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EXTENDED REPORT

Dysregulated CD4$^+$ T cells from SLE-susceptible mice are sufficient to accelerate atherosclerosis in LDLr$^{-/-}$ mice

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ABSTRACT

Objective Accelerated atherosclerosis is a major source of morbidity in systemic lupus erythematosus (SLE). However, the cause of SLE-accelerated atherosclerosis remains unclear.

Methods CD4$^+$ T cells from C57/Bl/6 (B6) or SLE-susceptible B6.Sle1.2.3 (B6.SLE) mice were transferred into LDLr$^{-/-}$, Rag$^{-/-}$ mice. T cells were examined for cytokine production and expression of interleukin-10 receptor (IL-10R) and functional markers. T cells were isolated based on FoxP3^{GFP} expression and transferred to LDLr$^{-/-}$, Rag$^{-/-}$ mice to establish a role for B6.SLE effector T cells (T_{eff}) in atherosclerosis.

Results Mice receiving whole B6.SLE CD4$^+$ T cells displayed no other SLE phenotype; however, atherosclerosis was increased nearly 40%. We noted dysregulated IL-17 production and reduced frequency of IL-10R expression by B6.SLE regulatory T cells (T_{reg}). Functional assays indicated resistance of B6.SLE T_{eff} to suppression by both B6.SLE and B6 T_{reg}. Transfer experiments with CD4$^+$FoxP3$^-$ T_{eff} and CD4$^+$FoxP3$^+$ T_{reg} from B6.SLE and B6 mice, respectively, resulted in increased atherosclerosis compared with B6 T_{eff} and T_{reg} recipients. T_{reg} isolated from mice receiving B6.SLE T_{eff} with B6 T_{reg} had increased production of IL-17 and fewer expressed IL-10R compared with B6 T_{eff} and T_{reg} transfer.

Conclusions Transfer of B6.SLE T_{eff} to LDLr$^{-/-}$, Rag$^{-/-}$ mice results in accelerated atherosclerosis independent of the source of T_{reg}. In addition, the presence of B6.SLE T_{eff} resulted in more IL-17-producing T_{reg} and fewer expressing IL-10R, suggesting that B6.SLE T_{eff} may mediate phenotypic changes in T_{reg}. To our knowledge, this is the first study to provide direct evidence of the role of B6.SLE T_{eff} in accelerating atherosclerosis through resistance to T_{reg} suppression.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterised by T and B cell dysfunction, autoantibody production and end organ damage, with 30% of deaths due to premature cardiovascular disease (CVD). Multiple studies demonstrate accelerated atherosclerosis in SLE patients, with risk of developing atherosclerosis increased, on average, 2-to 10-fold compared with healthy controls.[1–3] Although aetiology of SLE-accelerated atherosclerosis remains unknown, studies in patients and animal models implicate some likely suspects including the CD40–CD40L signalling pathway,[2 4–7] decreased TGF-β,[8] decreased regulatory T cells (T_{reg}),[9–12] increased proportions and activation of T_{h1} and T_{h17} cells[10 13–15] and production of autoantibodies against β2GPI–oxLDL complexes.[16]

T cell populations including T_{h1} and T_{h17} cells can be pro-atherogenic,[9 13 17–19] while T_{reg} have been shown to be atheroprotective, likely through production of anti-inflammatory cytokines including interleukin (IL)-10.[11 20–23] It is well established that, in both mice and humans, SLE T cells are hyperactivated and produce increased amounts of IFNγ and IL-17, inflammatory cytokines associated with T_{h1} and T_{h17} cells, respectively.[10 24–26] The B6.Sle1.2.3 (B6.SLE) mouse model contains three genetic loci (Sle1, Sle2 and Sle3) which confer SLE susceptibility.[27] When all three loci are present, mice display a fully penetrant lupus phenotype that develops with age and is similar to the human disease.[27 28] One aspect of this phenotype is T cell activation. Using a bone marrow transplant model, our laboratory has demonstrated that LDLr$^{-/-}$ mice receiving SLE bone marrow (LDLr.SLE) have increased atherosclerosis compared with mice receiving B6 bone marrow (LDLr.B6 controls).[24 29] This increased atherosclerosis was accompanied by an increase in the proportion of T cells within the atherosclerotic lesions, raising the question of whether SLE T cells are important for acceleration of lesion progression. Although, due to their role in autoantibody production, B cells are the focus of the majority of clinical trials of new SLE therapies, T cells also play a significant role in disease progression. Given the role of T cells in atherosclerosis, they may be an important target to consider when developing therapies to treat SLE and CVD. In the current study, we focus on the role of SLE CD4$^+$ T cells in accelerated atherosclerosis.

METHODS

Mice

C57BL/6J mice (B6), B6.129S7-*Rag1*^{tm1Mom}/J (Rag$^{-/-}$), B6;129S7-*Ldlr*^{tm1Her}/J (LDLr$^{-/-}$) and B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J mice were originally obtained from The Jackson Laboratory. Rag$^{-/-}$ and LDLr$^{-/-}$ mice were crossed to obtain LDLr$^{-/-}$, Rag$^{-/-}$ mice. The congenic strain B6.*Sle1.2.3* (B6.SLE), originally obtained from Edward K Wakeland UTSW, Dallas, Texas, USA, has been described.[24 27 28] B6.Cg-*Foxp3*$^{tm2(EGFP)Tch}$/J mice were crossed with the B6.SLE strain to obtain B6.SLE mice with FoxP3-driven GFP expression (B6.SLE$^{FoxP3-GFP}$). All mice are on the C57BL/6 background and maintained in our colony. Female mice were used

for all studies. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Statistical analyses

Statistical significance between two groups was calculated using a Student t test. Differences among three or more variables were calculated using a one-way analysis of variance (ANOVA) with a Bonferroni post-test. A p value of <0.05 was considered statistically significant. All calculations were performed and graphs generated using GraphPad Prism Software (Graph Pad Software, Inc, La Jolla, California, USA). More detailed methods can be found in the online supplementary material.

RESULTS

B6.SLE CD4$^+$ T cells are sufficient to induce SLE-accelerated atherosclerosis

An increased prevalence of atherosclerosis has been associated with SLE in both humans and mouse models.[1–3 24 29–32] To date, it is not clear whether one specific cell type is responsible for accelerated atherosclerosis in SLE. Results from our previous studies indicated that, along with larger atherosclerotic lesions, more CD4$^+$ T cells were present in the lesions of LDLr.SLE mice.[24 29 33] This suggests a role for CD4$^+$ T cells in SLE-accelerated atherosclerosis. Therefore, we hypothesised that B6.SLE CD4$^+$ T cells were sufficient to accelerate atherosclerosis. To test this hypothesis, we isolated CD4$^+$ T cells (>90% purity) from 6-month-old B6 and B6.SLE mice and adoptively transferred them to LDLr$^{-/-}$, Rag$^{-/-}$ mice, an atherosclerotic mouse model lacking functional B and T cells. Two weeks following transfer, all animals were placed on a Western diet for 10 additional weeks (see study design, figure 1A). B6.SLE donors had active lupus, confirmed by the presence of dsDNA autoantibody titres (figure 1B). At sacrifice, however, there were no differences between recipient mice for dsDNA autoantibody titres indicating that there was no significant transfer of B cells or autoantibody production in the recipient animals (figure 1B). Additionally, there was no difference in total spleen cell number, body weight, spleen to body weight ratio or urine protein grade (figure 1C and see online supplementary figure S1A, B and E). Numbers of CD4$^+$ T cells recovered from spleens were similar between B6 and B6.SLE recipients (figure 1D). Excitingly, atherosclerotic lesion area in the aortic root was increased 36% in mice receiving B6.SLE CD4$^+$ T cells (figure 1E). This increase in atherosclerosis was independent of serum cholesterol and triglyceride levels (see online supplementary figure S1C,D), supporting a specific role of CD4$^+$ T cells in lupus-accelerated atherosclerosis. Collectively, these data demonstrate that the CD4$^+$ T cells from B6.SLE mice are sufficient to accelerate atherosclerosis when transferred to a non-lupus, atherosclerosis-susceptible mouse.

B6.SLE T_{reg} have altered function and phenotype

T_{reg} dysfunction or reduced numbers of T_{reg} may contribute to the inflammatory capabilities of B6.SLE CD4$^+$ T cells (see online supplementary figure S2 and ref 28). Data from human and mouse studies are unclear as to whether T_{reg} are proportionally or functionally different in SLE patients versus healthy subjects.[34–37]

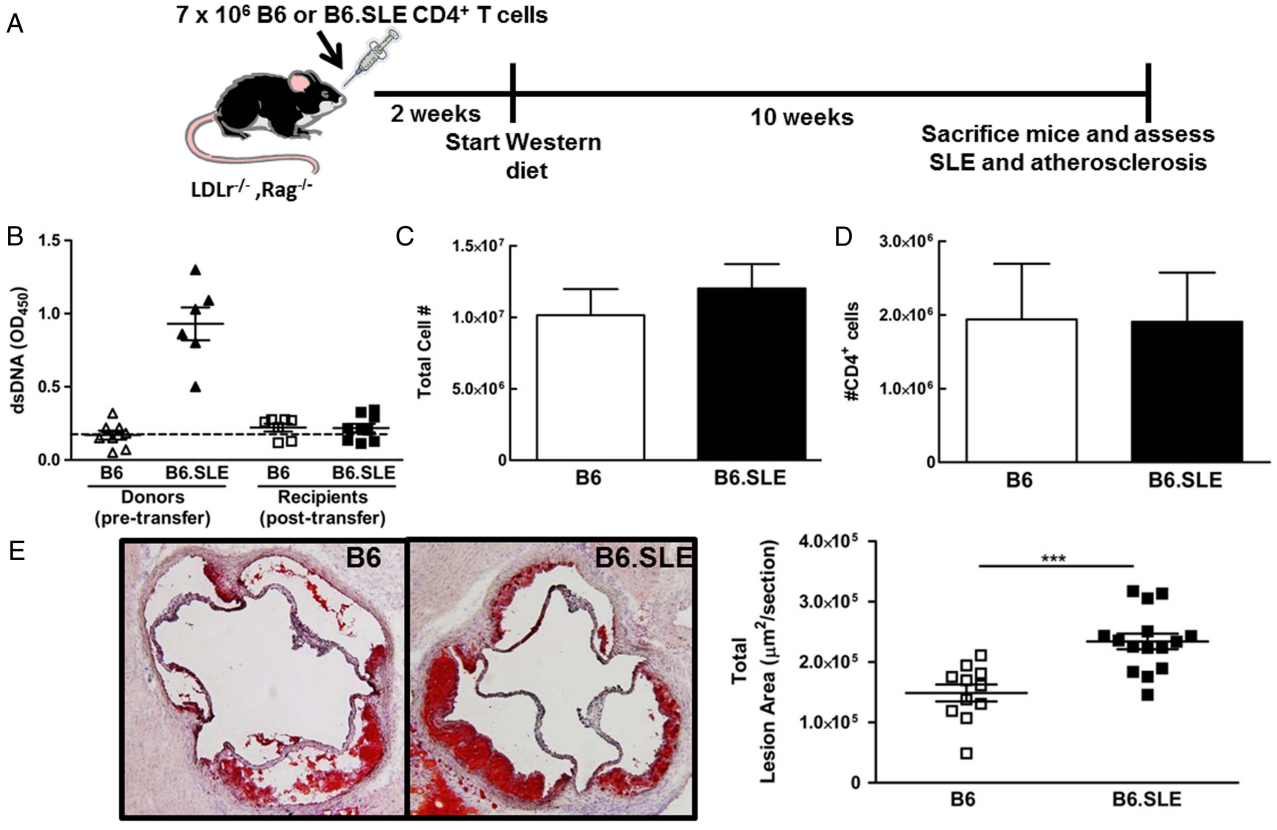

Figure 1 B6.SLE CD4$^+$ T cells are sufficient to accelerate atherosclerosis. (A) Study design. (B) dsDNA titres from donor mice (pretransfer) and recipient mice at the time of sacrifice. (C) Total number of cells in the spleens of recipient mice. (D) The total number of CD4 T cells recovered from the spleens of recipient mice. (E) Representative images of Oil Red O stained sections of the aortic root (left) and quantification of the total lesion area based on the Oil Red O staining (right). Data are representative of n=7–15 mice per group. ***Significant at p<0.001. SLE, systemic lupus erythematosus.

Although there are a number of effector T cells (T_{eff}) and T_{reg} markers, here we define T_{eff} as $CD4^+FoxP3^-$ and T_{reg} as positive for both CD4 and FoxP3. Examination of donor T_{reg} showed a twofold increase in the proportion of $CD4^+FoxP3^+$ T_{reg} in B6.SLE compared with B6 mice (figure 2A).

First, given the dysregulation of cytokine production by $CD4^+$ T cells in the B6.SLE model (see online supplementary figure S2D–F), and to determine whether SLE T_{reg} possessed qualitative differences in cytokine production compared with their B6 counterparts, we examined cytokine secretion by $CD4^+FoxP3^-$ T_{eff} and $CD4^+FoxP3^+$ T_{reg} after phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation of whole splenocytes. Not unexpectedly, B6.SLE $CD4^+FoxP3^-$ cells exhibited a 3.6-fold increase in the proportion of IL-17-producing cells compared with B6 mice (figure 2B, left panel). Surprisingly, however, B6.SLE $CD4^+FoxP3^+$ T_{reg} also exhibited a large increase in the percentage of $IL\text{-}17^+$ cells (figure 2B, right panel). Greater than 6% of T_{reg} produced IL-17; a twofold increase over unstimulated B6.SLE $CD4^+FoxP3^-$ T_{eff}. This percentage was further increased upon stimulation, with greater than 12% of T_{reg} secreting IL-17. These data suggest dysregulation of IL-17 production by $CD4^+FoxP3^+$ T_{reg} in B6.SLE mice.

T_{reg} exert their anti-inflammatory effects in part through IL-10.[38] IL-10 is classically anti-inflammatory but is associated with SLE disease activity.[39] $CD4^+FoxP3^-$ T cells in B6 and B6.SLE mice did not differ in terms of the proportion of $IL\text{-}10^+$ cells at baseline. However, after stimulation, B6.SLE $CD4^+FoxP3^-$ cells had a twofold increase in $IL\text{-}10^+$ cells compared with B6 (figure 2C, left panel), as would be expected in a model of SLE. Although both B6 and B6.SLE T_{reg} responded to stimulation by increasing the proportion of cells expressing IL-10, there was no significant difference between strains of mice (figure 2C, right panel).

B6.SLE T_{reg} have reduced levels of IL-10R expression

A recent report indicated that T_{reg} IL-10 receptor (IL-10R) expression facilitates suppression of T_{h17} responses.[40] Because we observed increased IL-17 production by T_{reg} (figure 2B) but also increased production of IL-10 in B6.SLE mice (figure 2C and see online supplementary figure S2F), we hypothesised that IL-10R expression might be reduced in B6.SLE. The proportion of $CD4^+FoxP3^-IL\text{-}10R^+$ cells was similar between B6 and B6.SLE (figure 3A,B). However, there was a 1.5-fold reduction of $IL\text{-}10R^+$ cells in the B6.SLE T_{reg} compartment compared with B6. Expression levels as measured by mean fluorescence intensity (MFI) were not different between B6 and B6.SLE (figure 3C).

B6.SLE T_{eff} are resistant to T_{reg}-mediated suppression

Despite the increased proportion of T_{reg}, T_{eff} activation persists (see online supplementary figure S2). This, in addition to increased IL-17 production and reduced IL-10R expression by B6.SLE T_{reg}, led us to hypothesise that these cells are dysfunctional in their ability to suppress T_{eff}. To test this hypothesis, we isolated T_{reg} and T_{eff} and performed in vitro functional assays. Because FoxP3 is an intracellular marker, we isolated these cells based on CD25 expression. $CD4^+CD25^+$ cells served as T_{reg} in this assay while T_{eff} were defined as $CD4^+CD25^-$. Suppressive function was determined as the ability of T_{reg} to suppress proliferation of T_{eff}. When challenged with strain-matched T_{eff}, B6.SLE T_{reg} were less suppressive compared with B6 T_{reg} (figure 4A,B).

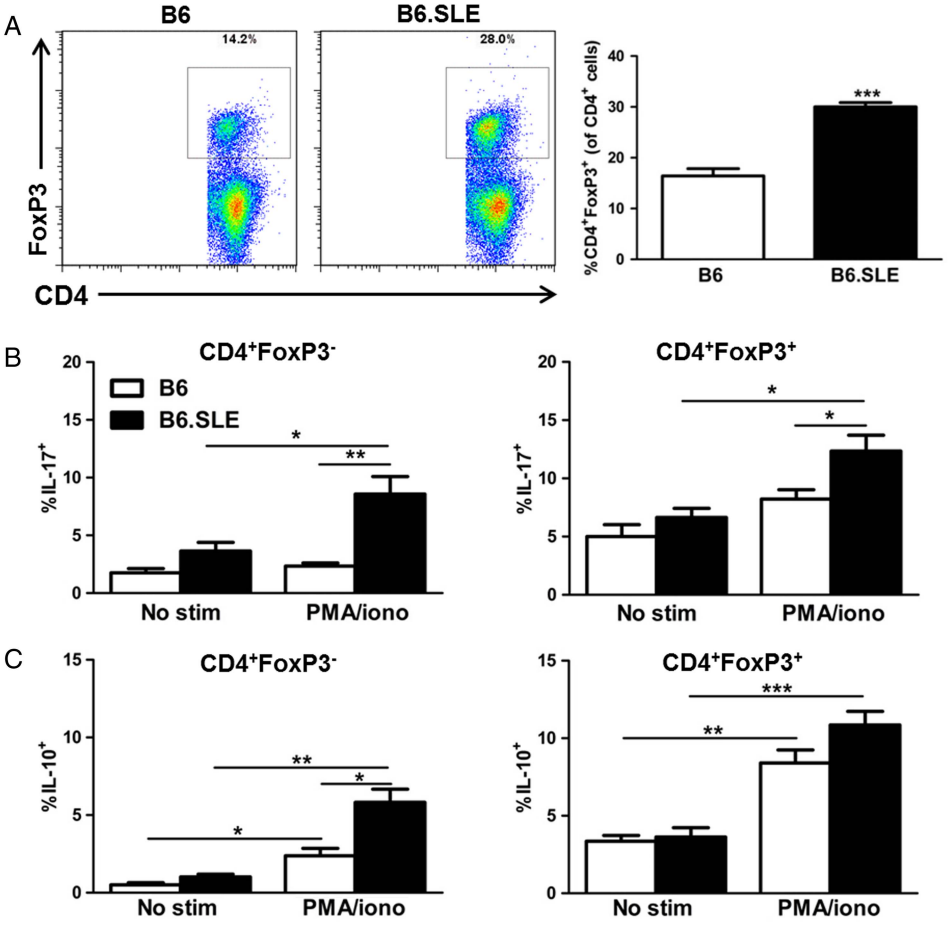

Figure 2 An increased proportion of B6.SLE T_{eff} and T_{reg} are $IL\text{-}17^+$. (A) Dot plots of FoxP3 versus CD4 (gated on $CD4^+$ cells), left panel and the resulting quantification of $CD4^+FoxP3^+$ cells, right panel. Whole splenocytes were cultured with or without anti-CD3/CD28 stimulation for 5 h and then subjected to intracellular staining for IL-17 (B) and IL-10 (C). The percentage of cytokine positive cells was measured by flow cytometry. (B) The percent of $IL\text{-}17^+$ cells in the $CD4^+FoxP3^-$ (left) and $CD4^+FoxP3^+$ (right) populations. (C) The percent of $IL\text{-}10^+$ cells in the $CD4^+FoxP3^-$ (left) and $CD4^+FoxP3^+$ (right) populations. Data are representative of n=4–6, 6-month-old mice per group. *, ** and *** indicate significance at p<0.05, 0.01 and 0.001, respectively. SLE, systemic lupus erythematosus.

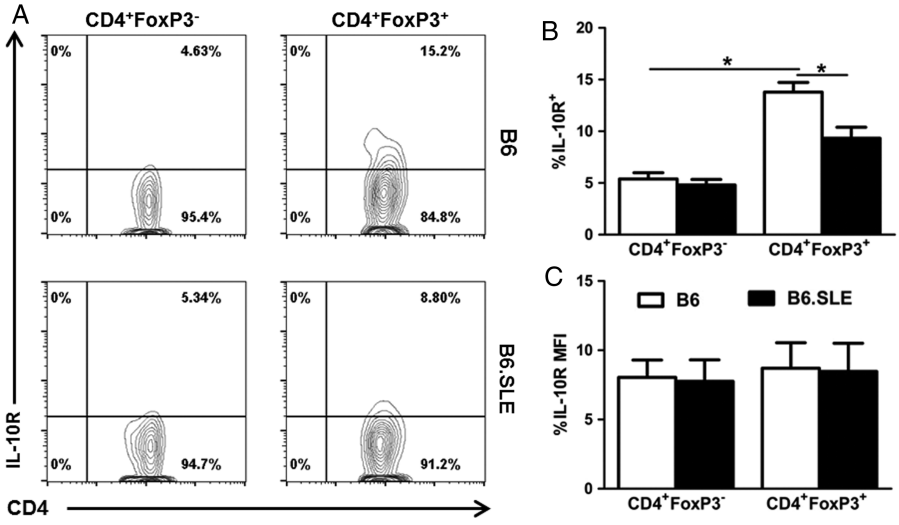

Figure 3 Reduced IL-10 receptor expression in B6.SLE T_{reg} and T_{eff} populations. Whole splenocytes were obtained from 6-month-old mice and examined for surface expression of the IL-10R by flow cytometry. Samples were gated on $CD4^+$ cells, and then the cell populations of interest were examined. (A) Contour plots showing IL-10R expression on $CD4^+FoxP3^-$ (left) and $CD4^+FoxP3^+$ (right) cells from B6 (top) and B6.SLE (bottom) mice. (B) Quantification showing the proportion of each cell population expressing IL-10R. (C) Quantification of the mean fluorescence intensity of IL-10R staining on T_{reg} and T_{eff} populations. Data are representative of at least six mice per group. * Indicates significance at $p<0.05$. SLE, systemic lupus erythematosus.

Surprisingly, when challenged with B6 T_{eff}, B6.SLE T_{reg} were as suppressive as B6 T_{reg}, suggesting that B6.SLE T_{eff} are resistant to suppression. To further confirm this, we incubated both B6 and B6.SLE T_{reg} with B6.SLE T_{eff}. The ability of T_{reg} from both strains to suppress B6.SLE T_{eff} proliferation was reduced (figure 4A,B) compared with their inhibition of B6 T_{eff}. These data suggest that, similar to results seen in some SLE patients,[37 41 42] B6.SLE T_{eff} are resistant to T_{reg}-mediated suppression.

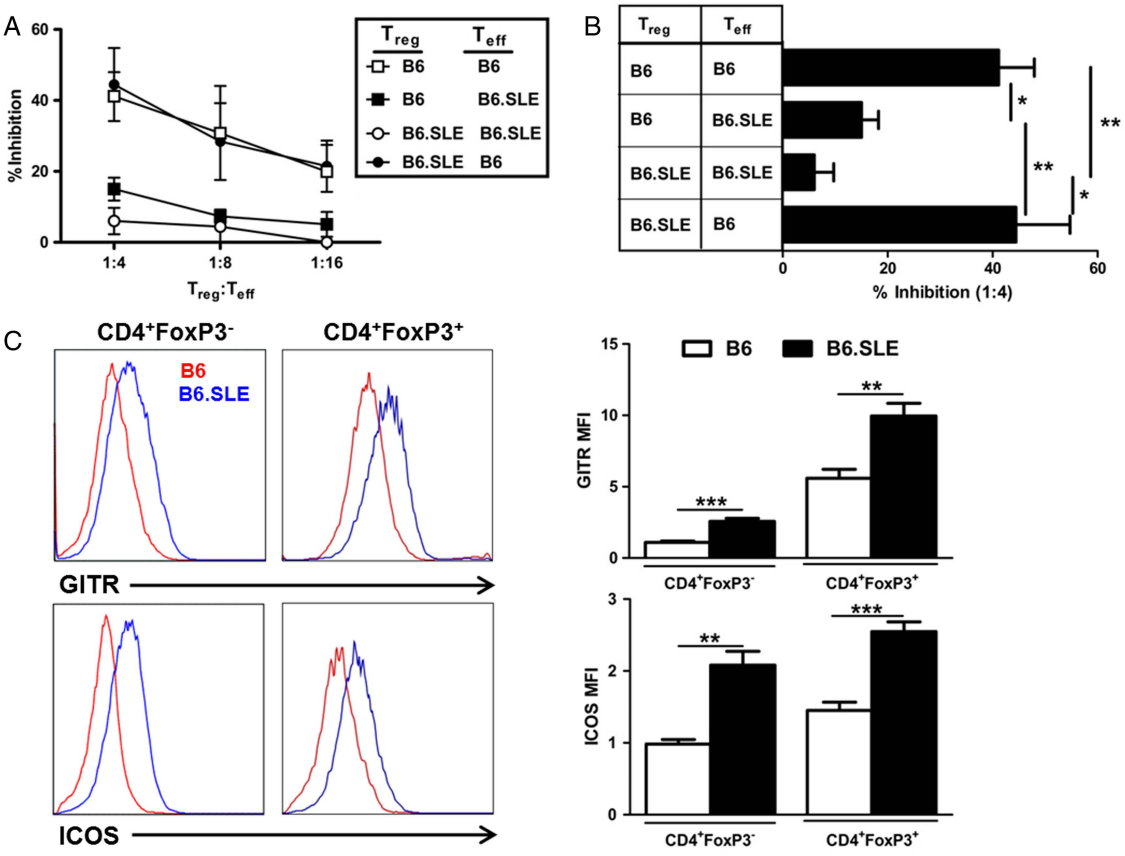

Figure 4 B6.SLE Teff are resistant to T_{reg}-mediated suppression. Suppression assays were performed from purified $CD4^+CD25^+$ (T_{reg}) and $CD4^+CD25^-$ (T_{eff}) cells. (A) Percent inhibition of proliferation by B6 and B6.SLE T_{reg} challenged with B6 or B6.SLE T_{eff}. (B) Comparison of inhibition at the 1:4 ratio of T_{reg} to T_{eff}. (C) All samples were gated based on $CD4^+$ T cells, and then the individual cell populations of interest were examined. Representative histograms of GITR expression (top panel) on $CD4^+$ cell populations in B6 (red line) and B6.SLE (blue line) mice; quantification of GITR expression based on mean fluorescence intensity (top right panel); representative histograms of inducible costimulatory molecule (ICOS) expression (bottom panel) on $CD4^+$ cell populations in B6 (red line) and B6.SLE (blue line) mice; Quantification of ICOS expression based on mean fluorescence intensity (bottom right panel). Data reflect n=6–8 mice per group from one of two representative experiments. *, ** and *** indicate significance at $p<0.05$, 0.01 and 0.001, respectively. GITR, glucocorticoid-induced TNFR-related protein; SLE, systemic lupus erythematosus.

We next measured surface expression of glucocorticoid-induced TNFR-related protein (GITR), a surface receptor upregulated on newly activated T cells. Surface expression of GITR on T_{eff} is thought to extend survival and confer resistance to suppression by T_{reg}.[43] Supporting the functional assay, B6.SLE $CD4^+$ T cell populations exhibited a twofold increase in GITR expression compared with B6 (figure 4C, top panel). Surface expression of inducible costimulatory molecule (ICOS), typically upregulated on the surface of activated T cells and important in Th2 responses,[44] was increased twofold on B6.SLE $CD4^+$ T cells compared with B6 (figure 4C, bottom panel). There were no differences between T_{eff} or T_{reg} cell populations. These data support results from figure 4A and online supplementary figure S1 indicating T cell activation and resistance of T_{eff} to T_{reg}-mediated suppression in the B6.SLE model.

B6 T_{reg} are not sufficient to prevent B6.SLE T_{eff}-mediated acceleration of atherosclerosis

Data thus far support the hypothesis that T_{eff} resistance to T_{reg}-mediated suppression in B6.SLE mice may accelerate atherosclerosis. To test this hypothesis, we performed a T cell transfer study in which $CD4^+FoxP3^-$ T_{eff} were isolated from 6-month-old $B6^{FoxP3-GFP}$ and $B6.SLE^{FoxP3-GFP}$ mice while $CD4^+FoxP3^+$ T_{reg} were isolated from $B6^{FoxP3-GFP}$ mice. A control group of $LDLr^{-/-}$, $Rag^{-/-}$ mice received both $CD4^+FoxP3^+$ T_{reg} and $CD4^+FoxP3^-$ T_{eff} from $B6^{FoxP3-GFP}$ mice (B6 TE:B6 TR) while the experimental group received B6.$SLE^{FoxP3-GFP}$ T_{eff} with $B6^{FoxP3-GFP}$ T_{reg} (B6.SLE TE:B6 TR). The study followed the timeline as shown in figure 1A. As in our previous transfer experiment, we noted no differences in body weight, spleen to body weight ratio, total number of splenocytes or in the total number of $CD4^+$ T cells in the spleens at sacrifice (see online supplementary figure S3A–D). Furthermore, there were no differences in urine protein grade, as all recipients had little to no urine protein (see online supplementary figure S3E). Upon examination of the aortic root, the total lesion area was increased 44% in the B6.SLE TE:B6 TR experimental group compared with the B6 TE:B6 TR mice (figure 5A). In addition, the percentage of $CD4^+$ T cells in the lesions was slightly, but significantly, higher in the B6.SLE T_{eff} recipients compared with control (figure 5B). When examining the T cell populations within the spleens of recipients, we discovered that, despite the presence of B6 T_{reg}, there was a greater than twofold increase in the percentage of T_{eff} in the SLE TE:B6 TR group which were producing IL-17 (figure 5C, left panel). Interestingly, in recipients of SLE TE:B6 TR, there was a 2.8-fold increase in the percentage of T_{reg} which were producing IL-17 (figure 5C, right panel). Accompanying this increase in the proportion of $IL\text{-}17^+$ cells was a significant reduction in the percentage of $IL\text{-}10R^+$ cells in the SLE TE:B6 TR group in both T_{reg} and T_{eff} (figure 5D). These data implicate B6.SLE T_{eff} as the primary player in SLE-accelerated atherosclerosis, and suggest T_{eff}-mediated dysregulation of T_{reg} IL-17 production and IL-10R expression as a potential mechanism by which T_{eff} cause this acceleration.

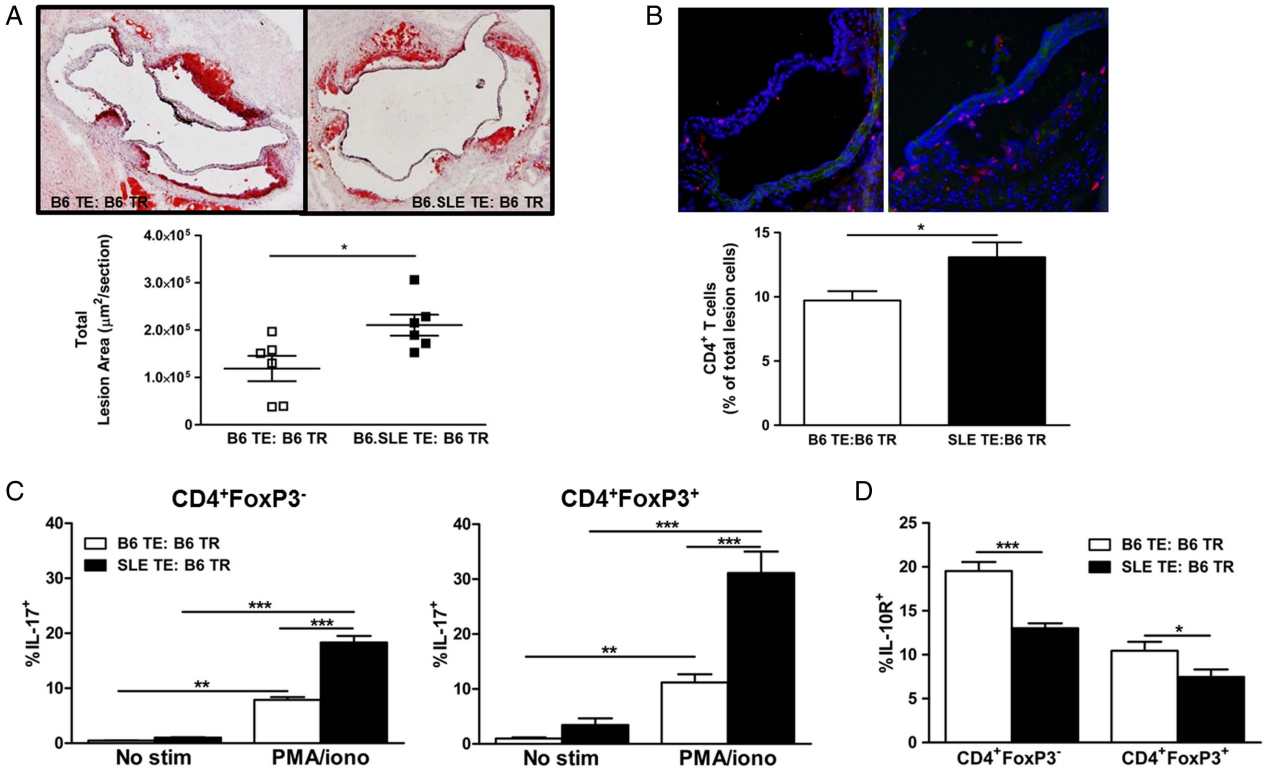

Figure 5 B6.SLE T_{eff} accelerate atherosclerosis independent of the source of T_{reg}. (A) Representative images of Oil Red O stained sections of the aortic root (top) and quantification of the total lesion area based on the Oil Red O staining (bottom). (B) Representative images of immunofluorescent staining for $CD4^+$ cells (red), with 4′, 6-diamidino-2-phenylindole (DAPI) staining (blue) for nuclei (top) and quantification of the proportion of total cells that were shown to express CD4 (bottom). (C) Whole splenocytes from recipients were cultured with or without anti-CD3/CD28 stimulation for 5 h and then subjected to intracellular staining for IL-17. The percentage of cytokine positive cells was measured by flow cytometry. The percent of $IL\text{-}17^+$ cells in the $CD4^+FoxP3^-$ (left) and $CD4^+FoxP3^+$ (right) populations. (D) IL-10R expression was measured on T_{eff} and T_{reg} from recipients. Cells were gated based on $CD4^+$ expression, then separated into T_{eff} and T_{reg} populations. IL-10R staining was measured by flow cytometry. Data reflect n=6 mice per group. *, ** and *** indicate significance at $p<0.05$, 0.01 and 0.001, respectively. SLE, systemic lupus erythematosus.

DISCUSSION

SLE and atherosclerosis are complex chronic inflammatory diseases characterised by immune dysfunction. Evidence supports interplay between the two diseases, with studies showing accelerated atherosclerosis in SLE. Increased activation of T cells has been demonstrated in both SLE patients and in mouse models of SLE including (NZB×NZW)F1,[45] MRL/MpJ-Fas(lpr/lpr)/J[46] and B6.SLE mice.[24 27 28] Given the important effector and regulatory functions of T cells in atherosclerosis and that accelerated CVD is a major cause of death for SLE patients, it has been suggested that T cells may be important to consider when developing therapies for SLE and SLE-accelerated atherosclerosis (reviewed in[47]). However, most clinical studies to date have focused on B cells due to their role in producing the autoantibodies that lead to immune complex formation and the resultant end organ damage in SLE. The current study was undertaken to determine the effects of B6.SLE $CD4^+$ T cells on atherosclerosis. While mice are not optimal for study of plaque vulnerability and rupture, common properties of atherosclerotic lesions in SLE patients, they provide a platform to study the complicated mechanisms by which the immune system contributes to plaque development. We chose the $LDLr^{-/-}$, $Rag^{-/-}$ model, allowing us to determine the direct effects of $CD4^+$ T cells on atherosclerosis, independent of other SLE immune cell populations including the autoantibody-producing B cells. Importantly, at the time of sacrifice, T cell recipients did not have an SLE phenotype, attributing any effects on atherosclerosis to the T cells. Here, we demonstrate a direct role for B6.SLE $CD4^+$ T cells in accelerated atherosclerosis.

While T_{eff} including T_{h1} cells are generally thought to be atherogenic[48 49] (and reviewed in[50]), T_{reg} have been shown to have antiatherogenic properties. Ait-Oufella *et al*[11] showed a negative correlation between T_{reg} number and atherosclerosis development (reviewed in[51]), and it has been directly demonstrated that depletion of T_{reg} results in increased atherosclerosis.[52] In our study, B6.SLE mice had an increased proportion of $CD4^+FoxP3^+$ T_{reg}, yet atherosclerosis was increased with $CD4^+$ T cell transfer from these mice. T_{reg} and T_{h17} dysregulation is a common feature in SLE. We made the unexpected observation that an increased proportion of B6.SLE T_{reg} was secreting IL-17, a cytokine typically attributed to T_{h17} cells. IL-17 contributes to tissue damage in SLE and is correlated with disease activity.[53 54] Interestingly, the proportion of these cells making IL-17 was higher than that of T_{eff}, suggesting that T_{reg} are a part of the IL-17 dysregulation observed in SLE and have the potential to contribute to tissue damage. IL-17 is also believed to play a role in atherosclerosis, although whether it contributes to or reduces atherosclerosis is unclear due to the existence of studies supporting both possibilities.[13 19 55] Since IL-17-producing cells likely play a role in atherosclerosis, the fact that significant proportions of B6.SLE T_{reg} secrete IL-17 identifies them as potential contributors to SLE-accelerated atherosclerosis. A report by Chaudhry *et al*[40] concluded that T_{reg} IL-10R expression is required to suppress inflammation mediated by T_{h17} cells. This led us to ask the question as to whether IL-10R expression was reduced in B6.SLE T cell populations. Reduced IL-10R expression could explain the persistence of inflammation despite increased IL-10 in the context of SLE and why an increase of this antiatherogenic cytokine does not lead to reduced atherosclerosis. Indeed, the proportion of $IL\text{-}10R^+$ cells in the T_{reg} populations from B6.SLE mice was reduced. These data led us to hypothesise that B6.SLE T_{reg} have reduced suppressive capacity. On the contrary, results from T_{reg} functional assays demonstrated intact suppressive function of B6.SLE $CD4^+CD25^+$ T_{reg} but that B6.SLE T_{eff} are resistant to T_{reg}-mediated suppression. These data are comparable with some studies in SLE patients which point to T_{eff} resistance rather than T_{reg} dysfunction.[37 56]

Given the damaging effects of IL-17 in SLE, uncovering the underlying mechanisms behind the dysregulation in IL-17 and IL-10R may be important for our understanding of the interplay between SLE and atherosclerosis. Indeed, a recent study in SLE patients with atherosclerosis has shed light on a potential atherosclerotic role for IL-17 in SLE by demonstrating that these patients have increased serum levels of IL-17 compared with SLE patients and healthy controls without atherosclerosis.[57] Importantly, results from our study implicate B6.SLE T_{eff} as being responsible for this dysregulation of T_{reg} IL-17 production and IL-10R expression. With transfer of B6.SLE T_{eff} and B6 T_{reg}, atherosclerosis was increased. This increase, despite the presence of wild-type T_{reg}, indicates that T_{eff} resistance to T_{reg}-mediated suppression may be a strong contributing factor in this mechanism. Not only did B6 T_{reg} fail to suppress B6.SLE T_{eff}-accelerated atherosclerosis, their phenotype appeared to be altered by the presence of B6.SLE T_{eff}, resulting in an increased proportion of B6 T_{reg} producing IL-17 and a reduced percentage of cells expressing IL-10R. This suggests that B6.SLE T_{eff} resistance may be a direct result of their ability to alter IL-10R expression and, subsequently, IL-17 production by T_{reg}. One limitation of this study, however, is our ability to definitively know whether the altered T_{reg} phenotype in SLE TE:B6 TR recipients was due to changes in the original B6 T_{reg} or conversion of B6.SLE T_{eff} to T_{reg} with a dysfunctional phenotype. We are currently developing the reporter mice which would allow us to map T_{reg} that become ex-T_{reg}[58] and these studies are the focus of future experiments.

With evidence mounting for the antiatherogenic role of T_{reg}, increased focus has been placed on these cells as a therapeutic target for atherosclerosis. Recent studies have identified a number of potential mechanisms involved in T_{reg} suppression that may prove useful for enhancement of suppression and/or T_{reg} numbers including MYD88 signalling in dendritic cells,[20] the IL-33–ST2 interaction,[59] CD39 and CD73 signalling, CTLA-4 and LAG-3.[60] However, data from the current study favour T_{eff} resistance over T_{reg}-mediated suppression as a major culprit in SLE and suggest that, at least in the context of SLE and atherosclerosis, targeting mechanisms of T_{eff} resistance may prove most effective. Given our data, we postulate that therapies targeting IL-17 production, and perhaps IL-10R expression, would prove effective in treatment of SLE-accelerated atherosclerosis.

Multiple aspects of T cell dysregulation in B6.SLE mice suggest these cells have the potential to accelerate atherosclerosis. To date, speculation regarding their role in SLE-accelerated atherosclerosis has far outweighed any direct evidence supporting this hypothesis. In the current study, we demonstrate (1) B6.SLE $CD4^+$ T cells are sufficient to accelerate atherosclerosis, (2) B6.SLE T_{eff} are resistant to T_{reg}-mediated suppression, (3) dysregulation of IL-17 production by T_{reg}, with a possible role for the IL-10R in this process and (4) B6.SLE T_{eff} are likely the primary T cell type involved in the observed accelerated atherosclerosis, potentially through their ability to alter T_{reg} IL-17 production and IL-10R expression. These data provide strong evidence for a causal role of $CD4^+$ T cells and, specifically, for B6.SLE T_{eff}, in SLE-accelerated atherosclerosis. To our knowledge, this is the first study to provide such evidence. These results emphasise the importance of considering T cell-targeted therapies when developing strategies to treat SLE and SLE-accelerated atherosclerosis and suggest that, in SLE, T_{eff} may be a more desirable target for therapy than T_{reg}.

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Contributors AJW designed, performed and interpreted experiments presented in this manuscript. She also wrote and edited most of the manuscript content. JPR performed the experiments and edited the text. NSW performed experiments presented in this manuscript. ASM designed, performed, interpreted the experiments and edited the text. She is also the principal investigator of the laboratory.

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Competing interests None.

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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.

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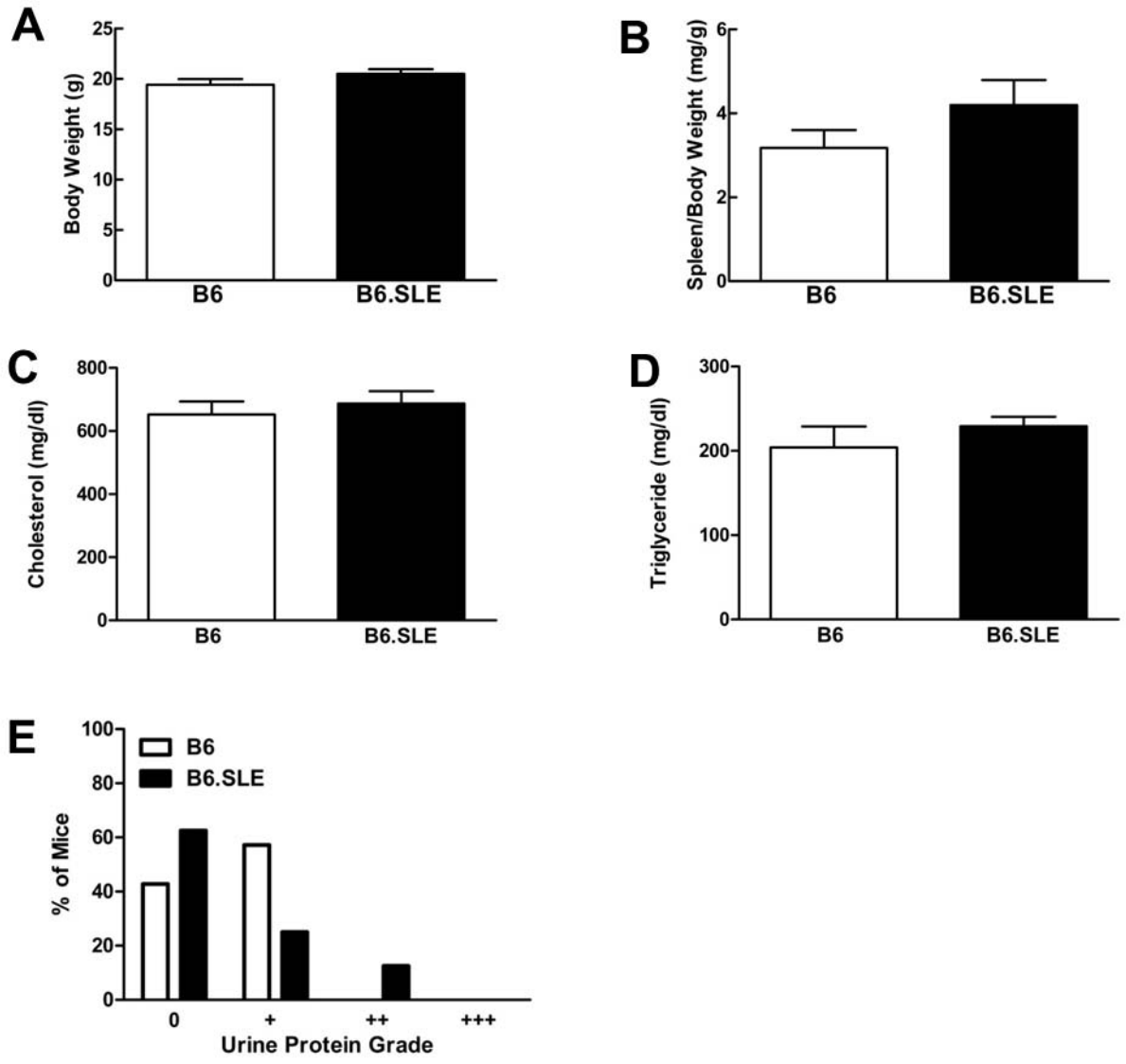
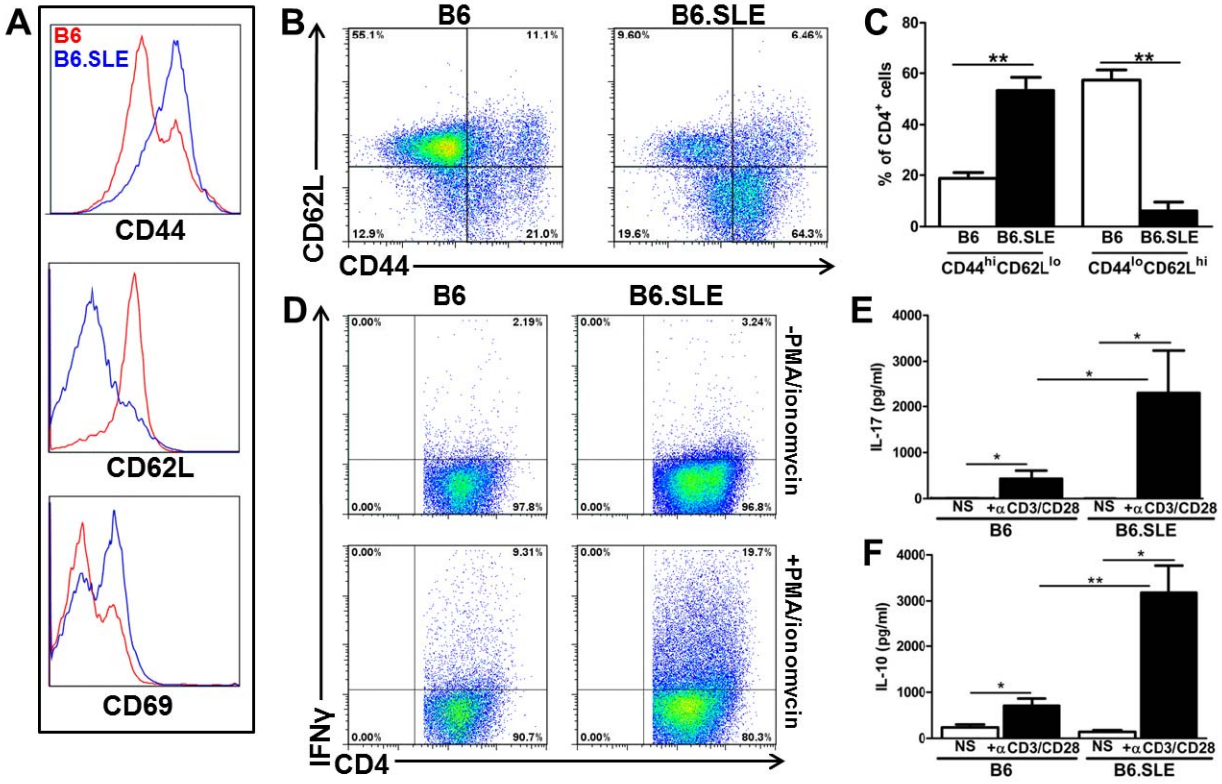
Supplemental Figures

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.



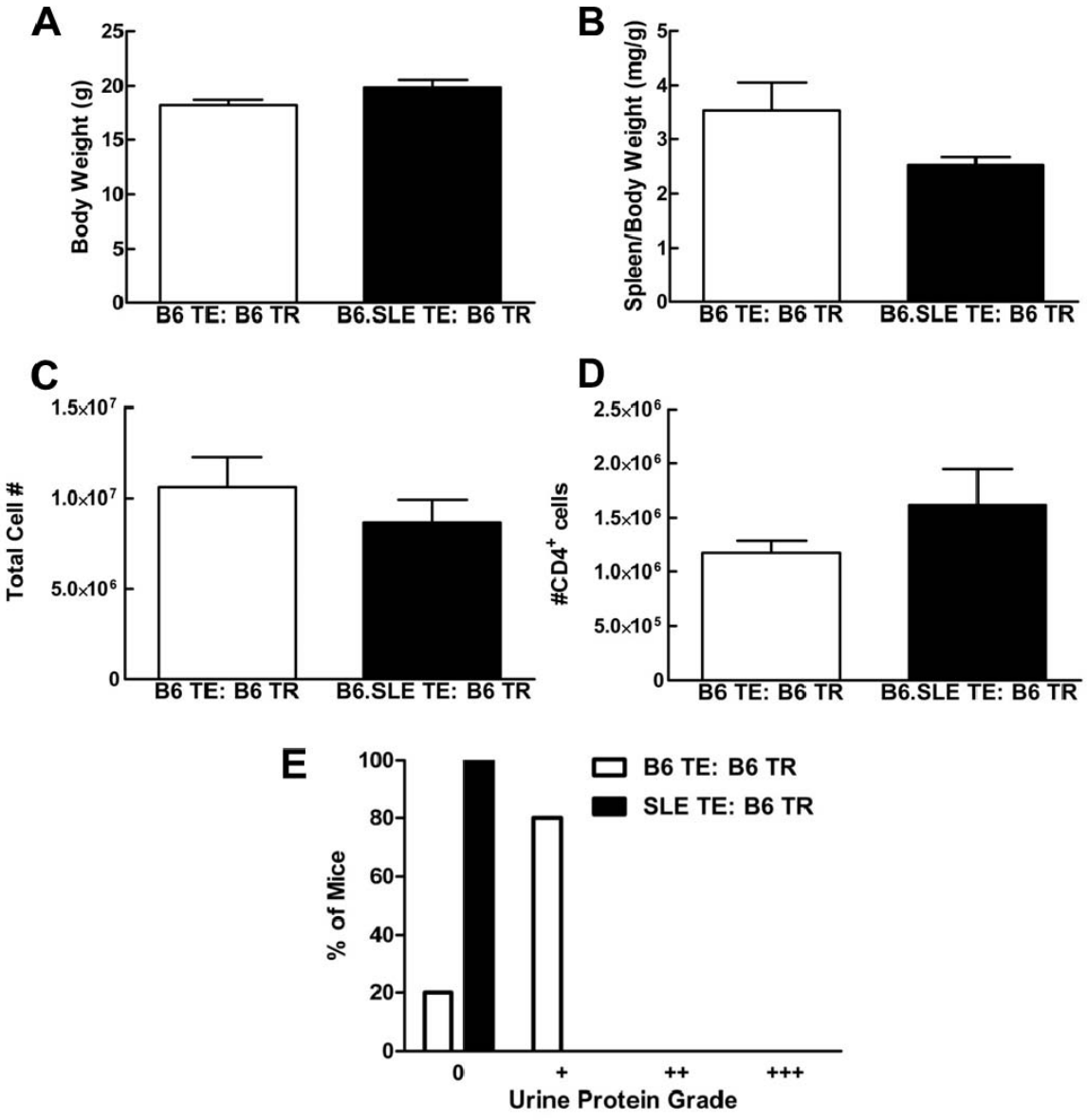
Supplemental Figure I.

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