Failure of CD25+ T Cells from Lupus-Prone Mice to Suppress Lupus Glomerulonephritis and Sialoadenitis

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The development of organ-specific autoimmune diseases in mice thymectomized on day 3 of life (d3tx mice) can be prevented by transferring CD4⁺CD25⁺ T cells from syngeneic, normal adult mice. Using a d3tx model, we asked whether CD4⁺CD25⁺ T cell deficiency contributes to glomerulonephritis (GN) in lupus-prone mice. New Zealand Mixed 2328 (NZM2328) mice spontaneously develop autoantibodies to dsDNA and female-dominant, fatal GN. After d3tx, both male and female NZM2328 mice developed 1) accelerated dsDNA autoantibody response, 2) early onset and severe proliferative GN with massive mesangial immune complexes, and 3) autoimmune disease of the thyroid, lacrimal gland, and salivary gland. The d3tx male mice also developed autoimmune prostatitis. The transfer of CD25⁺ cells from 6-wk-old asymptomatic NZM2328 donors effectively suppressed dsDNA autoantibody and the development of autoimmune diseases, with the exception of proliferative lupus GN and sialoadenitis. This finding indicates that NZM2328 lupus mice have a selective deficiency in T cells that regulates the development of lupus GN and sialoadenitis. After d3tx, the proliferative GN of female mice progressed to fatal GN, but largely regressed in the male, thereby revealing a checkpoint in lupus GN progression that depends on gender. The Journal of Immunology, 2005, 175: 944–950.

SLE is a complex multisystemic autoimmune disease, characterized by circulating Ab to nuclear and cytoplasmic Ags (9). Lupus glomerulonephritis (GN) affects ~50% of lupus patients and is associated with the deposition of immune complexes and complement components in the glomeruli, with infiltration of inflammatory cells and, in severe disease, glomerular and interstitial fibrosis, tubular atrophy, and renal failure (10). The lupus-prone New Zealand Mixed 2328 (NZM2328) mouse, a recombinant inbred strain that originated from the crosses among New Zealand Black and New Zealand White mice and their progenies also develops GN (11). NZM2328 mice have autoantibodies to nuclear Ags including dsDNA. At 12 mo of age, 72% of females and 25% of males develop severe proteinuria with lupus GN (12).

Previous studies have explored the presence of Treg cells for the dsDNA autoantibody response. See et al. (13) showed that the transfer of Treg cells into mice expressing anti-dsDNA BCRs abrogated the maturation process of transgenic B cells into Ab-forming cells. La Cava et al. (14) showed that Treg cells from (New Zealand Black × New Zealand White) F1 mice injected with a tolerogenic peptide from the CDR3 region of an anti-dsDNA Ab prevented B cell synthesis of dsDNA Ab. In these studies the question of whether CD25⁺ regulatory T cells can suppress lupus GN was not investigated.

In the present study we used the classical approach to induce autoimmune disease by thymectomy (tx) on day 3 of life (d3tx) (15, 16). Although the mechanism of d3tx-induced autoimmune diseases is not fully understood, the current belief is that d3tx results in preferential depletion of CD4⁺CD25⁺ Treg cells relative to the autoreactive CD25⁺ T effector population. The resultant state of regulatory and effector T cell imbalance is also exaggerated by homeostatic T cell expansion in the profoundly lymphopenic d3tx mice (17, 18). Regardless of the precise mechanism of disease induction, all d3tx-induced, organ-specific autoimmune diseases in nonlupus mice are readily prevented by early infusion of CD4⁺CD25⁺ T cells from normal syngeneic adults (19, 20).

In this study we have investigated the capacity of CD25⁺ regulatory T cells from lupus-prone NZM2328 mice to suppress autoimmune disease and autoantibody response in d3tx NZM2328 lupus mice. The study was possible because of the observation,
described below, that d3tx NZM2328 mice developed severe pro-
iferative GN of early onset, sialoadenitis, and accelerated autoan-
tobody response to dsDNA. As an important control, we studied the
capacity of the same population of CD25+ T cells from NZM2328
mice to suppress other organ-specific autoimmune diseases unre-
related to the lupus syndrome.

Materials and Methods

Mice
A colony of NZM2328 has been maintained and housed under specific
pathogen-free conditions at the University of Virginia Animal care facility.
All mice used were generated in this facility. The animal housing and
experimental protocols were performed in accordance with National Insti-
tutes of Health guidelines. Male and female NZM2328 mice were thymec-
tomized (tx) on days 2–4 after birth using established protocols and aseptic
procedure (21). Briefly, 2- to 4-day-old mice were anesthetized using hy-
pothesis, and the sternum was exposed through a midline incision. The
sternum and ribs were cut, and the thymus was aspirated using a Pasteur
pipette. The wound was closed using surgical glue. At the time of death,
tissue from the superior mediastinum was collected and studied by histol-
ogy for the presence of any residual thymus. Mice with residual thymus
were excluded from the study. Sham-tx mice underwent surgery on days
2–4 after birth without removal of the thymus.

Monitoring and autopsy

The mice were monitored for proteinuria, and tail blood was collected at
monthly intervals. At 20 or 30 wk of age, the mice were killed, and a complete autopsy was performed. Organs (kidneys, salivary glands, lacri-
mal glands, thyroid, ovary, testes, and prostate) were collected in Bouins’
fixative and processed for histopathology. One kidney was snap frozen in
liquid nitrogen and used for immunofluorescence staining and Ab elution
studies.

Histopathology, GN severity index and organ specific autoimmune

Four-micron sections were cut from Bouins-fixed, paraffin-embedded tis-
sues and stained with H&E. An observer blinded to the experimental de-
sign evaluated histopathology. The severity of acute GN was graded based
on the percentage of glomeruli involved, the severity of inflammatory cell
infiltration, and proliferative changes in mesangial and/or peripheral re-
gions. Glomeruli were measured at their maximum diameter using an oc-
ular micrometer, and numbers of nuclei in each glomerulus were counted.
The data for glomerular size and cellularity presented are the mean of
readings from 10 glomeruli for each mouse. Chronic GN was scored based on
glomerular fibrosis, interstitial fibrosis, and tubular atrophy. Each of the
above-mentioned changes was scored from 0 to 4, with 0 indicating no
pathology and 4 being maximum pathology. The severity index represents
a cumulative score. Severity of organ-specific disease was scored based on
the presence of inflammatory infiltrates in different tissues, with 0 as nor-
mal, 1–3 indicating increasing severity of infiltration, and 4 associated with
destruction of normal architecture.

Estimation of anti-dsDNA IgG and total IgG by ELISA

Ninety-six-well plates were coated with streptavidin (1 mg/ml), followed
by biotinylated plasmid DNA as previously described (22). After blocking
with 3% BSA in PBS, the plates were incubated with sera at 1/100
dilution. Bound Abs were detected with HRP-conjugated goat anti-mouse
IgG, followed by o-phenylene diamine and hydrogen peroxide in citrate
phosphate buffer, pH 5.0. The reaction was stopped with 2.5 N sulfuric
acid, and the OD was read at 490 nM in an ELISA reader. An mAb to
dsDNA (R4A; gift from Dr. B. Diamond, Albert Einstein College of Med-
icine, Bronx, NY) was used as the standard. IgG levels in sera and kidney
eluates were estimated using a sandwich ELISA as previously described
(21). Purified mouse IgG at different concentrations (4–500 ng/ml) was
used to construct a standard curve.

Detection of renal immune complexes by direct immunofluorescence

Immune complexes in renal glomeruli were detected by direct immuno-
fluorescence (21). Five-micron sections of snap-frozen kidney were fixed
in ice-cold acetone for 10 min. The sections were rinsed with PBS and
blocked with normal goat serum in 3% BSA/PBS (1/10, v/v) for 20 min.
The sections were then incubated with FITC-conjugated goat anti-mouse
IgG (Southern Biotechnology Associates; 1/50 dilution) for 45 min. After
two rinses in PBS, the tissue sections were mounted with Vectashield
mounting fluid (Vector Laboratories), and fluorescence intensity was
graded from 0 to 4 in a blinded fashion.

Acid elution of Igs from kidney

Igs were eluted from the kidney as previously described (23). Briefly, each
individual frozen kidney was weighed and homogenized in cold PBS on
ice. The homogenate was centrifuged at 1200 X g for 10 min at 4°C. The
pellets were washed by resuspension in cold PBS, followed by vortexing
and centrifugation to remove serum proteins. The washing was repeated
until the OD260 of the supernatant was <0.05. The pellet was then sus-
pended in 0.1 M glycine with 1% BSA, pH 2.8 (10 ml/g kidney), and
mixed gently at 4°C for 20 min. The suspension was centrifuged, and
supernatant was immediately neutralized with 1 M Tris base.

Purification of CD25+ and CD25− T cells

Lymph nodes were harvested from NZM2328 mice and enriched for T
cells by negative selection on T cell enrichment columns (R&D Systems).
The cells were incubated with biotinylated T4 Ab to CD25 (BD Pharm-
ingen). After washing, the cells were incubated with streptavidin-PE
(Rockland), followed by anti-PE magnetic beads (Miltenyi Biotec). The
cells were washed and then passed over a magnetic column using the
double-positive selection mode in an autoMACS cell separation system
(Miltenyi Biotec). The unlabeled CD25− cells were collected in the flow-
through fraction. The double-positive selected CD25+ fraction of >90% purity was used for passive transfer experiments as well as in vitro assays.

Transfer of CD25+ T cells into d3tx mice

Seven- to 10-day-old d3tx mice were given a single injection of purified
CD25+ T cells (105 cells/mouse i.p.) Tail blood was collected from the
recipient mice at 13, 15, 17, and 20 wk. The mice were killed at 20 wk, and
kidneys were studied. A cohort of mice was killed at 30 wk and also
studied for organ-specific disease.

Statistical analysis

Methods for statistical analysis include Student’s t test, Mann-Whitney U
test, χ2 analysis, and ANOVA using PRISM version 3.02 (GraphPad).

Results

D3tx accelerated dsDNA autoantibody production and renal
glomerular immune complex deposition in male and female
NZM2328 mice

D3tx NZM2328 male and female mice were studied for serum
dsDNA Ab and serum IgG at 5, 9, 13, and 20 wk. Compared with
sham-tx mice, the dsDNA Ab response of d3tx mice was greatly
accelerated (Fig. 1). A significant increase in total serum IgG was
also seen in d3tx mice (females, p = 0.04; males, p < 0.0001; data
not shown). To control for hypergammaglobulinemia, the data are
expressed as the dsDNA Ab titer per U of serum IgG in Fig. 1.

In addition, there was a dramatic increase in glomerular immune
complex deposition in 20-wk-old d3tx mice, evident as mesangial
IgG (Fig. 2A and B) represented by IgG1, IgG2a, and IgG2b, plus
complement component C3 (data not shown). This represented im-
une complex related to the lupus autoantibody response, because
significant enrichment of dsDNA Ab was found in the renal acid
eluates (Fig. 2C). Thus, the dsDNA Ab response was accelerated

FIGURE 1. Kinetics of anti-dsDNA Ab in serum of d3tx (●) and
sham-tx (○) male (A) and female (B) NZM2328 mice. Data are presented as
dsDNA Ab units per microgram of serum IgG. Each data point repre-
sents the mean ± SEM of 10 mice/group.
in d3tx NZM2328 mice, and this was accompanied by increased accumulation of immune complexes in renal glomeruli.

**D3tx induced proliferative GN in 20-wk-old male NZM2328 mice and greatly accelerated glomerular disease in female NZM2328 mice**

D3tx also induced severe GN in NZM2328 mice. At 20 wk, untreated male and female NZM2328 mice were devoid of renal histopathology. Their kidneys were indistinguishable from those of NZM2328 mice sham-tx (Fig. 3, A and E). In contrast, severe proliferative GN was found in both d3tx male and female NZM2328 mice at 20 wk (Fig. 3, B, C, and F). The glomerular cellularity was diffusely increased, glomeruli were globally enlarged, and the Bowman space became obliterated (Fig. 4, A and B). Numerous cells were present within capillary lumens and inside the mesangial spaces, and this was confirmed by findings on electron microscopy (Fig. 5). In addition, numerous large, electron-dense deposits, typical of immune complexes, were detected mainly in the mesangium. The endothelial cells were swollen, and the epithelial cell foot processes were partially effaced. There was no evidence of glomerular or interstitial fibrosis, and renal tubules were not altered.

The d3tx male and female mice had comparable incidence of proliferative GN (87 vs 90%, respectively) of comparable severity (Fig. 4). However, in 43% of d3tx females, in addition to the proliferative GN described above, there was evidence of chronic GN, including glomerulosclerosis, epithelial cell crescents, interstitial inflammation, and atrophy of renal tubules (Figs. 3D, 4D, and 5C).

Therefore, d3tx greatly accelerated the development of lupus GN in NZM2328 mice. In male mice, the changes included exclusively an early-onset proliferative GN characterized by profound glomerular cell proliferation and accumulation of mesangial immune complexes. In female NZM2328 mice, the d3tx effect represents an accelerated natural disease, with progression of the glomerular disease process as a whole, leading to high prevalence of both proliferative GN and chronic GN.

**FIGURE 2.** Immune complex deposition in kidney of 20-wk-old NZM2328 d3tx male mice (A) detected by immunofluorescence. Quantitative estimation of IgG (B) and anti-dsDNA Ab (C) eluted from kidneys of sham-tx and d3tx male and female mice at 20 wk of age is shown.

**FIGURE 3.** Histopathology of GN in NZM2328 mice. A. Photomicrographs of a sham-tx female kidney showing normal glomeruli (arrows) surrounded by tubules. The inset shows normal glomerulus at higher magnification. B and C, D3tx female at 20 wk with changes of acute proliferative GN with increased size and cellularity of glomerulus, infiltrated with inflammatory cells. The disease progresses to chronic GN (C) showing glomerulosclerosis and severe interstitial inflammatory infiltration. Sham-tx male mice at 20 wk have no pathologic changes in the kidney (E), whereas age-matched d3tx male mice show severe proliferative GN (F). Magnification: A and B, ×100; inset A and C–F, ×200.
D3tx induces or enhances extrarenal autoimmune disease in NZM2328 mice

In addition to GN, d3tx NZM2328 mice developed autoimmune disease that affected other organs (Fig. 6). Prostatitis was detected in 73% of d3tx males. Thyroiditis of greater severity and Ab to thyroid Ags of high frequency were detected in d3tx mice, with greater severity in females (data not shown). Similarly, inflammation of the salivary glands (sialoadenitis) and lacrimal glands (dacryoadenitis), detectable in many sham tx mice, was significantly more frequent and more severe in d3tx mice (Fig. 6). The tissue inflammation, first detected at 20 wk, reached high incidence at 30 wk when disease incidence was recorded (Fig. 7).

CD25⁺ T cells from young adult NZM2328 donors suppressed d3tx-induced autoimmune disease of prostate, thyroid, and lacrimal glands, but not proliferative GN or sialoadenitis

To determine the capacity of CD25⁺ T cells to suppress autoimmune disease in d3tx NZM2328 mice, CD25⁺ T cells were obtained from 6- to 8-wk-old untreated NZM2328 donors that exhibited no detectable renal disease or serum dsDNA Abs. The cells were transferred into 7- to 10-day-old d3tx NZM2328 recipients, and each recipient’s renal pathology was evaluated at 20 wk. A second cohort was killed at 30 wk to study the pathology of organs other than the kidneys.

Transfer of 10⁶ CD25⁺ T cells to d3tx NZM2328 mice completely suppressed prostatitis and significantly reduced the severity of inflammation in thyroid and lacrimal gland (Fig. 7). In contrast, the transfer of CD25⁺ T cells had no observable effect on the proliferative GN at 20 wk (Fig. 8). In both males and females, the extent of proliferative GN in d3tx mice that received CD25⁺ T cells was not reduced compared with that in d3tx mice. The disease was also comparable to that in another group of d3tx mice given purified CD25⁺ T cells (10⁶/mouse) at the same time as CD25⁺ T cells (data not shown). Similar to GN, sialoadenitis in d3tx mice was not affected by CD25⁺ T cell transfer (Fig. 7). Therefore, CD25⁺ T cells from NZM2328 mice, which strongly prevented the development of prostatitis, thyroiditis, and dacryoadenitis in d3tx mice, had little or no influence on the accelerated development of lupus GN and sialoadenitis in NZM2328 mice.

Although CD25⁺ T cells had no effect on proliferative GN development, they abolished the accelerated dsDNA Ab responses in d3tx NZM2328 mice between 13 and 20 wk (Fig. 9, A and C). Suppression was not due to nonspecific suppression of the polyclonal B cell response, because the hypergammaglobulinemia in these animals was not altered by CD25⁺ T cell transfer (Fig. 9, B and D).

Progression from acute to chronic GN in NZM2328 mice depends on gender

To determine the fate of proliferative GN in d3tx mice, we studied a cohort of d3tx mice at 30 wk and compared their data to those obtained at 20 wk. A dramatic difference was seen in the disease progression between male and female mice (Fig. 10A). Many d3tx or sham-tx female NZM2328 mice developed severe chronic GN by 30 wk. In contrast, only rare d3tx NZM2328 males progressed to chronic GN; indeed, most male d3tx mice were completely free of renal histopathology at 30 wk. Interestingly, despite the absence of renal pathology, d3tx male NZM2328 mice continued to have high levels of serum dsDNA Abs (data not shown), and the renal glomeruli continued to harbor large quantities of IgG immune complexes (Fig. 10B) with C3 deposition (data not shown). Thus, the progression of lupus GN evident in d3tx NZM2328 mice was strongly influenced by gender, but this was independent of the dsDNA Ab response.

Discussion

Mice tx between days 3–5 of life develop organ-specific autoimmune disease. The organ susceptibility (stomach, thyroid, ovary, prostate, and lacrimal gland) is mouse strain dependent (BALB/c, C3H, and A/J, respectively) (24). Evidence for the mechanisms of d3tx disease induction suggest three major factors: 1) a differential ontogeny of autoreactive T effectors and inhibitory T regulators (19), 2) depletion of thymus-derived CD25⁺ Treg cells, and 3)
d3tx-induced lymphopenia and the resultant homeostatic proliferation (17). In this study we have developed an experimental model of accelerated lupus GN in lupus-prone NZM2328 mice by d3tx. With this model we have investigated the in vivo function of thymus-derived CD4<sup>+</sup>CD25<sup>+</sup> T cells in the regulation of spontaneous systemic autoimmune disease.

The systemic autoimmunity in NZM2328 mice is not associated with a global deficiency in functional CD25<sup>+</sup> Treg cells. CD25<sup>+</sup> T cells purified from 2- and 5-mo-old NZM2328 females were found to efficiently prevent the proliferation of Con A-stimulated CD25<sup>-</sup> T cells (data not shown). Coculture of purified CD25<sup>+</sup> from 2- and 5-mo-old NZM2328 females with CD25<sup>-</sup> T cells in a 1:1 ratio was able to inhibit proliferation of CD25<sup>-</sup> T cells by 96 and 95%, respectively. Moreover, the CD25<sup>+</sup> Treg cells of NZM2328 mice were able to suppress the development of organ-specific autoimmune disease of the prostate, thyroid, and lacrimal glands, diseases that also developed in d3tx NZM2328 mice, but were unrelated to the lupus manifestation. In contrast, infusion of CD25<sup>+</sup> T cells did not influence the development of lupus GN.

Detection of global Treg abnormality is the focus of most clinical research on Treg cells in human autoimmune diseases. Given the general integrity of Treg in NZM2328 mice, the finding that they failed to suppress the d3tx-accelerated GN is of considerable interest. This result is consistent with the hypothesis that abnormal immunoregulation in lupus-prone NZM2328 mice is due to a selective deficiency of Treg cells that control the development of autoimmune GN and sialoadenitis in these animals. Defective Ag-specific Treg cells may thus contribute to the pathogenesis of lupus GN.

There is clear evidence for Ag-specific regulation of other autoimmune diseases. In d3tx mice and rats, autoimmune prostatitis and autoimmune thyroiditis suppression were more efficient if the CD4<sup>+</sup> T cells were derived from donors that harbored the target Ag (25, 26). Induction of insulin-dependent diabetes mellitus by diabetogenic T cells in lymphopenic hosts could be prevented by cotransfer of large numbers (10–20 million) of Treg cells from naive NOD compared with only 2 million islet Ag-specific Treg cells when cotransferred with diabetogenic T cells (reviewed in Ref. 27). Recent studies show that exposure to ovarian or prostate Ags in vivo resulted in enhanced regulatory capacity of CD25<sup>+</sup> T cells in prevention of oophoritis or prostatitis, respectively (E. S. Samy and K. S. K. Tung, unpublished observations; Y. Y. Seitady, K. Ohno, and K. S. K. Tung, unpublished observations). Thus, efficient regulation of autoimmune disease by CD25<sup>+</sup> regulatory T cells is determined by Ag-specific suppression.

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FIGURE 6. Organ-specific autoimmune disease in d3tx mice. A complete autopsy was performed at the time of death, and organs were studied by histopathology. Photomicrographs are of prostate (A and B), thyroid (C and D), salivary gland (E and F), and lacrimal gland (G and H) from sham-tx mice (A, C, E, and G) with normal glands (g), follicles (f), and ducts (d). The bottom panel (B, D, F, and H) shows lymphocytic infiltration (L) with destruction of normal architecture in d3tx mice. Lung, liver, stomach, small intestine, adrenal gland, ovary, testes, and heart did not show inflammatory changes.

FIGURE 7. CD25<sup>+</sup> Treg cells can block d3tx-induced, organ-specific autoimmune disease studied at 30 wk of age. The severity of inflammation in prostate, thyroid, lacrimal glands, and salivary glands in sham-tx, d3tx, and d3tx mice given Treg cells is shown. Data presented for thyroid, lacrimal, and salivary gland disease are pooled from female and male mice.

FIGURE 8. CD25<sup>+</sup> Treg cells do not prevent accelerated onset of GN in d3tx mice. D3tx male and female NZM2328 were injected with CD25<sup>+</sup> T cells (10<sup>6</sup>/mouse) on days 7–10 and killed at 20 wk of age. The severity of acute proliferative GN compared with sham-tx and d3tx mice is shown.
GN and sialoadenitis may be related to the cell dose used in suppression; namely, if more cells were transferred to the d3tx recipients at different ages, perhaps disease suppression would be detectable. Nevertheless, our finding is consistent with the conclusion that with a single experimental protocol, the CD25+ T cells from lupus-prone mice exhibited a differential threshold of suppression for organ-specific autoimmune diseases vs lupus GN and sialoadenitis.

SLE is a complex systemic autoimmune disease, and the pathogenesis of tissue injury is not completely understood. For the pathogenesis of lupus GN, the emphasis has continued to focus on the role of autoantibodies and the attendant glomerular immune complexes that invariably accompany lupus GN, although recent studies have begun to address T cell-mediated immunity independent of Th cells for autoantibody response (28). In d3tx NZM2328 studies have begun to address T cell-mediated immunity independent of Th cells for autoantibody response (28). In d3tx NZM2328 male mice, accelerated anti-dsDNA Ab and immune complex deposition was not accompanied by accelerated end-stage kidney disease. Secondly, infusion of CD25+ Treg cells in d3tx mice had little or no effect on accelerated lupus GN, but strongly suppressed the early spontaneous dsDNA autoantibody response in NZM2328 mice. These findings point to the dissociation between autoantibody production and target organ injury in NZM2328 mice and raise questions about the pathogenic requirement of dsDNA autoantibody in lupus GN. This is supported by genetic studies in NZM2328 mice (12, 29).

In the present study, additional experimental evidence was obtained that dissociated acute from chronic lupus GN. At 20 wk, d3tx results in an accelerated onset of acute GN in males normally resistant to GN. Significantly, in contrast to d3tx females, this acute GN does not progress to chronic GN. Thus, acute GN represents a checkpoint between inflammation and disease or loss of function that is influenced by gender. The lack of progression to chronic GN in d3tx male NZM2328 mice is similar to the progression from peri-insulitis to insulin and diabetes mellitus in NOD mice (30) and is relevant to the human disease. Indeed, a report by Pollock and Pirani (31) identified a significant number of lupus patients with a histopathological diagnosis of mild glomerulitis and failed to progress over an 8-year follow-up period.

The findings in d3tx NZM2328 mice have raised important questions fundamental to the pathogenetic mechanism of lupus GN and sialoadenitis and suggest a future direction for the investigation of experimental and human lupus.

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**Disclosures**

The authors have no financial conflict of interest.

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