The Journal of Immunology



# 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. Daikh

*J Immunol* 2006; 177:1451-1459; ;
doi: 10.4049/jimmunol.177.3.1451
http://www.jimmunol.org/content/177/3/1451



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Suppression of Disease in New Zealand Black/New Zealand White Lupus-Prone Mice by Adoptive Transfer of Ex Vivo Expanded Regulatory T Cells[1]

**Kenneth J. Scalapino,* Qizhi Tang,† Jeffrey A. Bluestone,† Mark L. Bonyhadi,‡ and David I. Daikh[2]***

**An increasing number of studies indicate that a subset of $CD4^+$ T cells with regulatory capacity (regulatory T cells; $T_{regs}$) can function to control organ-specific autoimmune disease. To determine whether abnormalities of thymic-derived $T_{regs}$ play a role in systemic lupus erythematosus, we evaluated $T_{reg}$ prevalence and function in (New Zealand Black × New Zealand White)$F_1$ (B/W) lupus-prone mice. To explore the potential of $T_{regs}$ to suppress disease, we evaluated the effect of adoptive transfer of purified, ex vivo expanded thymic-derived $T_{regs}$ on the progression of renal disease. We found that although the prevalence of $T_{regs}$ 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 $CD4^+CD25^+CD62L^{high}$ B/W $T_{regs}$ 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 $T_{regs}$ 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)[3] 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 ($T_{regs}$) 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 $T_{regs}$ are associated with the development of immune-mediated organ damage (3–6). Restoration or supplementation of $T_{reg}$ function in some of these models has been shown to inhibit autoimmunity (4–10). The ability of $T_{regs}$ to modulate peripheral immune responses and the association of $T_{reg}$ abnormalities with some forms of organ-specific autoimmune disease suggest the possibility that a $T_{reg}$ defect may contribute to the development of SLE.

Several studies have evaluated $T_{regs}$ in lupus and correlated low numbers of these cells with disease. Evaluation of $T_{reg}$ prevalence in (New Zealand Black × New Zealand White)$F_1$ (B/W) and (SWR × New Zealand Black)$F_1$ ($SNF_1$) lupus-prone mice found a lower frequency of these cells than in non-autoimmune strains (4, 11), similar to the reported $T_{reg}$ 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 exam $T_{reg}$ frequency in mice with active disease (12). Two recent studies of humans with SLE have reported that the frequency of $CD4^+CD25^+$ $T_{regs}$ in the peripheral circulation of SLE patients is reduced compared with healthy controls (13, 14). Murine models in which $T_{regs}$ are experimentally depleted by deleting the entire $CD4^+CD25^+$ population have produced conflicting data. Thymectomy studies in $SNF_1$ mice produce the expected loss of $T_{regs}$ 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 NZM2328 lupus model accelerated both autoantibody production and glomerulonephritis (16). Overall, these studies suggest that a $T_{reg}$ 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 $T_{regs}$ in healthy young and older diseased B/W lupus-prone mice. We expand on prior reports to characterize changes in $T_{reg}$ prevalence and distribution with the onset of disease. We demonstrate that although young B/W mice

*Department of Medicine, Division of Rheumatology, University of California San Francisco and San Francisco Veterans Affairs Medical Center, San Francisco, CA 94121; †Department of Medicine, University of California San Francisco Diabetes Center, University of California San Francisco, San Francisco, CA 94121; and ‡Xcyte, Seattle, WA 98104

Received for publication October 13, 2005. Accepted for publication April 28, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the University of California San Francisco Autoimmunity Center of Excellence, Department of Veterans Affairs, and the Rosalind Russell Arthritis Research Center.

[2] Address correspondence and reprint requests Dr. David I. Daikh, University of California San Francisco, Arthritis/Immunology 111R, 4150 Clement Street, San Francisco, CA 94121. E-mail address: daikh@itsa.ucsf.edu

[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; $T_{reg}$, regulatory T cell; $T_{eff}$, effector T cell; LN, lymph node; SI, suppression index.



0022-1767/06/$02.00

have a low prevalence of $T_{regs}$, 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 $T_{regs}$ can enhance control of autoreactive lymphocytes in lupus-prone B/W mice, we evaluated the effect of adoptive transfer of exogenously expanded, $CD4^+CD25^+CD62L^{high}$ $T_{regs}$ on disease. We demonstrate that supplementation of the endogenous $T_{reg}$ 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 cellar reagents*

FITC-conjugated mAb against CD4 (GK1.5) was purified in our laboratory. PerCP-Cy5.5-conjugated mAb to $CD4^+$ (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-$\beta$ (1D11) were purchased from R&D Systems. Anti-Fc 2.4G2 and anti-CD3 (2C11) mAb were purified in our laboratory. CFSE was purchased from Molecular Probes/Invitrogen 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 $T_{reg}$ 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 Biotec).

### *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 $CD4^+CD25^+CD62L^{high}$ and $CD4^+CD25^-$ fractions. Purity checks of sorted cells routinely demonstrated >98% purity (Fig. 1, demonstrating $T_{reg}$ purification).

### *$T_{reg}$ culture*

Sorted $CD4^+CD25^+CD62L^{high}$ $T_{regs}$ and $CD4^+CD25^-$ cells were cultured using the protocol described previously (17). Briefly, purified cells were maintained at a concentration of $0.7–1 \times 10^6$ 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 $\mu$M 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 Lympholyte medium (Cedarlane Laboratories) at $230 \times g$ for 25 min. Expanded $T_{regs}$ were routinely assessed for expression of $CD4^+CD25^+CD62L^{high}$, 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; $T_{eff}$) 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 $T_{effs}$ 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 $\mu$g/ml in each well. Freshly isolated or expanded $T_{regs}$ from B/W and BALB/c mice were then added to $T_{eff}$ + APCs (triplicate wells) with a $T_{eff}$:$T_{reg}$ ratio of 25:1. Cells were cultured for 60–70 h before addition of 1 $\mu$Ci/well of [$^3$H]thymidine. Cells were harvested 12 h later, and tritium incorporation was measured on a MicroBeta Scintillation Counter (Wallac). A suppression index (SI), defined as tritium incorporation in the mixed $T_{eff}$:$T_{reg}$ well divided by incorporation in the $T_{eff}$ well, was calculated for each experiment as a measure of $T_{reg}$ suppressive capacity. The SI for freshly isolated and expanded $T_{regs}$ was also measured in the presence

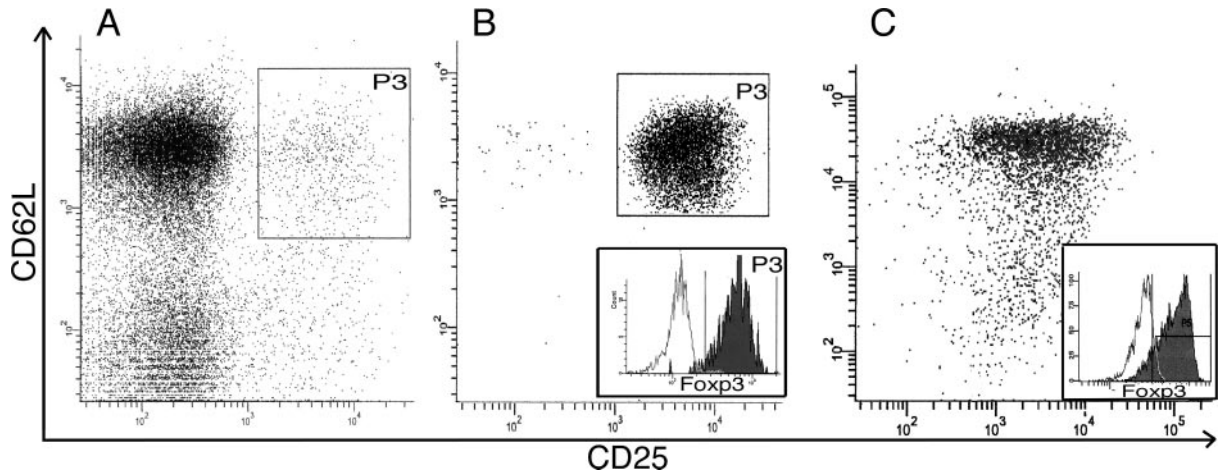


**FIGURE 1.** Purification and expansion of $CD4^+CD25^+CD62L^{high}Foxp3^+$ $T_{regs}$. *A*, Unsorted $CD4^+$ lymphocytes demonstrating ~3% prevalence of $CD4^+CD25^+CD62L^{high}$ cells (gate P3). *B*, Postsort analysis demonstrating 98.2% purity of the $CD4^+CD25^+CD62L^{high}$ cell fraction (gate P3). *Inset*, Histogram of P3-gated cells; 92% of cells express Foxp3 with IgG2a isotype control Ab shown in unfilled histogram. *C*, Expanded $CD4^+CD25^+CD62L^{high}$ cells from gate P3 demonstrating persistence of $CD25^+$ and $CD62L^{high}$ 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.

of neutralizing Abs to IL-10 or TGF-$\beta$ at a concentration of 10 $\mu$g/ml. Control suppression assays used exogenously expanded nonregulatory $CD4^+CD25^-$ cells in place of $T_{regs}$.

### *Adoptive transfer*

Expanded $T_{regs}$ were depleted of Xcyte beads as described above and washed repeatedly in sterile saline. Cells were concentrated in sterile PBS before transfer of $6 \times 10^6$ 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 $6 \times 10^6$ $CD4^+CD25^-$ T cells concentrated in sterile PBS. Two separate treatment experiments using exogenously expanded $T_{regs}$ 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 $T_{regs}$ were suspended in 2.5 $\mu$M CFSE solution for 5 min before washing and transfer of $5–6 \times 10^6$-labeled cells via tail-vein injection. Distribution and survival of CFSE-labeled $T_{regs}$ in the LNs, spleen, and renal parenchyma was evaluated in mice with active disease up to 23 days after transfer by FACS analysis. $T_{reg}$ expansion following transfer was evaluated by labeling $25 \times 10^6$ 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 $T_{reg}$ 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*

$T_{reg}$ frequency was compared using Student's *t* test. Differences in $T_{reg}$ 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 $\chi^2$ analysis using the Yates correction.

## Results

### *$T_{reg}$ prevalence*

We compared the prevalence of $CD4^+CD25^+CD62L^{high}Foxp3^+$ $T_{regs}$ in cohorts of 5–10 young healthy B/W mice, older sick B/W mice with persistent marked proteinuria, and age-matched non-autoimmune BALB/c mice. We assessed the prevalence of $T_{regs}$ in the spleen and in four regional LN distributions; cervical, axillary plus inguinal, mesenteric, and renal LNs. Intracellular expression of Foxp3 was consistently detected in >90% of cells expressing the combination of cell surface markers $CD4^+CD25^+CD62L^{high}$ (Fig. 1) and did not vary with mouse age or breed. Young B/W mice without proteinuria had significantly lower percentages of $T_{regs}$ 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 $T_{reg}$ 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 $T_{regs}$, 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 $T_{regs}$ from young and old mice. In contrast, there was a further decline in the prevalence of $T_{regs}$ 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 $T_{regs}$ in each distribution including the spleen increases with disease onset despite a decline in splenic $T_{reg}$ 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 $T_{reg}$ prevalence population was observed in both the LNs and the spleens of older mice.

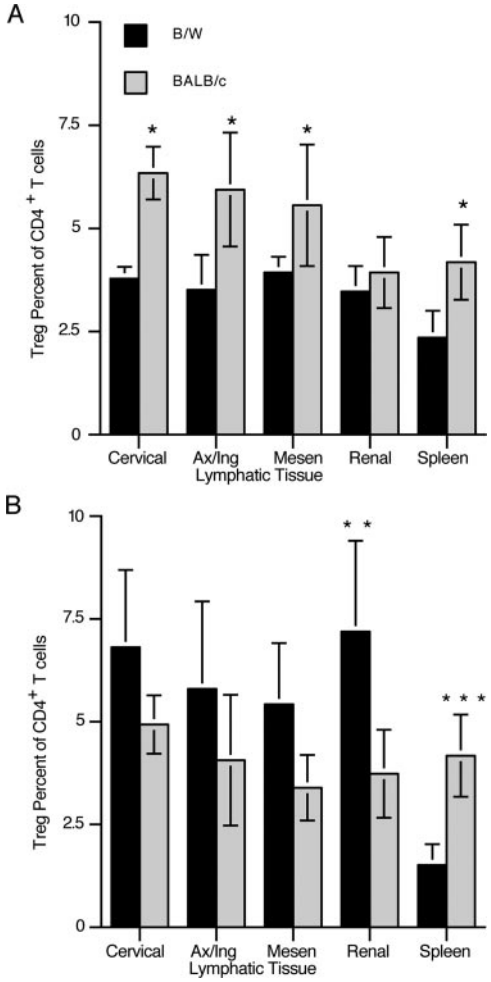


**FIGURE 2.** Lymphatic and splenic prevalence of $CD4^+CD25^+$ $CD62L^{high}$ $T_{regs}$ in B/W and age-matched BALB/c mice. *A*, Eight- to 10-wk-old mice demonstrating significantly fewer $T_{regs}$ in B/W mice (■) compared with BALB/c (□) in all distributions (*, $p < 0.05$) except for the renal LNs ($p = 0.31$). *B*, Forty-eight- to 52-wk-old mice demonstrating expansion of $T_{reg}$ in LNs and a decrease in splenic $T_{regs}$ in diseased B/W mouse. As compared with BALB/c, B/W mice with active renal disease have more renal LN $T_{regs}$ (**, $p = 0.04$) and fewer splenic $T_{regs}$ (***, $p = 0.04$). Compared with young B/Ws, the old B/W mice demonstrate significantly more $T_{regs}$ in all LN distributions ($p \leq 0.03$) except mesenteric LN ($p = 0.07$) and significantly fewer splenic $T_{regs}$ ($p = 0.011$).

### *$T_{reg}$ function*

To determine whether B/W $T_{regs}$ exhibit normal suppressor function, we evaluated their ability to suppress T cell proliferation compared with $T_{regs}$ purified from non-autoimmune mice. Three to five mice per experiment were evaluated. $T_{eff}$ were mixed with $T_{regs}$ at a ratio of 25:1 for each experiment. Freshly isolated $T_{regs}$ from young and old B/W mice produced a similar SI of 0.33 and 0.26, respectively ($p = 0.12$; Fig. 3*A*). Expanded B/W $T_{regs}$ 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. 3*A*). The suppression capacities of both freshly isolated and expanded BALB/c $T_{regs}$ were comparable to those of B/W $T_{regs}$ (freshly isolated SI = 0.30; and expanded SI = 0.16) and did not vary with the age of the mouse (Fig. 3*B*). The SI of all expanded cells was enhanced compared with freshly harvested $T_{regs}$ ($p = 0.04$), a characteristic of exogenously expanded $T_{regs}$ previously reported in NOD mice (17). The suppression capacity of freshly isolated and expanded $T_{regs}$ was not diminished by the addition of neutralizing Abs to IL-10 and TGF-$\beta$ ($p > 0.5$ for all assays) (Fig. 4).

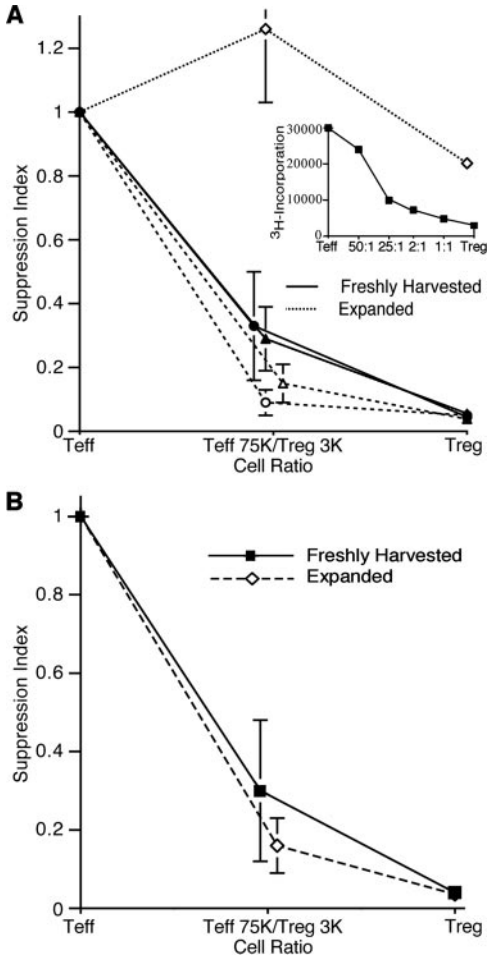


**FIGURE 3.** $T_{reg}$ suppressor function. *A*, Suppression of $T_{eff}$ proliferation by B/W $T_{regs}$ isolated from young healthy and older diseased mice. Suppression by both freshly isolated (solid lines) and expanded (dashed lines) $T_{regs}$ is shown for experiments at a $T_{eff}$:$T_{reg}$ ratio of 25:1 (average of 3 mice/group). Freshly isolated $T_{regs}$ from both young healthy (●) and diseased older (▲) B/W mice produce equivalent inhibition ($p = 0.48$). Expanded $T_{regs}$ from young (○) and diseased older (△) produce similar suppression ($p = 0.14$). Expanded $T_{regs}$ have enhanced suppression as compared with freshly isolated $T_{regs}$ ($p < 0.05$). Expanded nonregulatory $CD4^+$ T cells do not exert measurable suppression in this assay (◇). *Inset*, Titration curve demonstrating typical suppression over a range of $T_{eff}$:$T_{reg}$ concentrations. *B*, Suppression by both freshly isolated (solid line) and expanded (dashed line) BALB/c $T_{regs}$. There were no significant differences in suppression between young and old BALB/c mice.

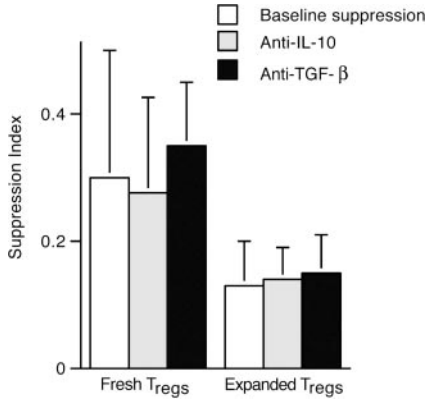


**FIGURE 4.** $T_{reg}$ suppressive function following cytokine neutralization. Suppression at a $T_{eff}$:$T_{reg}$ ratio of 25:1 before and after addition of blocking Abs to IL-10 and TGF-$\beta$. The suppressor function of fresh and expanded $T_{regs}$ is not dependent on secreted IL-10 or TBF-$\beta$ ($p > 0.5$ for all assays).

We next evaluated the possibility that an impaired $T_{reg}$ proliferation capacity might contribute to a loss of peripheral tolerance in B/W mice. To determine the proliferation capacity of $T_{regs}$, $CD4^+CD25^+CD62L^{high}$ cells purified from both young healthy and older sick B/W mice (5 mice/group) were expanded in vitro. $T_{regs}$ isolated from both healthy and sick B/W mice expanded 80-fold over a 10-day period, independent of whether the $T_{regs}$ were isolated from the LNs or spleen. This proliferation was equivalent to the 80-fold expansion of $T_{regs}$ purified from age-matched BALB/c obtained in parallel experiments. Expanded cells maintained expression of $CD4^+CD25^+CD62L^{high}$ and Foxp3 (>90% for cells isolated from both young and old mice) when the purity of the initial $T_{reg}$ culture exceeded 98% (Fig. 1).

### *Adoptive transfer experiments*

To determine whether exogenously expanded $T_{regs}$ survived and expanded following adoptive transfer, $T_{regs}$ 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 $T_{regs}$ population can suppress the development of murine lupus, we assessed the effects of transferring exogenously expanded $T_{reg}$ 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 $6 \times 10^6$ $T_{regs}$ via tail vein injection based on prior reports indicating a large number of $T_{regs}$ are required to influence organ-specific disease (10, 17). Following adoptive transfer of expanded $T_{regs}$, mice in the active treatment group had a significant delay in progression to severe

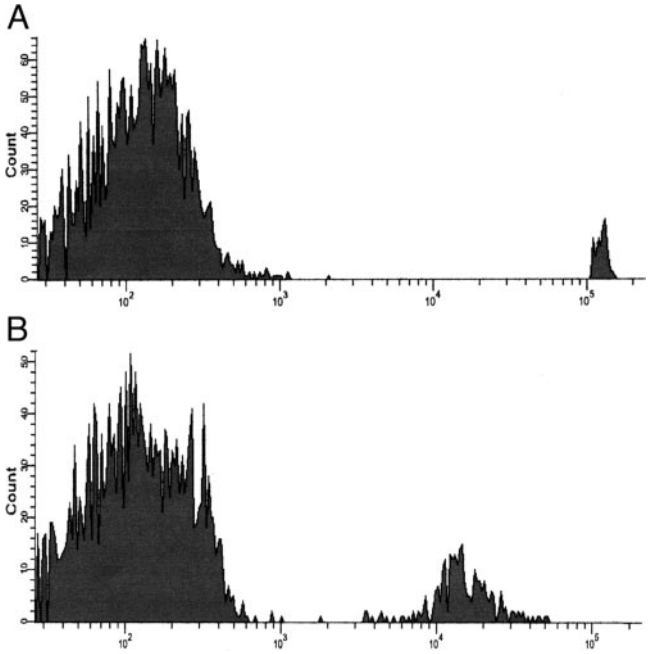


**FIGURE 5.** Survival and proliferation of adoptively transferred $T_{regs}$. Exogenously expanded, CFSE-labeled $T_{regs}$ were transferred to aged B/W mice. *A*, FACS analysis of CFSE bright cells 12 h after adoptive transfer, indicating successful transfer and early cell survival. *B*, CFSE bright $T_{regs}$ detected 23 days after transfer, demonstrating CFSE dilution consistent with survival and proliferation of transferred $T_{regs}$.

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. 6*A*). 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 $T_{reg}$ transfer ($6 \times 10^6$ cells) or PBS. Following this second transfer, the progression of renal disease in the $T_{reg}$ recipients was again slowed, while the prevalence of proteinuria continued to increase in the control animals (Fig. 6*A*). 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 $T_{regs}$ resulted in a significant reduction in mortality ($p < 0.01$ at week 13) that persisted for the duration of the experiment (Fig. 7*A*). This experiment was terminated when the surviving mice (all from the $T_{reg}$ 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 $T_{regs}$ 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. 6*B*; $p < 0.01$ 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 $T_{regs}$ 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

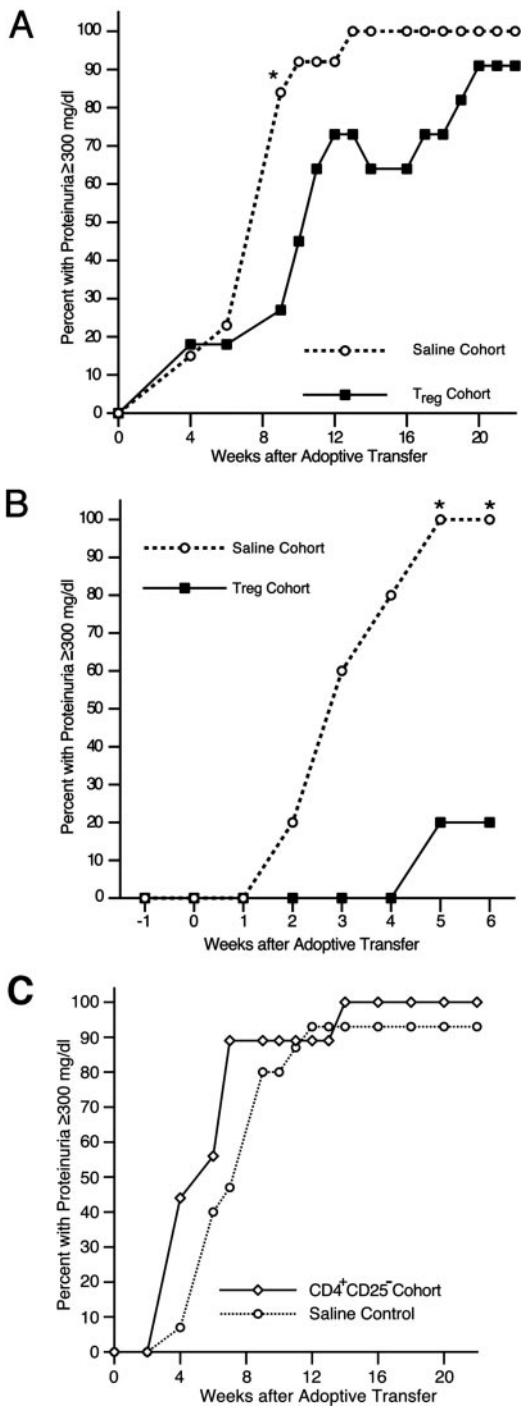


**FIGURE 6.** Delayed onset and progression of chronic proteinuria in B/W mice following adoptive transfer of $T_{regs}$. *A*, Twenty-four- to 29-wk-old mice received $CD4^+CD25^+CD62L^{high}$ $T_{regs}$ (■, 11 mice) or PBS (○, 13 mice). Percentage of each mouse cohort with proteinuria ≥300 mg/dl following adoptive transfer of $CD4^+CD25^+CD62L^{high}$ $T_{regs}$ at weeks 0 and 12 (*, $p < 0.01$ compared with control group). *B*, Second cohort of B/W mice treated at age 33 wk, demonstrating significantly delayed proteinuria (*, $p < 0.02$) in $T_{reg}$ recipients (■, 5 mice) as compared with PBS controls (○, 5 mice) before sacrifice for histologic evaluation. *C*, Progression to severe proteinuria in 29-wk-old mice receiving $CD4^+CD25^-$ control cells (8 mice) vs PBS (10 mice) ($p > 0.07$ at all time points).

pathology present in the kidneys of treated animals was consistent with the absence of marked proteinuria in this group (Fig. 6*B*).

Because a prior study has indicated that a weak regulatory capacity could be exerted by the $CD4^+CD25^-$ cell fraction (19)

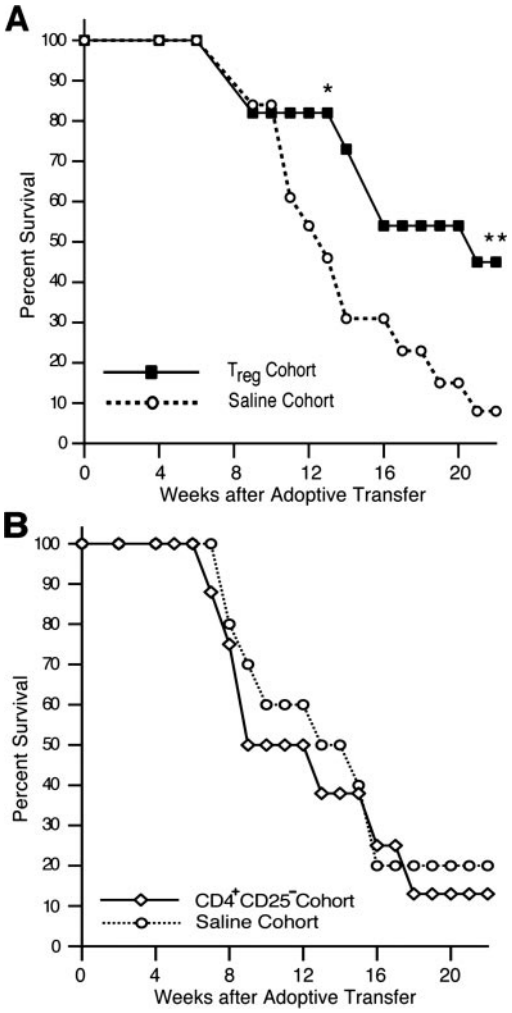


**FIGURE 7.** Survival of B/W mice following adoptive transfer. *A*, Mice (24 mice from Fig. 6*A*) received $CD4^+CD25^+CD62L^{high}$ $T_{regs}$ (■) or PBS (○). Percentage of survival of each cohort following adoptive transfer at weeks 0 and 12. Improved survival in the $T_{reg}$-treated group is significant starting at week 13 (*, $p < 0.01$; **, $p < 0.05$). *B*, Survival following transfer of $CD4^+CD25^-$ control cells vs PBS (18 mice from Fig. 6*C*) ($p > 0.2$ at all time points).

when these cells were used to reconstitute an immune system, we also compared the effect of $CD4^+CD25^-$ T cell transfer with PBS injection. Eighteen 29-wk-old B/W mice without renal disease received either PBS or $6 \times 10^6$ $CD25^+$ depleted, exogenously expanded $CD4^+$ T cells via tail vein injection. In this case, mice receiving $CD4^+CD25^-$ T cells developed similar onset of proteinuria and mortality as compared with the PBS controls over the 24 wk following transfer (Figs. 6*C* and 7*B*). We also measured Abs against dsDNA (dsDNA Ab) in all treatment and control mice. No dsDNA Abs were detectable at baseline in either the treatment or control groups, consistent with prediseased animals. Abs measured monthly thereafter demonstrated development of dsDNA Abs in both the $T_{reg}$ and control cohorts, but no difference between the groups was observed (data not shown).

## Discussion

The capacity of $T_{regs}$ to suppress peripheral lymphocyte activation and proliferation has led to extensive investigation of the role of $T_{regs}$ in the pathogenesis and treatment of immune-mediated disease. In this study, we expand on prior studies of $T_{regs}$ in lupus to evaluate prevalence, distribution, and function of these cells in B/W lupus-prone mice before and after the onset of disease. These studies show that $T_{regs}$ increase in number during the development of disease, and they indicate that there is not an intrinsic abnormality in suppressive function or proliferative capacity of thymic-derived $T_{regs}$ in B/W mice. The apparent absence of an intrinsic defect in these cells, as well as the relative deficiency of these cells before disease onset suggested the possibility that augmentation of the regulatory cell population could result in improved control of autoimmune disease in lupus-prone mice. We therefore evaluated the therapeutic potential of adoptive transfer of in vitro expanded $T_{regs}$ on the development and progression of murine lupus. Adoptive transfer of $T_{regs}$ was undertaken before the onset of significant clinical disease to assess whether lupus could be prevented by supplementation of $T_{reg}$ peripheral surveillance. This intervention resulted in a decreased progression of proteinuria and markedly decreased mortality compared with control animals. The slowing of the progression of renal disease was transient, but resulted in a survival benefit that was evident by 13 wk after adoptive transfer. After a second $T_{reg}$ transfer, treated mice again exhibited a slowing of disease progression. Overall, the delayed progression to severe proteinuria resulted in significantly enhanced survival over almost 6 mo among the mice receiving $T_{reg}$ supplementation. This beneficial impact of $T_{reg}$ supplementation was observed in a second experiment, in which we also found that adoptive $T_{reg}$ transfer inhibited progression of kidney pathology.

The mechanisms regulating the frequency of $T_{regs}$ in healthy and diseased mice are not known. Two recent studies indicate that production of functional $T_{regs}$ occurs in the thymus in the absence of IL-2, but peripheral $T_{reg}$ survival and expansion is greatly reduced in the absence of IL-2 (20, 21). Correlation between reduced $T_{reg}$ prevalence in the peripheral circulation or spleen and autoimmunity has been reported in several systems including the B/W mouse. Previous studies have noted a decreased prevalence of $T_{regs}$ in lupus-prone mice, implying that a lack of $T_{regs}$ may contribute to the loss of peripheral tolerance. However, we show in this study that while before the onset of clinical disease B/W mice have a low prevalence of $T_{regs}$ in most LN distributions and spleen compared with age-matched BALB/c mice, the prevalence of these cells increases in all regions with the development of disease. Whether the decreased numbers of $T_{regs}$ in young B/W mice represent an intrinsic regulatory defect or a simple strain difference is not clear. The increased prevalence of $T_{regs}$ in diseased B/W mice suggests that this population can expand in response to the onset of autoreactivity, either via local proliferation or increased trafficking, and that this response is wide spread, consistent with the systemic disease that characterizes SLE. Interestingly, the most dramatic expansion of $T_{regs}$, a 2-fold increase in LNs draining the salivary glands and kidneys, correlates with the development of significant cellular infiltration and inflammation of these tissues in B/W mice with advanced disease. Nevertheless, this endogenous expansion of $T_{regs}$ does not prevent end-organ damage in B/W mice.

A possible explanation for the failure of the naturally occurring B/W $T_{reg}$ expansion to prevent lupus is that their proliferation is insufficient for the degree of autoreactivity present. It has previously been reported that $T_{regs}$ require IL-2 stimulation to proliferate (22). As demonstrated in more recent studies, $T_{reg}$ activation and proliferation in the setting of impaired IL-2 signaling occurs, but at 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 $T_{reg}$ expansion in diseased B/W mice is substantial relative to healthy age-matched BALB/c mice, it is possible that expansion of BALB/c $T_{regs}$

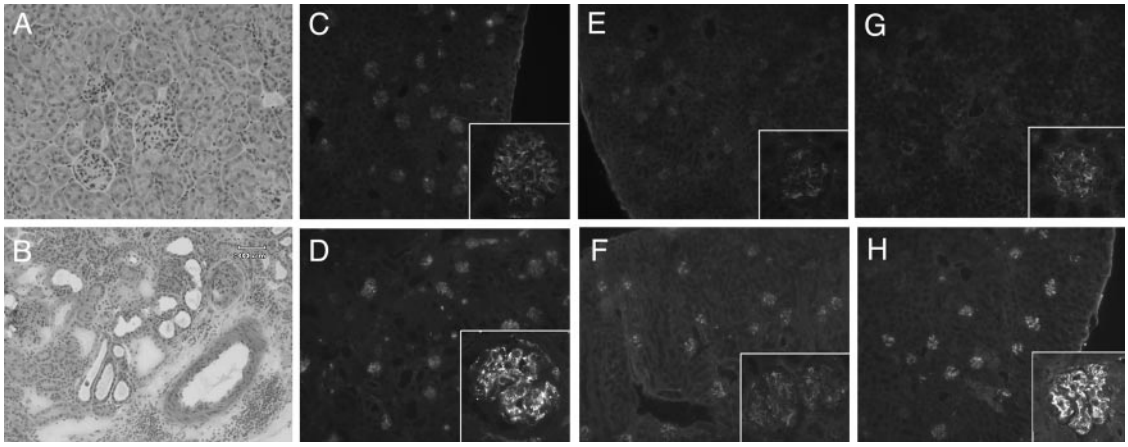


**FIGURE 8.** Renal immunohistopathology. Characteristic findings from age-matched mice (10 mice detailed in Fig. 6*B*) comparing kidneys from $T_{reg}$-treated mice (*top panels*) with control mice (*bottom panels*). *A* and *B*, Low-power view of H&E-stained kidney. *A*, Shows nearly normal kidney histology in $T_{reg}$-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 $T_{reg}$-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 $T_{reg}$-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 $T_{reg}$-treated mice as compared with marked C3 deposition in the glomeruli of control mice.

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 $T_{regs}$ to be comparable to that of $T_{regs}$ from non-autoimmune-prone BALB/c mice when exposed to in vitro stimulation that included exogenous IL-2. In contrast to the increased $T_{reg}$ prevalence in LNs of diseased mice, the splenic $T_{reg}$ 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 $T_{regs}$ was comparable to LN-derived cells in vitro. The significance of a decreased frequency of splenic $T_{regs}$ during disease progression in B/W mice is unclear.

Failure of the endogenous $T_{reg}$ response to control disease in B/W mice could also reflect a defect in $T_{reg}$ function. In this study, we evaluated the suppressive function of both freshly isolated and exogenously expanded $T_{regs}$. We found the suppressive capacity of freshly isolated $T_{regs}$ 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 suppression capacity of B/W and BALB/c $T_{regs}$ derived from young and old mice was equally enhanced by in vitro activation and expansion, a characteristic observed in $T_{regs}$ 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 $T_{regs}$, the suppressor phenotype of B/W $T_{reg}$ was not dependent on soluble cytokines. Blockade of IL-10 or TGF-$\beta$ did not decrease the efficiency of $T_{regs}$ as measured by in vitro suppression assay of either freshly isolated or expanded cells. Thus, $T_{reg}$-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 $T_{reg}$ suppression of autoimmunity might also be due to resistance of the autoreactive lymphocytes to $T_{reg}$ suppression. Resistance of $T_{eff}$ from young MRL/Mp lupus-prone mice to suppression by $T_{regs}$ 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 $CD4^+$ effector cells purified from young B/W mice to identify differences in $T_{reg}$ function independent of variations in $T_{eff}$ responsiveness with age. Testing at the high $T_{eff}$:$T_{reg}$ ratio of 25:1 was chosen after titration experiments indicated this ratio optimized detection of differences in $T_{reg}$ suppression. Under these conditions we measured highly efficient suppression of B/W $T_{eff}$, with maximal suppression consistently exceeding in 90%. We did find that $T_{regs}$ could suppress $T_{eff}$ derived from older, sick B/W mice, but a significantly reduced maximal proliferation rate of old $T_{eff}$ (15 times lower proliferation than young $T_{eff}$) prevented comparison between these assays. This abnormal proliferation of old $T_{eff}$ is consistent with the known T cell activation abnormality that develops in older B/W mice (23).

Although several $T_{reg}$ subsets have been described, we chose to evaluate the $CD4^+CD25^+CD62L^{high}$ $T_{reg}$ 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 $CD4^+CD25^+CD62L^{low}$ 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 $T_{regs}$ have been characterized in humans, and a protocol for isolation and expansion of functional human $T_{regs}$ 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 $T_{reg}$ transfer in relation to saline controls, because this provides the most conservative estimate of the benefit of $T_{reg}$ transfer on disease onset and survival. In previous transfer studies, Ag-specific

$T_{regs}$ 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 characterize by a wide range of reactivity and autoantibody production to self-Ags. A recent study in $SNF_1$ mice 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 $T_{regs}$ (28) demonstrated that $\alpha_e\beta_7$-expressing $T_{regs}$, corresponding to the $CD4^+CD25^+CD62L^{low}$ subset, preferentially colocalize in tissue with effector/memory T cells. The $T_{reg}$ subset not expressing $\alpha_e\beta_7$, 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 $\geq$300 mg/dl before the second transfer) was actually reversed in two mice. Proteinuria dropped to $\leq$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 $T_{regs}$, by a greater or more frequent supplementation of $T_{regs}$, 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.

## Acknowledgments

We are grateful to Dr. David Wofsy for critical review of this manuscript and Mingying Bi and Gail Cassafer for technical assistance with cell preparation, culture, and histologic slides.

## Disclosures

The authors have no financial conflict of interest.

## References

1. Itoh, M., T. Takahashi, M. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a function of the thymus in maintaining immunologic self-tolerance. *J. Immunol.* 162: 5317–5326.
2. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nature* 3: 253–257.
3. Lepault, F., and M. C. Gagnerault. 2000. Characterization of peripheral regulatory $CD4^+$ T cells that prevent diabetes onset in nonobese diabetic mice. *J. Immunol.* 164: 240–247.
4. Wu, A., S. Hua, S. Munson, and H. McDevitt. 2002. Tumor necrosis factor-$\alpha$ regulation of $CD4^+CD25^+$ T cell levels in NOD mice. *Proc. Natl. Acad. Sci. USA* 99: 12287–12292.
5. Kohm, A. P., P. A. Carpentier, H. A. Anger, and S. D. Miller. 2002. $CD4^+CD25^+$ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J. Immunol.* 169: 4712–4716.
6. Bach, J., and L. Chatenoud. 2001. Tolerance to islet autoantigens and type 1 diabetes. *Annu. Rev. Immunol.* 19: 131–161.
7. Taylor, P. A., C. J. Lees, and B. R. Blazar. 2002. The infusion of ex vivo activated and expanded $CD4^+CD25^+$ immune regulatory cells inhibit graft-versus-host disease lethality. *Blood* 99: 3493–3499.
8. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. 2002. The subpopulation of $CD4^+CD25^+$ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol.* 169: 2461–2465.
9. Hoffmann, P., J. Ermann, M. Edinger, C. G. Fathman, and S. Strober. 2002. Donor-type $CD4^+CD25^+$ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Exp. Med.* 196: 389–399.
10. Mekala, D. J., and T. L. Geiger. 2005. Immunotherapy of autoimmune encephalomyelitis with redirected $CD4^+CD25^+$ T lymphocytes. *Blood* 105: 2090–2092.
11. Wu, H. Y., and N. A. Staines. 2004. A deficiency of $CD4^+CD25^+$ T cells permits the development of spontaneous lupus-like disease in mice, and can be reversed by induction of mucosal tolerance to histone peptide autoantigen. *Lupus* 13: 192–200.
12. Monk, C. R., M. Spachidou, F. Rovis, E. Leung, M. Botto, R. I. Lechler, and O. A. Garden. 2005. MRL/Mp $CD4^+CD25^-$ T cells show reduced sensitivity to suppression by $CD4^+CD25^+$ regulatory T cells in vitro. *Arthritis Rheum.* 52: 1180–1184.
13. Crispin, J. C., A. Martinez, and J. Alcorcer-Varela. 2003. Quantification of regulatory T cells in patients with systemic lupus erythematosus. *J. Autoimmun.* 21: 273–276.
14. Liu, M. F., C. R. Wang, L. L. Fung, and C. R. Wu. 2004. Decreased $CD4^+CD25^+$ T cells in peripheral blood of patients with systemic lupus erythematosus. *Scand. J. Immunol.* 59: 198–202.
15. Bagavant, H., C. Thompson, K. Ohno, Y. Setiady, and K. Tung. 2002. Differential effect of neonatal thymectomy on systemic and organ-specific autoimmune disease. *Int. Immunol.* 14: 1397–1406.
16. Bagavant, H., U. Deshmukh, F. Gaskin, and S. Fu. 2004. Lupus glomerulonephritis revisited 2004: Autoimmunity and end-organ damage. *Scand. J. Immunol.* 60: 52–63.
17. Tang, Q., K. Henriksen, M. Bi, E. B. Finger, G. Szot, J. Ye, E. L. Masteller, H. McDevitt, M. Bonyhadi, and J. A. Bluestone. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J. Exp. Med.* 199: 1455–1465.
18. Cunnane, G., O. Chan, G. Cassafer, S. Bridis, E. Kaufman, T. Benedict Yen, and D. Daikh. 2004. Prevention of renal damage in murine lupus nephritis by CTLA-4Ig and cyclophosphamide. *Arthritis Rheum.* 50: 1539–1548.
19. Stephens, L. A., and D. Mason. 2000. CD25 is a marker for $CD4^+$ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function

are found in both $CD25^+$ and $CD25^-$ subpopulations. *J. Immunol.* 165: 3105–3110.
20. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6: 1142–1151.
21. D'Cruz, L. M., and L. Klein. 2005. Development and function of agonist-induced $CD25^+Foxp3^+$ regulatory T cells in the absence of interleukin 2 signaling. *Nat. Immunol.* 6: 1152–1159.
22. Papiernik, M., M. L. de Moraes, C. Pontoux, F. Vasseur, and C. Penit. 1998. Regulatory CD4 T cells: expression of the IL-2R$\alpha$ chain, resistance to clonal deletion and IL-2 dependency. *Int. Immunol.* 10: 371–378.
23. Altman, A., A. N. Theofilopoulos, R. Weiner, D. H. Katz, and F. J. Dixon. 1981. Analysis of T cell function in autoimmune murine strains: defects in production of and responsiveness to interleukin 2. *J. Exp. Med.* 154: 791–808.
24. Fu, S., A. C. Yopp, X. Mao, D. Chen, N. Zhang, D. Chen, M. Mao, Y. Ding, and J. S. Bromberg. 2004. $CD4^+CD25^+CD62^+$ T-regulatory cell subset had optimal suppressive and proliferative potential. *Am. J. Transplant.* 4: 65–78.
25. Earle, K. E., Q. Tang, X. Zhou, W. Liu, M. Bonyhadi, and J. A. Bluestone. 2005. In vitro expanded human $CD4^+CD25^+$ regulatory T cells suppress effector T cell proliferation. *Clin. Immunol.* 115: 3–9.
26. Kang, H.-K., M. A. Micheals, B. R. Berner, and S. K. Datta. 2005. Very low-dose tolerance with nucleosomal peptides controls lupus and induces potent regulatory T cell subsets. *J. Immunol.* 174: 3247–3255.
27. La Cava, A., F. M. Ebling, and B. H. Hahn. 2004. Ig-reactive $CD4^+CD25^+$ T cells from tolerized (NZB $\times$ NZW)$F_1$ mice suppress in vitro production of antibodies to DNA. *J. Immunol.* 173: 3542–3548.
28. Huehn, J., K. Siegmund, J. C. U. Lehmann, C. Siwert, U. Haubold, M. Feuerer, G. F. Debes, J. Lauber, O. Frey, G. K. Przybylski, et al. 2004. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like $CD4^+$ regulatory T cells. *J. Exp. Med.* 199: 303–313.
29. Bagavant, H., and K. S. K. Tung. 2005. Failure of $CD25^+$ T cells from lupus-prone mice to suppress lupus glomerulonephritis and sialoadenitis. *J. Immunol.* 175: 944–950.