulatory T cells (Tregs) with the capacity to modulate peripheral lymphocyte activation and expansion are increasingly recognized as an essential component of the normal murine and human immune systems (1, 2). A number of murine models of organ-specific autoimmunity have demonstrated that deficiencies in either naturally occurring or experimentally derived Tregs are associated with the development of immune-mediated organ damage (3–6). Restoration or supplementation of Treg function in some of these models has been shown to inhibit autoimmunity (4–10). The ability of Tregs to modulate peripheral immune responses and the association of Treg abnormalities with some forms of organ-specific autoimmune disease suggest the possibility that a Treg defect may contribute to the development of SLE.

Several studies have evaluated Treggs in lupus and correlated low numbers of these cells with disease. Evaluation of Treg prevalence in (New Zealand Black × New Zealand White)F₁ (B/W) and (SWR × New Zealand Black)F₁ (SNF₁) lupus-prone mice found a lower frequency of these cells than in non-autoimmune strains (4, 11), similar to the reported Treg deficiency in diabetic-prone NOD mice (4, 6). A third strain, MRL/Mp, was found to have a normal frequency of CD4⁺ CD25⁺ cells before disease onset, but this report did not examine Treg frequency in mice with active disease (12). Two recent studies of humans with SLE have reported that the frequency of CD4⁺ CD25⁺ Tregs in the peripheral circulation of SLE patients is reduced compared with healthy controls (13, 14). Murine models in which Tregs are experimentally depleted by deleting the entire CD4⁺ CD25⁺ population have produced conflicting data. Thymectomy studies in SNF₁ mice produce the expected loss of Tregs followed by accelerated dsDNA Ab production, expansion of autoreactive T cells, and diffuse organ inflammation (15). Surprisingly, glomerulonephritis, a hallmark of human SLE, did not occur in this model despite immune complex deposition in the kidneys. In contrast to this study, thymectomy in the NZM2238 lupus model accelerated both autoantibody production and glomerulonephritis (16). Overall, these studies suggest that a Treg deficiency might contribute to the immune defect that results in loss of self-tolerance and the development of lupus in genetically susceptible mice.

In this study, we detail the prevalence, in vitro expansion, and functional capacity of thymic-derived Tregs in healthy young and older diseased B/W lupus-prone mice. We expand on prior reports to characterize changes in Treg prevalence and distribution with the onset of disease. We demonstrate that although young B/W mice...
have a low prevalence of Treg, these cells expand with the development of disease, maintain a stable suppressive phenotype, and have a robust capacity to proliferate when isolated from either young healthy or older sick mice. Finally, to determine whether augmentation of a functional population of Treg can enhance control of autoactive lymphocytes in lupus-prone B/W mice, we evaluated the effect of adoptive transfer of exogenously expanded, CD4+ CD25+ CD62Lhigh Treg on disease. We demonstrate that supplementation of the endogenous Treg population in lupus-prone B/W mice with exogenously expanded cells has the capacity to slow the rate of disease progression and significantly decrease mortality in these mice.

Materials and Methods

Mice

B/W mice and BALB/c mice were purchased from The Jackson Laboratory and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited San Francisco Veterans Affairs Medical Center (VAMC) Animal Care Facility under the supervision of a licensed veterinarian. Animal protocols were reviewed and approved by the VAMC Institutional Animal Care Use Committee.

Abs and other cellular reagents

FITC-conjugated mAb against CD4 (GK1.5) was purified in our laboratory. PerCP-Cy5.5-conjugated mAb to CD41 (RM4-5), allophycocyanin-conjugated mAb to CD62L (MEL-14), and neutralizing Abs to IL-10 (JES5-16E3) were purchased from BD Pharmingen. FITC-conjugated mAb to Foxp3 (FJK-16s) was purchased from eBioscience. FITC-conjugated mAb to B220 (RA3-6B2), biotinylated goat anti-mouse IgG (catalog no. M30215), goat anti-mouse IgM (catalog no. M31515), and FITC-conjugated streptavidin (SA1001) were purchased from Caltag Laboratories. R-PE-conjugated anti-CD25 mAb (7D4) was purchased from Southern Biotechnology Associates. Neutralizing Abs to TGF-β (1D11) were purchased from R&D Systems. Anti-Fc γRII/III (2.4G2 and anti-CD3 (2C11) mAb were purified in our laboratory. CFSE was purchased from Molecular Probes/In VitroGen Life Technologies. Biotinylated rat anti-mouse C3 (11H9) was purchased from HyCult Biotechnology.

Lymphocyte isolation

Animals were sacrificed, and lymph nodes (LNs) from four regions (cervical, axillary/inguinal, mesenteric, and renal), as well as the spleen were isolated into petri dishes containing DMEM with 2% FCS. Single-cell suspensions were obtained from each group. An aliquot from each LN distribution and spleen was used for assessment of CD4+ T cell and Treg frequency at the time of sacrifice. Before cell sorting, the remaining splenocytes underwent enrichment of the CD4+ T cell fraction using a negative selection autoMACS protocol (Miltenyi Biotech).

FACS analysis and cell sorting

Lymphocytes from the spleen and LNs were blocked with 2.4G2 mAb. Individual aliquots from each spleen and LN group were incubated with the PerCP-Cy5.5-CD4+, R-PE-CD25+, and allophycocyanin-CD62L. Cells were fixed and permeabilized before intracellular staining with FITC-Foxp3 (eBioscience). Remaining lymphocytes were labeled with FITC-CD4+, PE-CD25+, and allophycocyanin-CD62L in preparation for sorting. FACS analysis of fixed aliquots for CD4+, CD25+, CD62L, and Foxp3 expression was performed on a FACSaria (BD Biosciences) using FACS-Diva software. Purity-prioritized sorting of the unfixed cells was conducted on the FACSaria to obtain the CD4+ CD25+ CD62Lhigh and CD4+ CD25+ fractions. Purity checks of sorted cells routinely demonstrated >98% purity (Fig. 1, demonstrating Treg purification).

Treg culture

Sorted CD4+ CD25+ CD62Lhigh Treg and CD4+ CD25- cells were cultured using the protocol described previously (17). Briefly, purified cells were maintained at a concentration of 0.7–1 × 106 cells/ml over a 10-day culture period in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (BioSource International), rhIL-2 (2000 IU/ml) (Hoffmann-LaRoche; provided by the National Cancer Institute), 5 mM HEPEs (Sigma-Aldrich), nonessential amino acid, 0.5 mM sodium pyruvate, 1 mM glutamax (all obtained from Invitrogen Life Technologies), and 55 μM 2-ME (Sigma-Aldrich). Cells were stimulated with anti-CD3 and anti-CD28-coupled Xcyte beads/Xcyte Therapeutics. Following expansion, cells were separated from the Xcyte beads by centrifugation in Lymphocyte medium (Cedarlane Laboratories) at 230 × g for 25 min. Expanded Treg were routinely assessed for expression of CD4+, CD25+ CD62Lhigh, Foxp3 (Fig. 1), and suppressive function. No detectable Foxp3 was observed in the expanded CD4+ CD25+ cell fraction.

Suppression assay

CD4+ T cells (effector T cell; Teff) were isolated from splenocytes of young B/W by autoMACS separation as described above. The remaining splenocytes were irradiated at 2000 rads and served as APCs. A total of 75,000 Teff and 75,000 APCs were combined into wells of U-bottom 96-well plates. Soluble anti-CD3 was added to obtain a final concentration of 4 μg/ml in each well. Freshly isolated or expanded Treg from B/W and BALB/c mice were then added to Teff + APCs (triplicate wells) with a Teff:Treg ratio of 25:1. Cells were cultured for 60–70 h before addition of 1 μCi/well of [3H]thymidine. Cells were harvested 24 h later, and tritium incorporation was measured on a MicroBeta Scintillation Counter (Wallac). A suppression index (SI), defined as tritium incorporation in the mixed Teff:Treg well divided by incorporation in the Teff well, was calculated for each experiment as a measure of Treg suppressive capacity. The SI for freshly isolated and expanded Treg was also measured in the presence of CD4+ CD25- CD62Lhigh cells from gate P3 demonstrating persistence of CD25+ and CD62Lhigh following expansion. Inset, Histogram of the entire expanded cell population (C) demonstrating Foxp3 expression in >80% of expanded cells. Isotype control for expanded cells shown in unfilled histogram.

![FIGURE 1. Purification and expansion of CD4+ CD25+ CD62LhighFoxp3+ Treg. A. Unsorted CD4+ lymphocytes demonstrating ~3% prevalence of CD4+ CD25+ CD62Lhigh cells (gate P3). B. Postsort analysis demonstrating 98.2% purity of the CD4+ CD25+ CD62Lhigh cell fraction (gate P3). Inset, Histogram of P3-gated cells: 92% of cells express Foxp3 with IgG2a isotype control Ab shown in unfilled histogram. C. Expanded CD4+ CD25+ CD62Lhigh cells from gate P3 demonstrating persistence of CD25+ and CD62Lhigh following expansion. Inset, Histogram of the entire expanded cell population (C) demonstrating Foxp3 expression in >80% of expanded cells. Isotype control for expanded cells shown in unfilled histogram.](http://www.jimmunol.org/)
of neutralizing Abs to IL-10 or TGF-β at a concentration of 10 μg/ml. Control suppression assays used exogenously expanded nonregulatory CD4+ CD25+ cells in place of Treg.

Adaptive transfer

Expanded Treg were depleted of X-cyte beads as described above and washed repeatedly in sterile saline. Cells were concentrated in sterile PBS before transfer of 6 × 10^6 cells/mouse via tail-vein injection to mice in the treatment protocol. Control mice received tail vein injections of an equivalent volume of sterile PBS or 6 × 10^6 CD4+ CD25+ T cells concentrated in sterile PBS. Two separate treatment experiments using exogenously expanded Treg were conducted. The first experiment was designed to evaluate progression of proteinuria and survival. A follow-up experiment compared renal pathology in treatment and control animals. In additional experiments, expanded Treg were suspended in 2.5 μM CFSE solution for 5 min before washing and transfer of 5–6 × 10^6-labeled cells via tail-vein injection. Distribution and survival of CFSE-labeled Treg in the LNs, spleen, and kidneys were evaluated in mice with active disease up to 23 days after transfer by FACS analysis. Treg expansion following transfer was evaluated by labeling 25 × 10^6 expanded cells before dividing them into equal aliquots and transferring to age-matched mice with 4+ proteinuria. Mice were sacrificed 1, 10, 15, and 23 days after transfer, and the prevalence and fluorescence intensity of labeled cells from LNs, spleen, and renal parenchyma were compared.

Assessment of lupus disease activity and survival

Renal disease was assessed by measurement of proteinuria using Uristix (Bayer) and by evaluation of renal immunohistopathology. Anti-dsDNA Ab concentration in individual mice was assessed by an ELISA already established in our laboratory (18). The first cohort of 24 mice received either Treg transfer or PBS and was then followed for the development of proteinuria and survival. Mice were assessed daily to determine development of a premorbid condition defined as persistent weight loss >25% associated with decreased activity level and feeding behavior. A second cohort of 10 mice underwent a similar treatment protocol and was sacrificed for assessment of renal histology. A third cohort of 18 mice received either CD4+ CD25+ transfer or PBS and was followed as described above for development of proteinuria and survival.

Immunohistochemistry

Cryopreserved sections of kidney tissue were stained with H&E for evaluation of renal pathology. Indirect immunofluorescence was used to visualize deposition of IgG and IgM immune complexes and C3. Tissue damage and immunofluorescence was graded by a blinded reader using a scoring system previously established in our laboratory (18).

Statistical analysis

Treg frequency was compared using Student’s t test. Differences in Treg proliferative capacity was determined by Wilcoxon rank sum test. Suppression assays were performed in triplicate, and the mean thymidine incorporation was assessed by Student’s t test. In treatment studies, the concentration of anti-dsDNA Abs in individual mice was compared using the Student’s t test. Development of marked proteinuria, defined as ≥300 mg/dl on serial testing, and survival were compared by χ^2 analysis using the Yates correction.

Results

Treg prevalence

We compared the prevalence of CD4+ CD25+ CD62L^hi/Foxp3+ Treg in cohorts of 5–10 young healthy B/W mice, older sick B/W mice with persistent marked proteinuria, and age-matched nonautoimmune BALB/c mice. We assessed the prevalence of Treg in the spleen and in four regional LN distributions; cervical, axillary, mesenteric, and renal LNs. Intracellular expression of Foxp3 was consistently detected in >90% of cells expressing the combination of cell surface markers CD4+ CD25+ CD62L^hi (Fig. 1) and did not vary with mouse age or breed. Young B/W mice without proteinuria had significantly lower percentages of Treg in both the LNs and spleen as compared with age-matched BALB/c mice (combined LN prevalence 3.67 vs 5.44%, p = 0.002; spleen 2.35 vs 4.18%, p = 0.009). This reduced prevalence in young B/W mice was evident in each LN region except the renal LNs, where Treg prevalence was comparable to that of BALB/c (Fig. 2). Interestingly, older B/W mice with active lupus characterized by persistent proteinuria ≥300 mg/dl demonstrated a significant increase in the percentage of LN Treg, with the most dramatic rise (>2-fold) occurring in nodes draining salivary glands and kidneys (Fig. 2). Cellular expression of Foxp3+ as measured by mean fluorescent intensity was similar between Treg from young and old mice. In contrast, there was a further decline in the prevalence of Treg in the spleens of sick animals (healthy B/W 2.35% vs old B/W 1.51%; p = 0.014). In both the LNs and spleen of sick B/W mice, there is an increase in the total number of CD4+ CD8+, and B lymphocytes, and thus the total number of Treg in each distribution including the spleen increases with disease onset despite a decline in splenic Treg prevalence. These changes with age were not found in BALB/c mice, in which lymphatic hypertrophy does not develop, and only a small, nonsignificant decline in the Treg prevalence population was observed in both the LNs and the spleens of older mice.

FIGURE 2. Lymphatic and splenic prevalence of CD4+ CD25+ CD62L^hi/Foxp3+ Treg in B/W and age-matched BALB/c mice. A, Eight- to 10-wk-old mice demonstrating significantly fewer Treg in B/W mice (■) compared with BALB/c (□) in all distributions (*, p < 0.05) except for the renal LNs (p = 0.31). B, Forty-eight- to 52-wk-old mice demonstrating expansion of Treg in LNs and a decrease in splenic Treg in diseased B/W mouse. As compared with BALB/c, B/W mice with active renal disease have more renal LN Treg (⋆⋆, p = 0.04) and fewer splenic Treg (⋆⋆⋆, p = 0.04). Compared with young B/Ws, the old B/W mice demonstrate significantly more Treg in all LN distributions (p ≤ 0.03) except mesenteric LN (p = 0.07) and significantly fewer spleen Treg, (p = 0.011).
To determine whether B/W Tregs exhibit normal suppressor function, we evaluated their ability to suppress T cell proliferation compared with Tregs purified from non-autoimmune mice. Three to five mice per experiment were evaluated. Teff were mixed with Tregs at a ratio of 25:1 for each experiment. Freshly isolated Tregs from young and old B/W mice produced a similar SI of 0.33 and 0.26, respectively (p = 0.12; Fig. 3A). Expanded B/W Tregs from both young and old also demonstrated equivalent suppression (SI = 0.09 and 0.15, respectively; p = 0.13). In control assays, expanded nonregulatory CD4+ T cells did not exert suppression (Fig. 3A). The suppression capacities of both freshly isolated and expanded BALB/c Tregs were comparable to those of B/W Tregs (freshly isolated SI = 0.30; and expanded SI = 0.16) and did not vary with the age of the mouse (Fig. 3B). The SI of all expanded cells was enhanced compared with freshly harvested Tregs (p = 0.04), a characteristic of exogenously expanded Tregs previously reported in NOD mice (17). The suppression capacity of freshly isolated and expanded Tregs was not diminished by the addition of neutralizing Abs to IL-10 and TGF-β (p > 0.5 for all assays) (Fig. 4).

We next evaluated the possibility that an impaired Treg proliferation capacity might contribute to a loss of peripheral tolerance in B/W mice. To determine the proliferation capacity of Tregs, CD4+CD25+CD62Lhigh cells purified from both young healthy and older sick B/W mice (5 mice/group) were expanded in vitro. Tregs isolated from both healthy and sick B/W mice expanded 80-fold over a 10-day period, independent of whether the Tregs were isolated from the LNs or spleen. This proliferation was equivalent to the 80-fold expansion of Tregs purified from age-matched BALB/c obtained in parallel experiments. Expanded cells maintained expression of CD4+CD25+CD62Lhigh and Foxp3 (>90%) for cells isolated from both young and old mice) when the purity of the initial Treg culture exceeded 98% (Fig. 1).

Adoptive transfer experiments
To determine whether exogenously expanded Tregs survived and expanded following adoptive transfer, Tregs were labeled with CFSE and injected into the tail vein of recipient B/W mice with active glomerulonephritis (proteinuria ≥300 mg/dl). Dividing CFSE-labeled cells remained detectable over the 23 days evaluated (Fig. 5). Cells were evident in all four LN distributions evaluated (cervical, axillary/inguinal, mesenteric, renal) as well as the spleen and renal parenchyma of recipient mice. No differences in the prevalence of labeled cells were evident between these tissues at 23 days, at which point cells with detectable CFSE fluorescence comprised <1% of the total tissue lymphocytes.

To determine whether augmentation of the endogenous Tregs population can suppress the development of murine lupus, we assessed the effects of transferring exogenously expanded Tregs on the development and progression of disease in B/W mice. Eleven 24- to 29-wk-old B/W mice without clinical renal disease, defined by serial proteinuria of ≥30 mg/dl, were compared with 13 PBS-injected littermate controls. We transferred 6 × 10⁶ Tregs via tail vein injection based on prior reports indicating a large number of Tregs are required to influence organ-specific disease (10, 17). Following adoptive transfer of expanded Tregs, mice in the active treatment group had a significant delay in progression to severe glomerulonephritis.
renal disease, such that 9 wk after cell transfer, only 27% of animals in the treatment group had developed marked proteinuria compared with 85% in the control group. (p < 0.01; Fig. 6A). However, subsequently a number of mice in the treatment group began to develop significant proteinuria, at which time the groups received a second round of Treg transfer (6 × 10⁶ cells) or PBS. Following this second transfer, the progression of renal disease in the Treg recipients was again slowed, while the prevalence of proteinuria continued to increase in the control animals (Fig. 6A). Interestingly, previously sustained severe proteinuria transiently disappeared in two animals and remained below pretransfer levels for 6 wk following the second transfer. In contrast, proteinuria remained >300 mg/dl among control mice. The delayed progression of renal disease in the group of animals that received Tregs resulted in a significant reduction in mortality (p < 0.01 at week 13) that persisted for the duration of the experiment (Fig. 7A). This experiment was terminated when the surviving mice (all from the Treg treatment cohort) developed severe proteinuria and a premorbid state >20 wk after the first transfer. In a separate experiment, a cohort of 10 prediseased B/W mice was treated with either Tregs or PBS to evaluate the effect of transfer on renal pathology. These mice were evaluated by serial measurement of proteinuria for 6 wk following transfer, at which point a significant difference in the development of severe proteinuria (≥300 mg/dl) again developed between the treated and control mice (Fig. 6B; p < 0.01 at 6 wk). These mice were then sacrificed for evaluation of renal pathology. Fig. 8 demonstrates typical sections from treatment and control mice. Overall, mice that received Tregs had less glomerular damage than control animal (average glomerulosclerosis score 0.18 vs 0.93; p < 0.05) and fewer perivascular lymphocytic infiltrates (0.4 vs 2.0; p < 0.05). Treated animals also exhibited less prominent glomerular immune complex and C3 deposition than control mice (IgM, p < 0.02; IgG, p < 0.001; C3, p < 0.05). The less severe pathology present in the kidneys of treated animals was consistent with the absence of marked proteinuria in this group (Fig. 6B).

Because a prior study has indicated that a weak regulatory capacity could be exerted by the CD4⁺CD25⁺ cell fraction (19)
A possible explanation for the failure of the naturally occurring B/W Treg expansion to prevent lupus is that their proliferation is insufficient for the degree of autoactivity present. It has previously been reported that Tregs require IL-2 stimulation to proliferate (22). As demonstrated in more recent studies, Treg activation and proliferation in the setting of impaired IL-2 signaling occurs, but at a rate that appears to be insufficient to prevent lymphoproliferative disorders and autoimmunity (20). Interestingly, B/W mice are known to develop impaired in vivo T cell activation and reduced IL-2 production with age (23). Although the Treg expansion in diseased B/W mice is substantial relative to healthy age-matched BALB/c mice, it is possible that expansion of BALB/c Tregs
would be substantially greater if these non-autoimmune-prone mice were exposed to a similar level of inflammatory stimulus. Significantly, we found the proliferation capacity of B/W Tregs to be comparable to that of Treg from non-autoimmune-prone BALB/c mice when exposed to in vitro stimulation that included exogenous IL-2. In contrast to the increased Treg prevalence in LNs of diseased mice, the splenic Treg prevalence further declined in B/W mice with active disease due to expansion of nonregulatory lymphocytes in the hypertrophied spleens of mice with active lupus. Notably, proliferation of splenic-derived Tregs was comparable to LN-derived cells in vitro. The significance of a decreased frequency of splenic Tregs during disease progression in B/W mice is unclear.

Failure of the endogenous Treg response to control disease in B/W mice could also reflect a defect in Treg function. In this study, we evaluated the suppressive function of both freshly isolated and exogenously expanded Tregs. We found the suppressive capacity of freshly isolated Tregs from both B/W and BALB/c mice to be similar by in vitro assay and that this capacity was not altered by the age or disease state of the animal. Furthermore, the suppressive capacity of B/W and BALB/c Treg derived from young and old mice was equally enhanced by in vitro activation and expansion, a characteristic observed in Tregs from other mouse strains that does not appear to reflect an absolute abnormality in the in vivo suppressive function of these cells (17). Consistent with prior reports of thymic-derived Treg, the suppressor phenotype of B/W Treg was not dependent on soluble cytokines. Blockade of IL-10 or TGF-β did not decrease the efficiency of Treg as measured by in vitro suppression assay of either freshly isolated or expanded cells. Thus, Treg suppressive function is not affected by the disease state in B/W mice, and loss of intrinsic suppression capacity does not appear to be a significant contributor to the development of lupus in this model. Insufficiency of Treg suppression of autoimmunity might also be due to resistance of the autoreactive lymphocytes to Treg suppression. Resistance of Teff from young MRL/Mp lupus-prone mice to suppression by Treg has recently been reported using syngeneic cocultures (12). Due to differences in assay conditions and activation stimulus used, it is difficult to compare our results with those reported for the MRL/Mp mice. All suppression assays reported in our study used CD4+ effectors purified from young B/W mice to identify differences in Treg function independent of variations in Teff responsiveness with age. Testing at the high Tcell:Treg ratio of 25:1 was chosen after titration experiments indicated this ratio optimized detection of differences in Treg suppression. Under these conditions we measured highly efficient suppression of B/W Teff with maximal suppression consistently exceeding 90%. We did find that Tregs could suppress Teff derived from older, sick B/W mice, but a significantly reduced maximal proliferation rate of old Teff (15 times lower proliferation than young Treg) prevented comparison between these assays. This abnormal proliferation of old Teff is consistent with the known T cell activation abnormality that develops in older B/W mice (23). Although several Treg subsets have been described, we chose to evaluate the CD4+CD25+CD62Lhigh Treg subset based on the ability of these cells to ameliorate organ-specific autoimmune disease in murine models of autoimmune diabetes and experimental autoimmune encephalitis (3, 5, 17, 24). The use of this population was also intended to minimize transfer of potentially pathologic, activated nonregulatory CD4+ T cells that transiently express CD25+ in association with down-regulation of CD62L. Isolation and expansion of the CD4+CD25+CD62Llow cell fraction from B/W mice resulted in an expanded cell population containing 50% Foxp3+ CD4+ T cells, underscoring the mixed phenotype of this T cell subset and the potential for transfer of autoreactive cells. Furthermore, these thymic-derived Treg have been characterized in humans, and a protocol for isolation and expansion of functional human Treg has recently been published (25), making these cells of significant therapeutic interest. The expanded nonregulatory CD4+CD25− T cells used in one arm of our control experiments also contain autoreactive T cells, and thus this control might be expected to result in accelerated disease. Although we did not observe significant worsening of disease onset in this control group, we have reported the effect and statistical significance of Treg transfer in relation to saline controls, because this provides the most conservative estimate of the benefit of Treg transfer on disease onset and survival. In previous transfer studies, Ag-specific

**FIGURE 8.** Renal immunohistopathology. Characteristic findings from age-matched mice (10 mice detailed in Fig. 6B) comparing kidneys from Treg-treated mice (top panels) with control mice (bottom panels). A and B. Low-power view of H&E-stained kidney. A. Shows nearly normal kidney histology in Treg-treated mice as compared with extensive glomulosclerosis, tubular dilation, and perivascular infiltrates in the control mice. C and D, Indirect IgG immunofluorescence. Low-power view with high-power inset, demonstrating minimal deposition in glomeruli of Treg-treated mice as compared with marked deposition in glomeruli of control mice. E and F. Indirect IgM immunofluorescence. Low-power view with high-power inset, demonstrating minimal deposition in glomeruli of Treg-treated mice as compared with glomeruli of control mice. G and H, Indirect C3 immunofluorescence. Low-power view with high-power inset, showing minimal deposition in glomeruli of Treg-treated mice as compared with marked C3 deposition in the glomeruli of control mice.
T_{regs} were superior to an unselected polyclonal T_{reg} population in controlling diabetes and experimental autoimmune encephalitis (10, 17). However, the Ag specificity of the autoreactive T cells in B/W lupus-prone mice and in human SLE is unknown. In addition, while it is not entirely clear whether autoreactivity in lupus initially develops as a loss of tolerance to a single or limited set of self-Ags, active disease is characterized by a wide range of reactivity and autoantibody production to self-Ags. A recent study in SNF1 mouse in which murine lupus is accelerated by nucleosome peptide priming demonstrated that T_{regs}, isolated from an Ag-primed mouse protected a recipient mouse from disease acceleration when primed with the same Ag (26). Similarly, peptide-specific CD4^+CD25^+ T cells are induced in B/W mice by a tolerizing consensus peptide, and these cells inhibit production of dsDNA Abs in vitro (27). These interesting findings are consistent with the ability of T_{regs} to inhibit autoantigen-specific responses. However, the ability of an Ag-specific T_{reg} population to suppress the full range of autoreactivity in a systemic autoimmune disease like lupus is unclear. Thus, adoptive transfer of a polyclonal T_{reg} population, as accomplished in this study, may be required for the treatment of SLE. A recent study of integrin expression on Tregs (28) demonstrated that αββ_1-expressing T_{reg}, corresponding to the CD4^+CD25^+CD62L^low subset, preferentially colocalize in tissue with effector/memory T cells. The T_{reg} subset not expressing αββ_1, corresponding to CD4^+CD25^+CD62L^high cells, localize to LNs and thus may predominantly influence naive lymphocytes. Future studies will be required to provide insight into the role of these different T_{reg} subsets in control of lupus.

Interestingly, in this experiment the development of lupus in treated mice was slowed but not prevented, indicating that peripheral tolerance was not completely restored by the T_{reg} transfer protocol used. In the first treatment cohort, the slowing of the progression of renal disease in previously healthy mice was first evident 9 wk after transfer, but this effect was not sustained. Yet after a second transfer, a further impact of T_{reg} supplementation on the progression of active disease was evident within 2–3 wk. Notably, marked disease (sustained proteinuria ≥300 mg/dl before the second transfer) was actually reversed in two mice. Proteinuria dropped to ≤30 mg/dl in these two animals and remained at this level over a period of 6–8 wk following this second transfer. These results also suggest that adoptive transfer of T_{regs} can slow the progress of active murine lupus. The combined effect of two rounds of adoptive T_{reg} transfer produced a substantial survival advantage over control animals during the almost 6-mo-long study. In the second adoptive transfer experiment, T_{reg} treatment again clearly delayed proteinuria and corresponded to a reduction in histologic kidney damage. These findings differ from a recent report in which CD4^+CD25^+ T_{regs} failed to suppress glomerulonephritis in thymectomized NZM2328 mice (29). However, differences in T_{reg} subset, transfer size, and recipient strain, as well our use of ex vivo activation and expansion before transfer make it difficult to compare these studies, but likely contribute to the success of this approach in the B/W model. These results now raise the additional questions of whether disease can be completely prevented by earlier transfer of T_{reg} or by a greater or more frequent supplementation of T_{reg}, or by using a combination of other T_{reg} subsets in addition to the CD4^+CD25^+CD62L^high subset studied here.

Taken together, these results indicate that thymic-derived T_{regs} have a significant role in the control of autoimmunity in lupus-prone B/W mice. Why then do endogenous T_{regs} fail to maintain peripheral tolerance in B/W mice? Although their prevalence is low compared with age-matched non-autoimmune mice, they appear to expand in response to the development of autoimmune disease in vivo, have normal proliferative potential ex vivo, and exhibit normal suppressive function compared with non-autoimmune controls. Thus, if a T_{reg} abnormality contributes to the development of autoimmune disease in B/W mice, this defect is likely to be at the level of development and/or in the homeostatic mechanisms that regulate T_{reg} prevalence or activation rather than due to an intrinsic functional defect. This conclusion is strengthened by the finding that supplementation of the T_{reg} population with exogenously expanded B/W T_{regs} ameliorates disease in this model. Finally, these results suggest that augmentation of the endogenous regulatory population by autologous transfer may be an effective therapeutic approach to SLE.

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Disclosures

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