Methods

Flow cytometry. Single cell suspensions were obtained from spleens as described previously[1] . One million cells were incubated with the following anti-mouse antibodies for 30 minutes, then fixed in 2% paraformaldehyde in PBS: CD4, CD44, CD62L, CD69, and CD25 (BD Biosciences). For intracellular cytokine staining, cells were plated in 96 well round bottom plates at 2 x 10^5 cells per well (triplicate) in complete RPMI (cRPMI) containing 10% FBS, penicillin-streptomycin, l-glutamine and 2 mM β-mercaptoethanol. To stimulate cytokine production, PMA (10 ng/ml), ionomycin (1 μg/ml) and GolgiStop (BD Biosciences) were added and the cells cultured for 5 hours. Control wells received media with GolgiStop only. In the case of overnight stimulation with PMA and ionomycin or 5 hr stimulation for IL-17 staining, the GolgiStop was added in the last 3 hours of culture. After incubation, surface staining was performed as described above. Cells were then permeabilized in 1x permeabilization buffer (BD Biosciences) and incubated with anti-mouse IFNγ, IL-10 (BD Biosciences) or IL-17 (eBioscience) for 30 minutes.

T_{reg} staining was performed using the mouse regulatory T cell staining kit from eBioscience according to the manufacturer's instructions. Additional markers for T_{reg} function included: GARP (eBioscience), GITR, IL-10R (CD210) and ICOS (CD278) (BD Biosciences). All samples were acquired on a MACSQuant flow cytometer (Miltenyi Biotec) and data analyzed using FlowJo analysis software (Treestar).

$CD4^+$ T cell purification. $CD4^+$ cells were isolated from spleens of 6 month old B6 and B6.SLE mice using CD4 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. Briefly, cells were incubated with anti-CD4 MicroBeads for 15 minutes. Non-attached beads were washed off and the cell suspension was filtered through a column placed in a Miltenyi QuadroMACS separator. Cells with magnetic beads attached ($CD4^+$) remained in the column, while $CD4^-$ cells were collected as flow through. The column was removed from the separator and $CD4^+$ cells were cleared from the column using a plunger. Cell purity of >90% was confirmed by flow cytometry (data not shown).

Cytokine ELISA. Purified $CD4^+$ T cells (2 x 10^5 cells/well) were cultured in cRPMI with CD3 (1 μg/ml) and CD28 (1 μg/ml) stimulation. After 72 hours, supernatants were collected and assessed for IL-17 and IL-10 using ELISA kits from eBioscience and BD Biosciences, respectively. ELISAs were performed according to manufacturer's instructions.

Atherosclerosis studies. Six to eight week old female $Rag^{-/-}$, $LDLr^{-/-}$ mice received 7 x 10^6 purified $CD4^+$ cells from 6 month old B6 or B6.SLE mice via retro-orbital injection. Two weeks after transfer, mice were placed on a Western diet (21% fat, 0.15% cholesterol). After 10 weeks on diet, mice were sacrificed and aortic root lesion area

was evaluated as described previously[1]. At sacrifice, mice were examined for signs of graft versus host disease, a potential concern when receiving T cells from an inflammatory milieu. Mice displayed no signs of abdominal pain, diarrhea, intestinal inflammation or skin rash.

Isolation of $CD4^+FoxP3^-$ and $CD4^+FoxP3^+$ T cells for transfer and atherosclerosis study. $CD4^+$ T cells were purified from 6 month old $B6.FoxP3^{GFP}$ and $B6.SLE.FoxP3^{GFP}$ mice using magnetic beads as described above. Cells were then stained with anti-CD4 and were sorted using a BD FACS Aria (Vanderbilt Flow Cytometry Core Facility) into two populations: $CD4^+GFP^+$ ($CD4^+FoxP3^+$ T_{reg}) and $CD4^+GFP^-$ ($CD4^+FoxP3^-$ T_{eff}). Upon analysis, purity of these cell populations was better than 99%.

Six to eight week old female $Rag^{-/-}$, $LDLr^{-/-}$ mice received a total of 7 x 10^6 cells via retro-orbital injection. Control mice received B6 T_{eff} and B6 T_{reg} (B6 TE:B6 TR) in a 1 to 8.5 ratio. The experimental group received B6.SLE T_{eff} and B6 T_{reg} (B6.SLE TE: B6 TR) in the same ratio. The study design followed that of the above mentioned atherosclerosis experiments: two weeks after transfer, mice were placed on a Western diet (21% fat, 0.15% cholesterol). After 10 weeks on diet, mice were sacrificed and aortic root lesion area was evaluated as described previously[1].

Serum dsDNA ELISA. Prior to T cell transfer, presence of dsDNA antibody titers in B6.SLE donors was confirmed by ELISA. Serum was collected from donor B6 and B6.SLE mice by retro-orbital bleeding. dsDNA ELISA was performed as described previously[1,2].

Serum cholesterol and triglyceride analysis. Prior to sacrifice, $LDLr^{-/-}$,$Rag^{-/-}$ mice receiving either B6 or B6.SLE $CD4^+$ T cells were fasted for 4 hours and serum collected via retro-orbital bleeding. Serum cholesterol and triglycerides were measured by colorimetric assay.

Measurement of urine protein grade. Urine was collected from mice at the time of sacrifice and protein level was measured using Multistix 10 SG reagent strips (Siemens). The protein level was determined based on a color chart provided by the manufacturer. Trace or negative results were reported as 0, while + indicates 30-99 mg/dl, ++indicates 100-299 mg/dl and +++ indicates 300-1999 mg/dl.

Immunohistochemistry. Aortic root sections were subjected to immunofluorescent staining with anti-CD4 as described previously [1]**.** Nuclei were stained with DAPI. Based on DAPI staining, the total number of cells in the atherosclerotic lesion was determined. $CD4^+$ cells were then counted and the data expressed as %$CD4^+$ of total cells.

Regulatory T cell isolation and in vitro functional assays. $CD4^+CD25^+$ regulatory T cells (T_{reg}) were isolated from spleens of 6 month old female B6 and B6.SLE mice using

the $CD4^+CD25^+$ Regulatory T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. Briefly, non-$CD4^+$ cells were depleted from the sample, followed by incubation with anti-CD25 MicroBeads. Cells were then divided into two populations: $CD4^+CD25^-$ (effector T cells or T_{eff}) and $CD4^+CD25^+$ T_{reg}. T_{eff} were then labeled with CFSE using the CFSE cell proliferation kit (Invitrogen), according to the manufacturer's protocol.

T_{reg} and $CFSE^+$ T_{eff} were resuspended in cRPMI and plated in 96 well round bottom plates at ratios of T_{reg}:T_{eff} ranging from 1:2 to 1:16. The number of T_{eff} remained constant at 1 x 10^5 cells per well, while the number of T_{reg} changed according to the ratio. Cells were then stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml). Cells were incubated for 72 hours and then harvested for flow cytometry. Surface staining with anti-CD4 was performed as described above. Samples were acquired on a MACSQuant Flow Cytometer and data analyzed using Flow Jo software. Proliferation in T_{reg}:T_{eff} wells was compared to that in T_{eff} only wells to determine the percent inhibition of proliferation at each ratio. The percent inhibition was calculated as follows: **[(prolif. at ratio/prolif. T_{eff} only)*100].**

Results

Increased $CD4^+$ T cell inflammation in B6.SLE mice. To confirm activation of $CD4^+$ T cells in 6 month old B6.SLE female donor mice, we first measured expression of the activation markers CD44, CD62L and CD69 on the surface of $CD4^+$ T cells. As expected, $CD4^+$ T cells from B6.SLE mice exhibited increased expression of CD44 and CD69 and lower expression of CD62L (Supplemental Figure IIA) compared to controls. Furthermore, the proportion of $CD4^+$ cells which were $CD44^{hi}CD62L^{lo}$ effector/memory T cells was increased nearly 3-fold in B6.SLE mice, while the proportion of naïve $CD44^{lo}CD62L^{hi}$ T cells was reduced by ~9-fold compared to B6 (Supplemental Figures IIB and C). Increased production of IFNγ, IL-17 and IL-10 by B6.SLE CD4+ T cells was also confirmed (Supplemental Figure IID-F).

To measure T cell cytokine production, purified $CD4^+$ T cells were stimulated overnight with PMA and ionomycin. While there was a slight increase in the proportion of B6.SLE $CD4^+$ T cells producing IFNγ without stimulation (Supplemental Figure IID, top panel), the percentage of these cells secreting IFNγ was increased 2-fold upon stimulation compared to B6 (Supplemental Figure IID, bottom panel). To measure IL-17 and IL-10 production, purified $CD4^+$ T cells were cultured for 72 hours in the presence or absence of anti-CD3/CD28. While $CD4^+$ T cells from both mouse strains made negligible amounts of IL-17 in the absence of stimulation, B6.SLE $CD4^+$ T cell secretion of IL-17 was approximately 5-fold higher upon stimulation than that of B6 $CD4^+$ T cells (Supplemental Figure IIE). IL-10, typically thought of as an anti-inflammatory cytokine, has been shown to be increased in SLE, and levels of IL-10 correlate with disease

severity[3]. Perhaps not surprisingly, B6.SLE $CD4^+$ T cells produced 4.5-fold more IL-10 upon stimulation compared with their B6 counterparts (Supplemental Figure IIF). Collectively, these data indicate that, as in human SLE, B6.SLE $CD4^+$ T cells are hyperactivated and exhibit a heightened production of the cytokines IFNγ, IL-17 and IL-10.

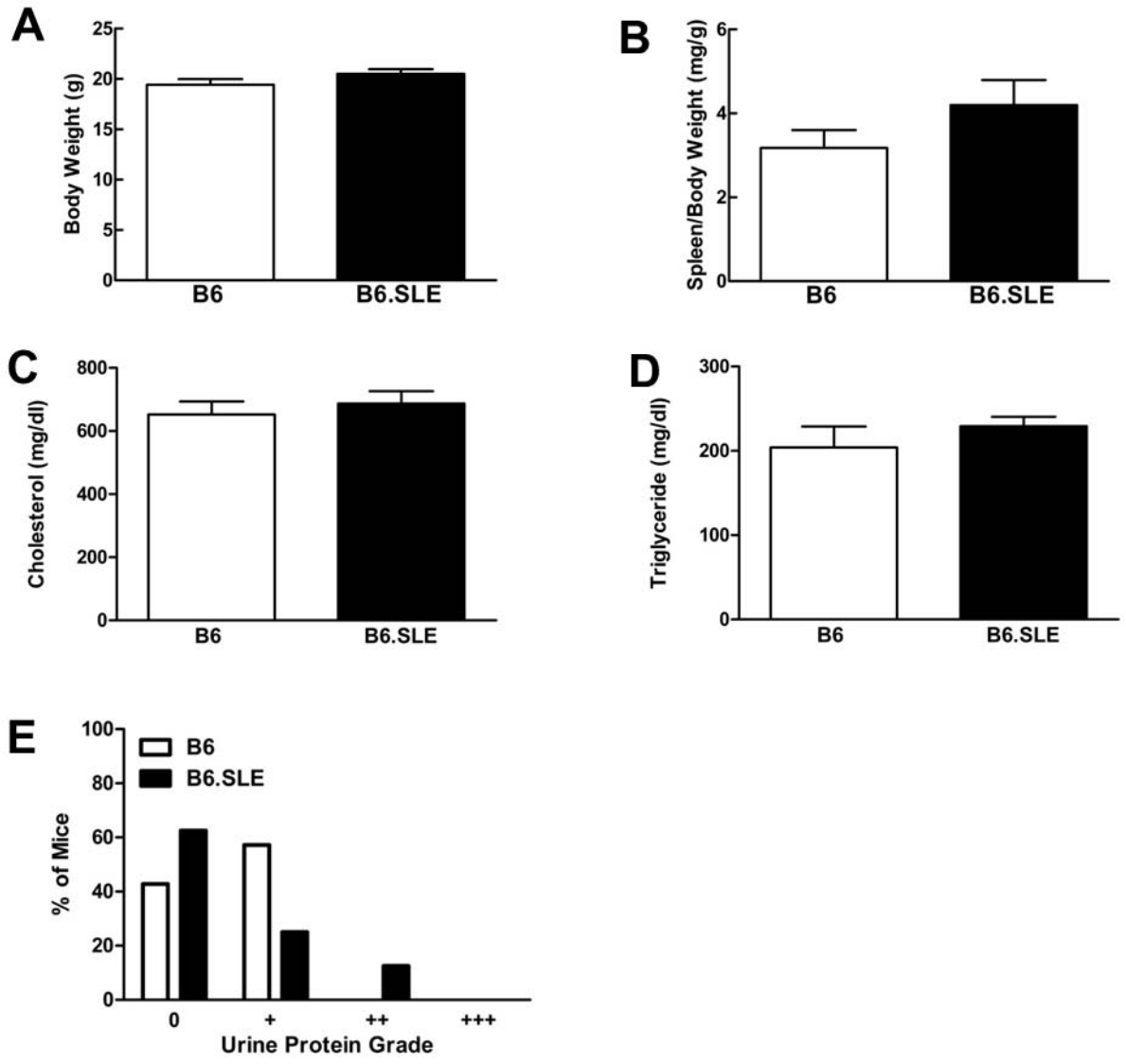
1. Stanic AK, Stein CM, Morgan AC, Fazio S, Linton MF, Wakeland EK, Olsen NJ, Major AS. Immune dysregulation accelerates atherosclerosis and modulates plaque composition in systemic lupus erythematosus. *PNAS*. 2006;103(18):7018–23.

2. Morel L, Croker BP, Blenman KR, Mohan C, Huang G, Gilkeson G, Wakeland EK. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. *PNAS*. 2000;97(12):6670–5.

3. Park YB, Lee SK, Kim DS, Lee J, Lee CH, Song CH. Elevated interleukin-10 levels correlated with disease activity in systemic lupus erythematosus. *Clin. Exp. Rheum*. 16(3):283–8.

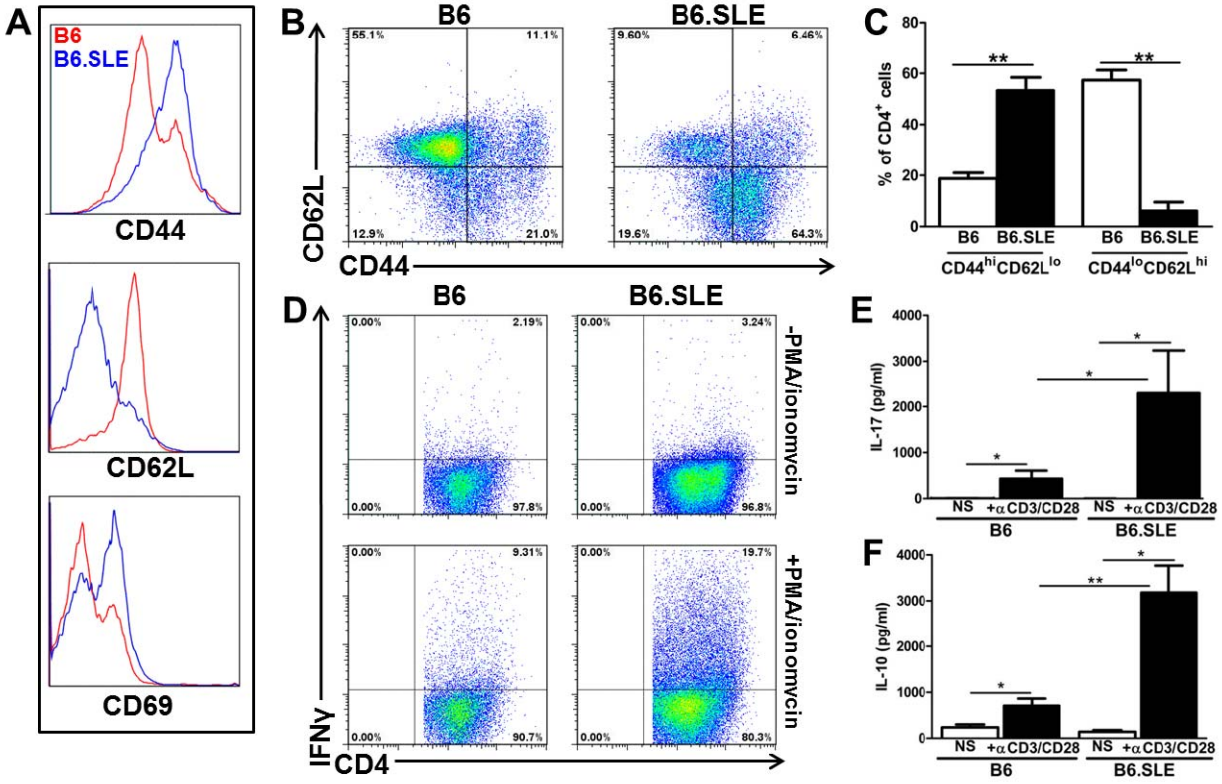
Supplemental Figures

Supplemental Figure I: Body and spleen weights and cholesterol and triglyceride data from CD4^{+} T cell transfer study. Recipient mice were sacrificed 10 weeks after starting western diet, as shown in Figure 1A. Data were obtained from recipient mice at the time of sacrifice. **A.** Body weights. **B.** Spleen to body weight ratio. **C and D.** Mice were fasted for 4 hours before collecting blood via retroorbital bleeding. **C.** total serum cholesterol. **D.** total serum triglycerides. **E.** Urine protein grade at sacrifice. Data represent a minimum of 6 mice per group.



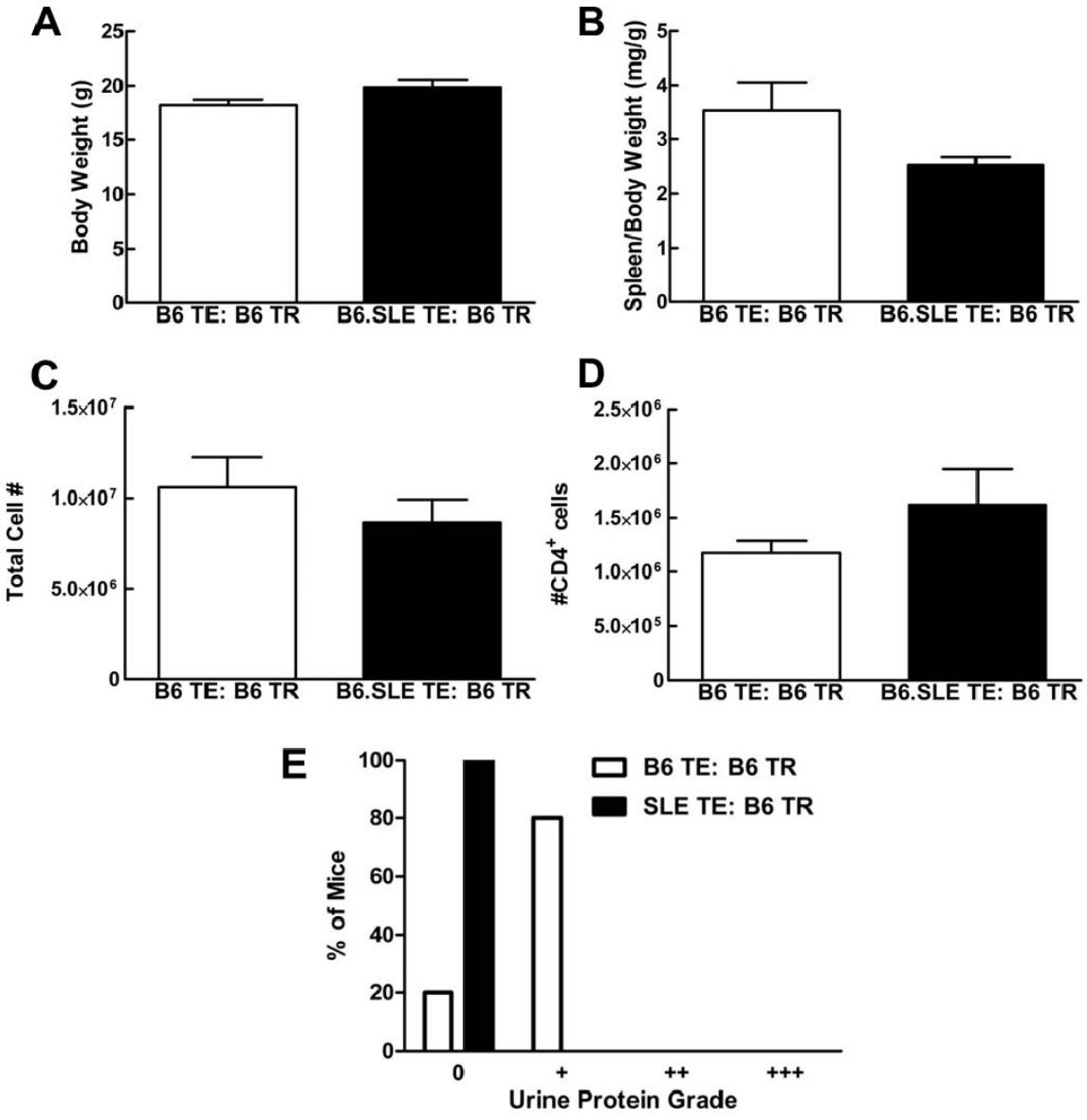
Supplemental Figure I.

Supplemental Figure II: Increased inflammatory marker expression and cytokine production by B6.SLE CD4$^+$ T cells. A. Histograms showing expression of CD44, CD62L and CD69 on CD4$^+$ cells in B6 (red) and B6.SLE (blue) mice (whole splenocytes, gated on CD4$^+$ cells). **B.** Dot plots showing CD62L vs. CD44 expression (gated on CD4$^+$ cells), and quantification, **C**. **D.** Representative dot plots showing intracellular staining for IFNγ in purified CD4$^+$ T cells from B6 and B6.SLE mice, with and without PMA/ionomycin stimulation. **E-F.** Purified CD4$^+$ T cells were stimulated with anti-CD3/CD28 for 72hrs. Supernatants were collected and ELISAs were performed to measure IL-17 (**E**) and IL-10 (**F**). Each panel is representative of n=6-8 mice per group. * and ** indicate significance at p<0.05 and 0.01, respectively.



Supplemental Figure II.

Supplemental Figure III. : Body and spleen weights and cell data from B6 TE:B6TR and B6.SLE TE:B6 TR transfer. Recipient mice were sacrificed 10 weeks after starting western diet, as in Figure 1A. Data were obtained from recipient mice at the time of sacrifice. **A.** Body weights. **B.** Spleen to body weight ratio. **C.** Total spleen cell number at sacrifice. **D.** Total number of $CD4^+$ cells recovered from the spleens of recipients. **E.** Urine protein grade at sacrifice. Data are representative of n=6 mice per group.



Supplemental Figure III