

## RESEARCH ARTICLE

# Double negative T cells, a potential biomarker for systemic lupus erythematosus

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## Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease that is a challenge to diagnose and treat. There is an urgent need for biomarkers to help define organ involvement, and more effective therapies. A unique population of T cells, the CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> (DNeg) cells, is significantly increased in lupus patients. Twenty-seven cases (53%) of pediatric SLE patients had elevated DNeg cells in their peripheral blood, which correlated with kidney function ( $R^2 = 0.54$ ). Significant infiltration of DNeg cells was observed in both adult and pediatric lupus kidneys by immunofluorescence. For the first time, this study provides direct evidence that DNeg cells facilitate kidney injury in preclinical 8-week-old MRL/lpr lupus mice. In lupus mice, the increase in DNeg cells tracked with worsening disease and correlated with kidney function ( $R^2 = 0.85$ ). Our results show that DNeg cells *per se* can cause kidney dysfunction, increase in number with increase in disease pathology, and could serve as a potential biomarker.

**Key words:** CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T cells; glomerulonephritis; inflammation; lupus

## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease<sup>1</sup> with a reported prevalence of 52 cases per 100 000 population in the USA and about 5 million globally. It mainly affects women, men at a ratio of 9:1. In addition, marked differences have been described between pediatric and adult lupus patients. Manifestations such as lupus nephritis, hematological disorders, photosensitivity, butterfly rash, and mucosal ulceration

were more acutely presented in pediatric patients, while neurological symptoms, polyarthritis, anti-SSA, anti-SSB and antiphospholipid antibodies were detected at significantly higher levels in adult-onset patients.<sup>2</sup>

The diverse clinical manifestations, the lack of insight into the underlying mechanisms, and the absence of reliable markers of disease activity make the disease a challenge to diagnose and treat.<sup>3</sup> Identification of specific cells that participate in the disease process, and

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mechanisms that are involved in causing the disease will help assign appropriate therapeutic modalities to this heterogeneous group of patients. A single agent is insufficient to control such a complex disease, and a paradigm that targets different pathways needs to be defined. In addition, biomarkers need to be identified that will define the pattern of organ involvement and whether the patient has responded to treatment.

T cells play a critical role in lupus by infiltrating target tissues, regulating B cell responses, and driving the production of antibodies.<sup>4–6</sup> In lupus, T cell populations are altered, skewed towards the inflammatory Th17 phenotype.<sup>7</sup> The CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> (DNeg) population of T cells (normally <5% of the circulating CD3<sup>+</sup> T cell population) is substantially increased in SLE patients and in murine models of the disease, and is a major producer of IL-17 and INF $\gamma$ .<sup>8,9</sup> More recently, an increase in DNeg cells was observed in pediatric SLE patients.<sup>10</sup> DNeg cells can be composed of diverse subsets such as those with TCR- $\alpha\beta$ , TCR- $\gamma\delta$ .<sup>11</sup> They can be derived from CD4<sup>(+)</sup> and CD8<sup>(+)</sup> T lymphocytes, or from a special cell lineage.<sup>12,13</sup> The DNeg cells display “yin/yang” behavior. Although they may be protective defending the hosts against bacteria,<sup>14,15</sup> they may also contribute to the pathogenesis of different immune-mediated diseases.<sup>16</sup>

T cells include those with suppressor activity such as Tregs that express CD25, the  $\alpha$ -chain of the IL-2 receptor.<sup>17</sup> Transfer of CD4<sup>+</sup>CD25<sup>-</sup> cells to immunodeficient mice can induce disease which is prevented by cotransfer of CD4<sup>+</sup>CD25<sup>+</sup> cells.<sup>18</sup> In addition, Tregs that express the forkhead/winged helix transcription factor, Foxp3, are crucial for preventing autoimmunity and keeping the immune system in homeostatic balance.<sup>19</sup> The adhesion molecule CD44 regulates T cells by enhancing signal transduction via the TCR/CD3 complex.<sup>20</sup> There is a significant increase of CD44 in SLE patients, which correlates with the disease activity.<sup>21–23</sup> CD69, a transiently expressed early activation marker on T cells<sup>24</sup> is increased in NZB/W lupus mice,<sup>25</sup> and functional studies suggest that it alters cytokine generation. As a number of alterations occur under the T cell umbrella, it is necessary to delineate and understand the role of each cell type in the lupus setting. This study attempts to gain a deeper insight into the role of DNeg cells in lupus and associated kidney dysfunction.

In brief, our results show for the first time that: (a) the DNeg T cell population increases with progressing disease in experimental lupus; (b) DNeg cells *per se* can cause disease pathology and play an important role in lupus nephritis; and (c) DNeg cells are present in kidneys from both adult and pediatric patients with lupus.

## Methods

### Mice and treatments

Experiments involving animals were reviewed and approved by the Animal Care and Use Committees from the Universities of Chicago and Buffalo (MED09122Y and

PROTO201800154), and were performed in compliance with current animal use guidelines. Mice used in this study were maintained under pathogen-free conditions with a 12-hour light and dark cycle. Male congenic control MRL<sup>+/+</sup> mice and MRL/lpr lupus mice (6–8 weeks) were purchased from the Jackson Laboratory and maintained in our facility.

### Reagents

C3 and IgG antibodies were obtained from Cappel, MP Biomedicals (Solon, OH, USA). C9 antibody was a kind gift from Dr Scott Barnum (Birmingham, AL, USA). Antibodies used for flow cytometry analysis were purchased from BD-Pharmingen unless indicated otherwise. All reagents, unless stated differently, were from Sigma Chemical Co. (St. Louis, MO, USA).

### Kidney function

Urine and serum were collected and analyzed for mouse urine albumin excretion using an ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) as described previously<sup>26</sup> and blood urea nitrogen (BUN) with a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA, USA), respectively. Urine albumin concentration was normalized to urine creatinine concentrations measured using the creatinine kit Stanbio Creatinine Procedure No. 0400 (Stanbio Laboratory, Boerne, TX, USA). Albumin excretion in normal mice is <0.025 mg/mg creatinine.

### Histology

To evaluate renal pathologic changes, kidneys were harvested from mice at 15 weeks of age or at 5 weeks post transfer, fixed in 4% paraformaldehyde and embedded in paraffin. Four-micrometer sections were stained with periodic acid-Schiff. Slides were scored in a blinded manner by a renal pathologist based on average percentages of high power fields for the extent of glomerulonephritis and interstitial nephritis using scales of 0–4 (in increments of 0.5) as described previously.<sup>27</sup>

### Immunofluorescence microscopy

For immunofluorescence (IF) microscopy, 4- $\mu$ m cryostat sections from mouse kidneys were fixed in 1:1 ether-ethanol and stained with FITC-anti-mouse C3 (Cappel Pharmaceuticals, Aurora, OH, USA) or Alexa 647-anti mouse IgG (Molecular Probes, Eugene, OR, USA). Antibodies were used at a dilution of 1:100.

Paraffin-embedded kidney biopsy sections were obtained from the Department of Pathology, University of Chicago. Sections from three patients were obtained for each condition: Lupus + inflammation, Lupus + no inflammation, and Control + inflammation. Inflammation versus no inflammation was assessed based on Hematoxylin & Eosin Periodic Acid Schiff using light microscopy. Lymphocytes/plasma cells infiltration = inflammation. The lupus nephritis

classification was based on the 2003 ISN/Renal Pathology Society lupus nephritis classification.

Sections were deparaffinized, and stained with anti-CD3, rat monoclonal antibody from ABD Serotec (MCA1477), anti-CD4, rabbit monoclonal from Abcam (ab13616), and anti-CD8, mouse monoclonal from Abcam (ab17147). Sections were visualized using Alexa 488, Alexa 594 and Alexa 647 second antibodies (Molecular Probes, Eugene, OR, USA) at a dilution of 1:250, respectively. Slides were viewed with an Olympus BX-60 IF microscope (Carter Valley, PA, USA). Representative photomicrographs were taken at identical settings with a Hamamatsu EM-CCD camera (Bridgewater, NJ, USA). All assays included negative controls where the primary antibody was omitted.

### Flow cytometry

With the development/identification of better markers and multi-color flow cytometry, it is possible to identify and define different populations of immune cells. Splenic and peripheral leukocytes were analyzed by flow cytometry. Spleen and blood cells were harvested as described previously. Briefly, spleens were forced through a 100  $\mu\text{m}$  cell strainer in 10 ml DMEM. Cells were collected by centrifugation, resuspended in 500  $\mu\text{l}$  ACK lysis solution (0.15 M  $\text{NH}_4\text{Cl}$ , 1 mM  $\text{KHCO}_3$ , and 0.1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.2) to lyse red blood cells, and washed with 9.5 ml FACS buffer (2% FBS, 0.05%  $\text{NaN}_3$ , and 2 mM EDTA in PBS). Cells were collected by centrifugation and resuspended in FACS buffer to a final concentration of  $10^7/\text{ml}$ . Whole blood was collected in EDTA followed by three successive lysis steps as stated above. After final centrifugation cells were resuspended in a volume of  $10^6/\text{ml}$  in FACS buffer. To prevent non-specific binding, 100  $\mu\text{l}$  of the splenocytes or peripheral blood cells were incubated with 1  $\mu\text{g}$  mAb 2.4G2 on ice for 5 minutes. Cells were then simultaneously stained for 1 hour with the following mAbs: Brilliant Violet 421  $\alpha\text{CD3}$  (145C211), APC-Cy7  $\alpha\text{CD19}$  (6D5), Alexafluor 647  $\alpha\text{CD8}$  (KT15), FITC  $\alpha\text{CD4}$  (W3/25), PE-Cy5 CD69, PE-Cy7 CD44, Alexafluor 700 CD25. Samples were washed and resuspended in FACS buffer and run on an LSRII contessa instrument (BD Biosciences). Analysis was performed with FlowJo software (Tree Star, Inc).

Combining the high throughput of flow cytometry with the resolution and informative value of fluorescence microscopy, ImageStream analysis allows visualization of marker expression on the different cells. Using ImageStream X II from Amnis, Millipore blood and splenic cell samples were analyzed to obtain both fluorescent signals similar to a regular FACS machine and fluorescence images. To determine whether these cells had any regulatory properties, we identified the presence of CD25 and FoxP3 by staining with the following labeled antibodies: Brilliant Violet 421  $\alpha\text{CD3}$  (145C211), APC-Cy7  $\alpha\text{CD19}$  (6D5), Alexafluor 647  $\alpha\text{CD8}$  (KT15), FITC  $\alpha\text{CD4}$  (W3/25), V500  $\alpha\text{FoxP3}$ , Alexa Fluor 700  $\alpha\text{CD25}$  cells.

### Double negative T cell adoptive transfer

DNeg cells were isolated from spleens of 19-week-old MRL/lpr mice and transferred to young MRL/lpr mice (8 week,  $n = 6$ ). Briefly, spleens were collected from 19-week-old MRL/lpr mice, minced, and subsequently filtered through a cell strainer (70  $\mu\text{m}$ ). The cell suspension obtained was subjected to red blood cell lysis using ACK lysis buffer (eBioscience, San Diego, CA, USA). Double negative T cells (>95% purity) were separated by cell sorting, using FACStar<sup>®</sup> (Becton Dickinson, Mountain View, CA, USA). Enriched DNeg cells were then washed three times in HBSS to remove serum from the preparation. Approximately  $8 \times 10^6$ – $8.5 \times 10^6$  pooled enriched DNeg cells were injected i.v. via tail vein into each 8-week-old MRL/lpr mouse. Similarly, splenic T cells isolated from 8-week-old MRL/lpr mice were injected into the control 8-week-old MRL/lpr mice ( $n = 6$ ). Tissues were harvested 5 weeks after transfer.

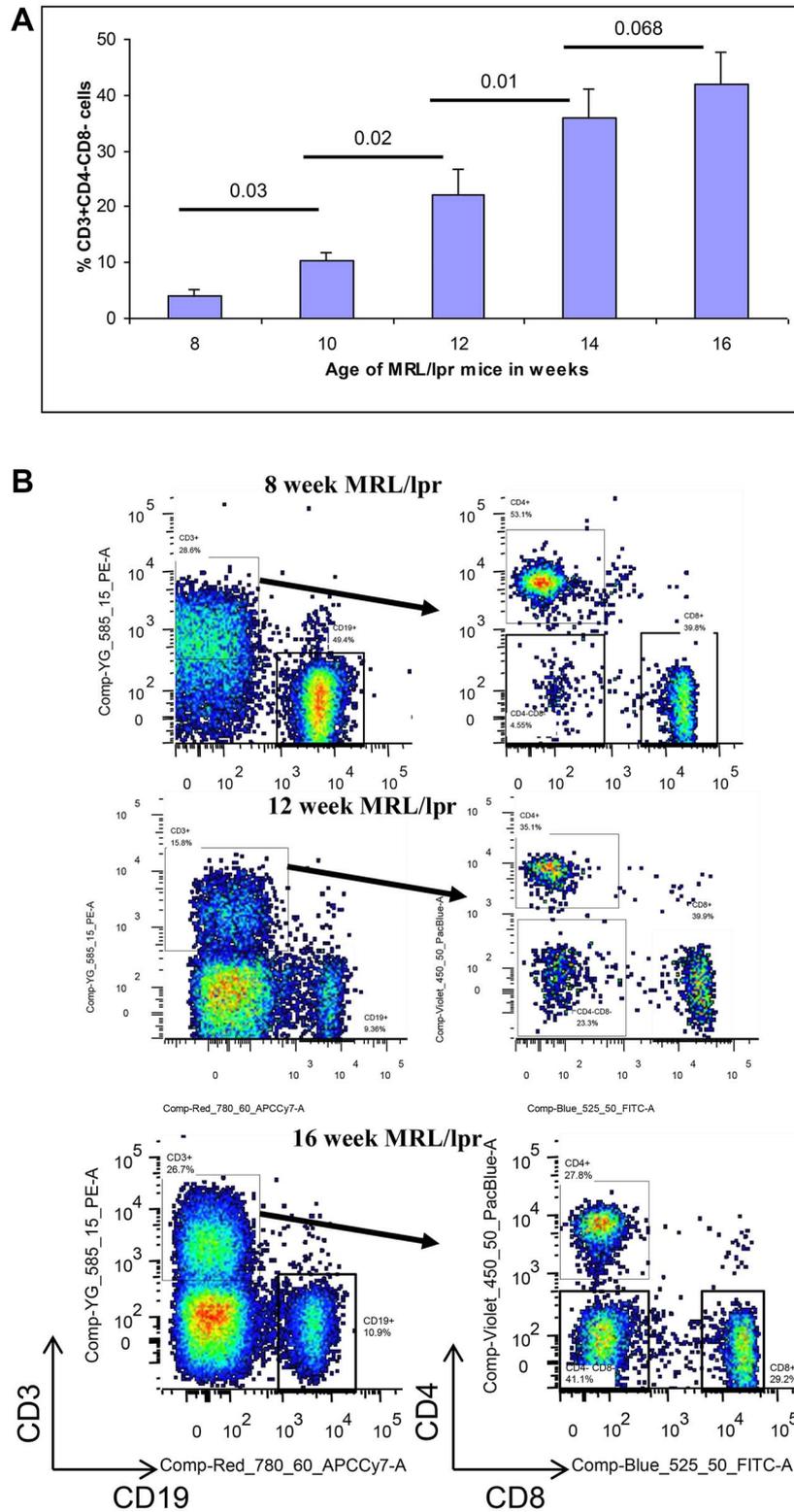
### Pediatric blood samples

Clinical samples ( $n = 50$ ) were obtained from boys and girls, aged 7–15 years, attending the pediatric rheumatology clinics at the Women and Children's Hospital of Buffalo. Samples were processed identically along with those from healthy controls as described above. Whole blood collected in EDTA was subjected to RBC lysis and resuspended in a volume of  $10^6/\text{ml}$  in FACS buffer. To prevent non-specific binding, 100  $\mu\text{l}$  of the splenocytes or PBCs were incubated with 1  $\mu\text{g}$  mAb 2.4G2 on ice for 5 minutes. Cells were then simultaneously stained for 1 hour with the following mAbs: Pacific Blue: anti-humanCD19 and anti-humanCD3 PE-Cy5/CD4 PE/CD8 FITC cocktail (Biolegend). Samples were washed and resuspended in FACS buffer and run on an LSRII contessa instrument (BD Biosciences). Analysis was performed using FlowJo software (Tree Star, Inc).

Approval to acquire and use clinical materials was reviewed and approved by the University of Buffalo Children and Youth Institutional Review Board (IRB). Informed consent was obtained from the parents of the children, and all subjects completed research assent documents. All research was performed in compliance with the IRB-approved protocol. Patients with SLE fulfilling four or more of the revised classification criteria for SLE from the American College of Rheumatology 1997 were included in this study.

### Statistical analysis

Numeric data from all experiments were first analyzed using the "graphical summary" function in Minitab 15 (State College, PA, USA) to determine data normality (Anderson-Darling test) and 95% CI for mean, median, and SD. Parametric and non-parametric data were analyzed by one-way ANOVA and Kruskal-Wallis tests, respectively. Data were expressed as means  $\pm$  SD. For paired data, Student's t-tests were used. Statistical significance was set at  $P < 0.05$ .



**Figure 1.** T cell population in blood from preclinical (8 weeks) to diseased (16 weeks) by FACS analysis. Flow cytometry was performed on peripheral blood mononuclear cells obtained from MRL/lpr mice from preclinical (8 weeks) to diseased (16 weeks) of age. At each time point, blood was obtained from six animals. Cells were stained with the following mAbs: Brilliant Violet 421  $\alpha$ CD3 (145C211), APC-Cy7  $\alpha$ CD19 (6D5), Alexa 647  $\alpha$ CD8 (KT15), FITC  $\alpha$ CD4 (W3/25). They were analyzed for different T cell populations. (A) The T cell population was expressed as a percentage of gated CD3-positive cells. The percentage of CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells increased with increasing age in MRL/lpr mice. (B) Representative data from time points 8, 12, and 16 weeks,  $n = 6$  in each group.



**Figure 2.** ImageStream analysis of CD25 and FoxP3 on T cells from spleen and blood from MRL/lpr mice. Cells from spleen and blood were subjected to analysis by ImageStream 100 instrument (AMNIS Corporation, Seattle, WA). During ImageStream data acquisition, single cells are first separated from debris or multicellular events and composite images of single cell fluorescence intensities are given. Cells were stained with Brilliant Violet 421  $\alpha$ CD3 (145C211), APC-Cy7  $\alpha$ CD19 (6D5), Alexa Fluor 647  $\alpha$ CD8 (KT15), FITC  $\alpha$ CD4 (W3/25),  $\alpha$ CD25, and  $\alpha$ FoxP3. CD25 was localized on the surface of CD4 cells and FoxP3 was cytosolic as expected. However, CD25 and FoxP3 were not expressed by either CD8 or CD4<sup>+</sup>CD8<sup>-</sup> cells.

## Results

### CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> (DNeg) cells increase with progressing disease

The circulating DNeg cell population was assessed in blood at different ages in lupus mice. At 8 weeks, the MRL/lpr mice do not show any clinical symptoms and the DNeg cells constitute only 4% of the CD3<sup>+</sup> population. In line with the progressive nature of the disease in our model, DNeg cells increase progressively with age, reaching >45% by 16 weeks of age (Fig. 1). The increase in DNeg cells was not significant at 16 weeks compared to 14 weeks, indicating that the cells may have reached a plateau and therefore at 16 weeks the experiment was terminated and tissues were harvested.

### Characterization of DNeg cells

In an attempt to further characterize the DNeg cells, they were analyzed by ImageStream analysis. The simultaneous use of mAbs specific for CD19, CD4, CD8, CD25, and FoxP3 allowed us to detect the different populations of T cells. The DNeg cells had CD3 on their surface, but not CD4 or CD8. In addition, these cells had no CD25 on their surface or FoxP3 internally. Based on the absence of these markers, we conclude that they are neither activated nor regulatory and have no T<sub>reg</sub> cell function. CD25 and FoxP3 were present on CD4<sup>+</sup> cells, but not on CD8<sup>+</sup> cells (Fig. 2).

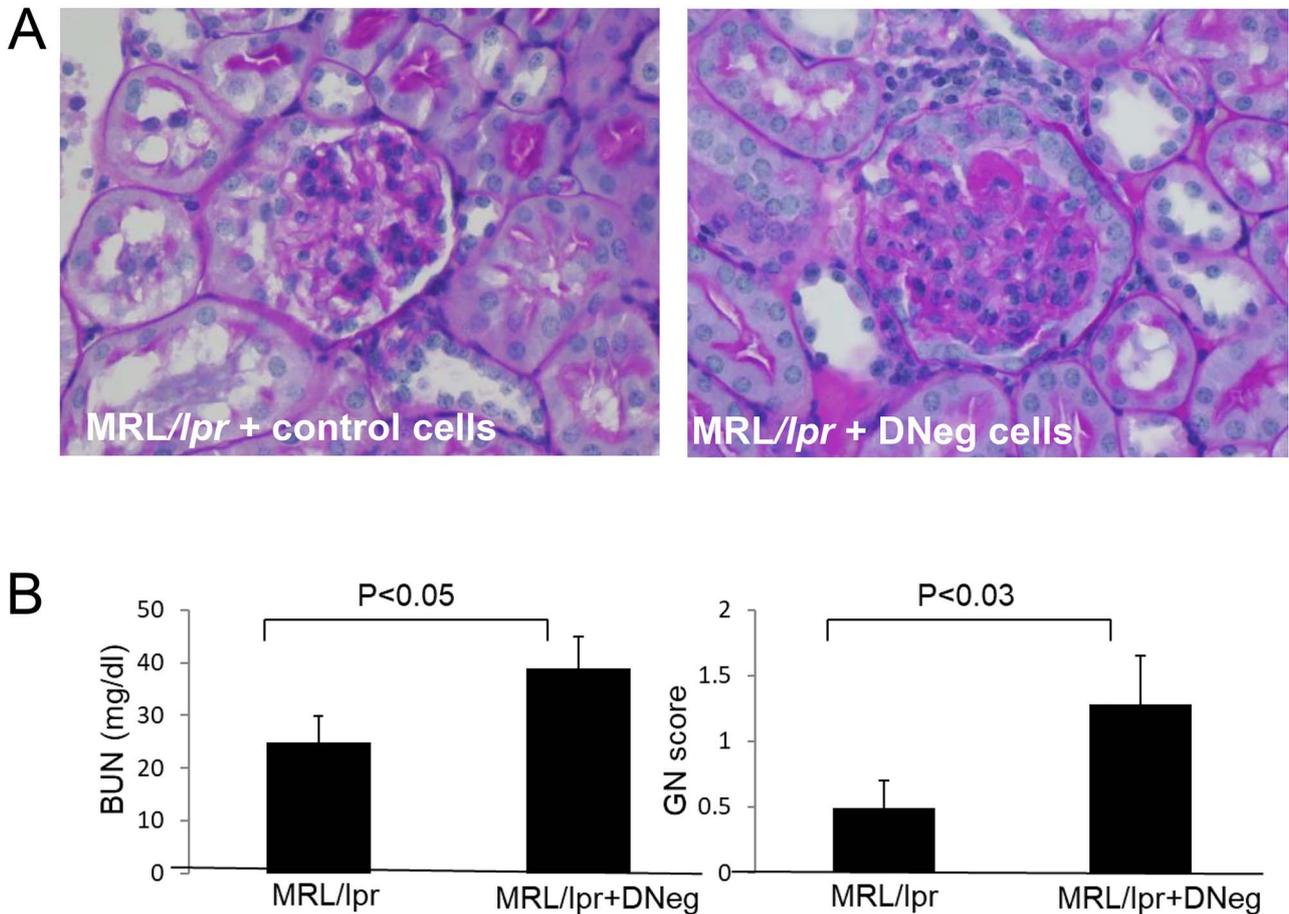
### DNeg cells negatively correlate with the CD19 population and with kidney function

The increase in the DNeg population was inversely proportional to the decrease in the CD19 cell population ( $R^2 = 89.7$ ). Surprisingly, there was a significant correlation between the change in DNeg cell population and kidney function assessed by BUN ( $R^2 = 0.852$ ) (Fig. 3).

### Adoptive transfer aggravates pathology in young MRL/lpr mice

Young, preclinical MRL/lpr mice ( $n = 16$ , 8 weeks of age) were separated into two groups. Group 1 was given DNeg cells isolated from spleens of MRL/lpr mice (19 weeks of age) by tail vein injections. They were compared to Group 2 mice that were injected with splenic cells from young mice of the same age. For each mouse,  $8 \times 10^6$ – $8.5 \times 10^6$  DNeg cells/mouse were adoptively transferred, and the mice were sacrificed after 2 weeks and 5 weeks. The mice that received DNeg cells from 19-week-old MRL/lpr mice (experimental) developed splenomegaly ( $0.23 \pm 0.06$ ,  $P < 0.05$ ) compared to MRL/lpr mice that received lymphocytes from 8-week-old mice (controls) ( $0.12 \pm 0.03$ ).

**Kidney histology.** To assess for evidence of pathology in recipient mice after receiving adoptive transfer of DNeg lymphocytes, kidneys were examined at 2 and 5 weeks after transfer. At 2 weeks, based on BUN and GN



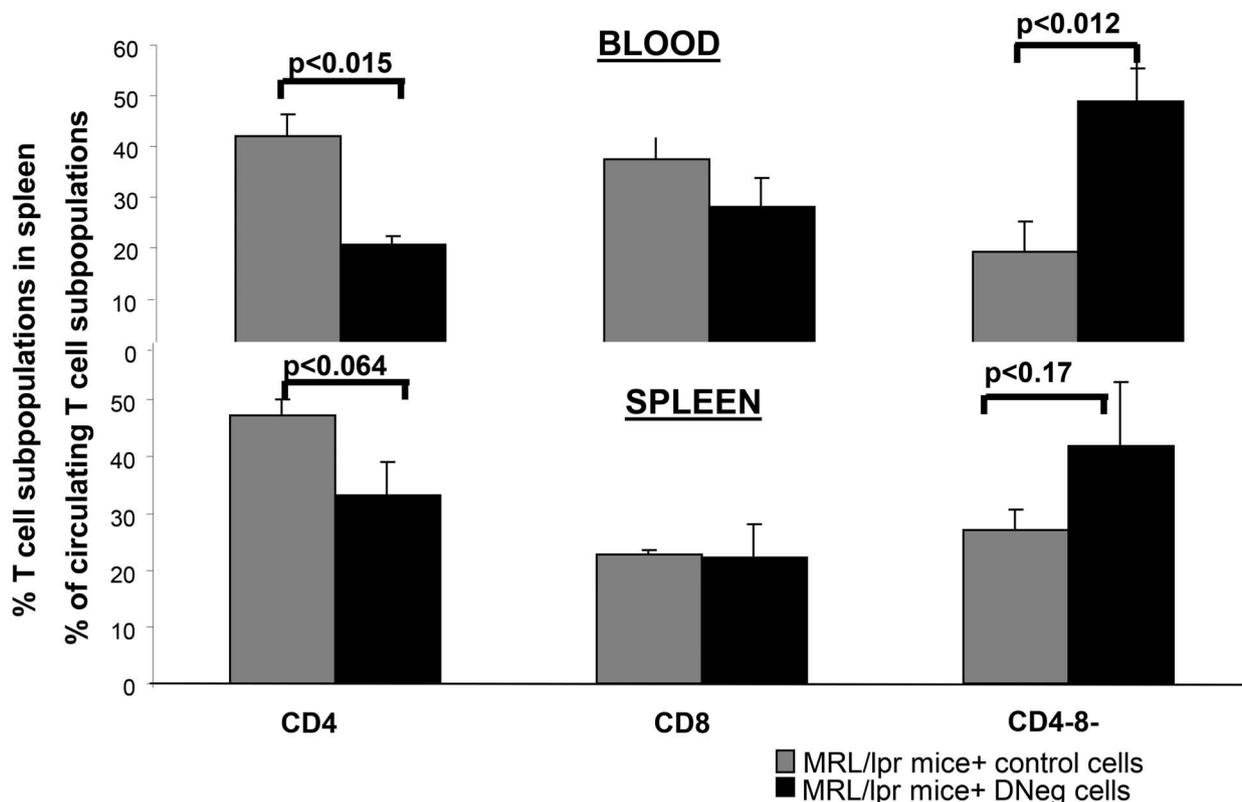
**Figure 3.** Effect of adoptive transfer of DNeg cells on kidneys in young MRL/lpr mice. Representative periodic acid-Schiff–stained kidneys from 8-week-old MRL-lpr mice that received DNeg cells from 20-week MRL/lpr mice (experimental) or splenic cells from 8-week MRL/lpr mice (control) (A) and tissues harvested 5 weeks later. Increased pathology GN scores (B) with increased infiltration of inflammatory cells is seen in the glomeruli of the experimental mice compared to controls. Original magnifications,  $\times 600$ . In line with the histology, kidney function assessed by BUN (B) was worsened in the experimental mice,  $P < 0.05$ .

assessments, the mice did not have significant renal pathologic changes (results not shown). However, at 5 weeks, the MRL/lpr mice that received DNeg cells developed diffuse lupus nephritis characterized by mesangial hypercellularity and increased infiltrating cells (Fig. 4) compared to the mice that received normal lymphocytes. With the use of semiquantitative evaluation, the scoring of MRL/lpr mice that received DNeg cells had significantly higher pathology than those that received control lymphocytes.

**Renal function.** To assess renal functional changes in the recipient mice 5 weeks after receiving DNeg cells or control lymphocytes, BUN and albumin were measured in the recipient mice. A significant increase in serum BUN was detected in the experimental group compared to the control mice at 5 weeks post transfer ( $63 \pm 7.8$  mg/dl in experimental mice vs.  $25.6 \pm 2.1$  mg/dl in control mice). At baseline (8 weeks of age) all mice had normal albumin levels ( $0.5 \pm 0.1$  mg/mg creatinine). At 13 weeks of age (5 weeks post transfer), the MRL/lpr

mice that received the DNeg cells developed significantly increased albuminuria ( $105 \pm 24.5$  mg/mg creatinine) compared with  $1.2 \pm 0.2$  in the control MRL/lpr mice ( $P = 0.032$ ).

**T cell profile.** To determine the changes that occur in the different populations, the recipient mice receiving splenocytes from 19 week or 8 week (control) MRL/lpr mice were euthanized, and their circulating mononuclear cells and splenocytes were isolated and analyzed by flow cytometry. The staining and gating approaches allowed us to detect different subsets of cells. The total CD3<sup>+</sup> cells remained the same in all the mice. The circulating CD3<sup>+</sup>CD4<sup>+</sup> cells ( $42 \pm 4$  vs.  $21 \pm 2$ ,  $P < 0.015$ ) were significantly reduced in the DNeg recipient mice, the CD3<sup>+</sup>CD8<sup>+</sup> population remained unchanged while the CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells ( $19 \pm 6$  vs.  $49 \pm 7$ ,  $P < 0.012$ ) were significantly increased. The splenic T cells also showed similar changes, although the changes did not reach statistical significance because of variability. In addition, most of the CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> were CD69<sup>-</sup>CD44hi (% in



**Figure 4.** Adoptive transfer of DNeg cells on CD4 and CD8 T cell populations in MRL/lpr mice. Single cell suspensions of spleen and blood depleted of RBCs were simultaneously stained for 1 hour with the following mAbs: Brilliant Violet 421  $\alpha$ CD3 (145C211), APC-Cy7  $\alpha$ CD19 (6D5), Alexa Fluor 647  $\alpha$ CD8 (KT15), FITC  $\alpha$ CD4 (W3/25) and analyzed by flow cytometry. The T cell population was expressed as a percentage of gated CD3-positive cells of total splenocytes. Mice that received DNeg cells from aged MRL/lpr mice showed significant decrease in CD4<sup>+</sup> and increase in DNeg cells, while CD8<sup>+</sup> cells remained the same both in circulation and spleen compared to control mice ( $n = 3$  in each group).

circulation— $81.8 \pm 10.4$  vs.  $92.1 \pm 6.6$ ; spleen— $31.6 \pm 2$  vs.  $44.1 \pm 5.8$ ).

### DNeg cells infiltrate kidneys of lupus patients

DNeg cells were increased in kidneys of patients with lupus by triple immunofluorescence staining. Representative kidney sections are given (Fig. 5A). A significantly higher number of DNeg cells (green) was observed in the sections taken from lupus patients with inflammation, whereas sections from lupus patients with no inflammation and non-lupus patients with inflammation had higher numbers of CD4 cells (yellow) and minimal DNeg cells.

### DNeg cells increased in pediatric patients with lupus and correlate with kidney disease

Blood was obtained from pediatric patients with lupus in Buffalo. Twenty-seven cases (53%) had elevated DNeg cells ( $>8\%$  of parent population) (Fig. 5A). The DNeg cell population correlated with kidney function (BUN,  $R^2 = 0.54$ ) (Fig. 5A).

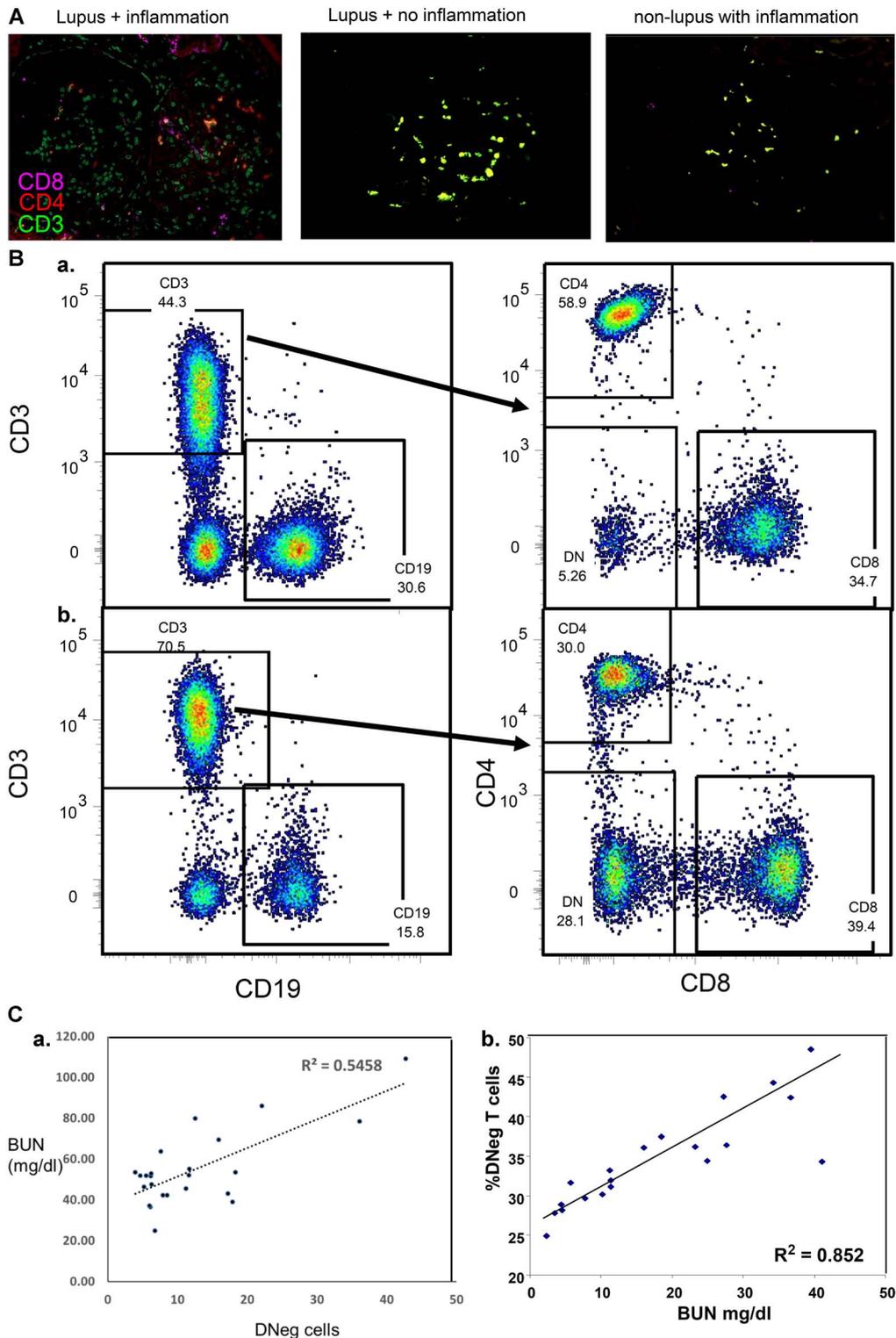
Mice allow assessments in controlled settings and allow us to assess disease progression in settings not

compounded by the use of medication, and variable environments. However, our studies show that the results obtained in mice are clinically relevant because these are in line with those observed in patient kidneys and in circulation.

## Discussion

For the first time, our studies demonstrate that the number of DNeg cells increase with worsening disease in experimental lupus and cause or exacerbate kidney disease in the same setting. In addition, our observation that DNeg cells from pediatric patients with SLE significantly correlate with kidney function supports the relevance of these studies. These results suggest that DNeg cells play an important role in lupus nephritis and suggest that they could potentially serve as a marker for lupus.

DNeg T cells are found in increased numbers in tissues of many chronic and progressive diseases,<sup>28</sup> and there is increasing awareness of the important role they play in lupus. T cells are not a uniform effector population, but exist in distinct subsets with differential susceptibility to the altered environment. Our results, along with several others studies<sup>29,30</sup> demonstrate that DNeg T cells, which constitute less than 5% of T cells in a normal



**Figure 5.** (A) DNeg cells are increased in kidneys from lupus patients. Representative confocal images of kidney biopsy specimens obtained from both adult and pediatric lupus were deparaffinized and stained with antibodies (CD3, green; CD4, red; and CD8, magenta) are shown. Scale bars: 50  $\mu$ m. Original magnification:  $\times 400$ . Images are representative of three biological replicates. (B) Circulating DNeg cells in pediatric lupus patients are increased compared to controls. Single cell suspensions from RBC depleted blood of pediatric patients were simultaneously stained for 1 hour with mAbs for CD3, CD19, CD8, CD4 and analyzed by flow cytometry. The T cell population is expressed as a percentage of gated CD3-positive cells. Normal subjects vs. lupus subjects =  $6.5 \pm 0.99$  vs.  $10 \pm 6.15$ . 53% of lupus subjects had increased (0.8% of parent T cell population). FACS images of (a) control and (b) lupus subjects (B). (C) Circulating DNeg cells correlate with kidney function. BUN and DNeg cells were assessed in the same cases. Correlation between DNeg cells and BUN was significant in pediatric lupus patients ( $R^2 = 0.5$ ) and in lupus mice ( $R^2 = 0.852$ ).

setting, are increased (50%–60%) in the lupus setting. DNeg T cells (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>) play a role in limiting chronic immune activation. Furthermore, the percentage of DNeg T cells increases (as a proportion of circulating T cells) as disease progresses. Our future studies will attempt to identify the identity of the cells from which DNeg cells originate in this setting. Furthermore, the expansion of the DNeg population correlated with rising BUN and albumin, supporting the idea that these cells play a direct role in the renal disease. Our results indicate that the DNeg cells in MRL/lpr mice had neither CD25 nor FoxP3, suggesting that they were not regulatory in function. It is well recognized that there is a persistence of lineage plasticity during B cell development and the potential to form either B or T cells is not lost until the CD19 proB stage.<sup>31</sup> An interesting observation in our study is the significant correlation between the DNeg cells and CD19 + ve cells. Further studies are needed to determine whether the DNeg cells have the same precursor/lineage as the CD19 + ve cells or whether the changes in the number of cells occurred through different mechanisms.

An elegant study demonstrated that recipient rat that received splenocytes 6 weeks after myocardial infarction developed myocardial inflammation and patchy fibrosis, but no functional changes as measured by echocardiography. We followed a similar experimental approach, and found that transfer of DNeg cells to young, preclinical 8-week-old MRL/lpr mice aggravated the functional impact of kidney, indicated by increased BUN, albumin and histological changes, suggesting that these cells directly participate in the development of progressive kidney injury.

In an autoimmune setting, including lupus, adherence and migration of T cells to the target organs is an important aspect of the pathobiology that sustains the inflammatory response, resulting in organ damage. Several studies have shown that CD44 can promote the recruitment of inflammatory cells to sites of chronic inflammation, a finding that is relevant to the current study, as infiltrating kidney T cells in SLE patients had increased expression of CD44 and correlated significantly with the disease. In line with these studies, CD44 expression was increased on T cells in circulation but not in spleen of MRL/lpr mice in our study (results not shown). CD44 can be upregulated by a variety of cytokines IL-15 and IFN- $\gamma$ , both of which are elevated in lupus. CD44 binds to its ligand and regulates a number of immunological processes, such as lymphocyte migration, extravasation, activation and cytolytic activity.

The increase in DNeg cells positively correlated with increasing BUN, indicating that they may have a direct role in the kidney disease in MRL/lpr mice. The aggravation of disease caused by adoptive transfer of DNeg cells from 20 week animals with lupus compared with control animals that received equal numbers of splenic cells from 8-week-old counterparts once again gives credence

to the possibility that the DNeg cells may be directly involved in kidney disease. To gain insight into the clinical relevance of our findings, we assessed the circulating DNeg cell population in pediatric patients. DNeg cells increased in 50% of the patients. An exciting finding was that the DNeg cells significantly correlated with BUN levels in these patients.

In summary, our results for the first time provide evidence that in lupus DNeg cells increased in parallel with worsening disease. The expanded population of DNeg T cells correlated with decrease in CD19 cells and kidney function. Transfer of DNeg cells from aged MRL/lpr mice to a young preclinical MRL/lpr mouse hastened kidney disease. Therefore, the increase in DNeg cells could serve as a marker for kidney disease in lupus. Further investigation in lupus patients is required to determine the correlation between these cells and stage of disease or organ affected and organ response to therapy.

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## Conflict of interest statement

None declared.

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