EXTENDED REPORT

Dysregulated CD4+ T cells from SLE-susceptible mice are sufficient to accelerate atherosclerosis in LDLr−/− mice

Ashley J Wilhelm,[1] Jillian P Rhoads,[2] Nekeithia S Wade,[3] Amy S Major[1,2]

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]
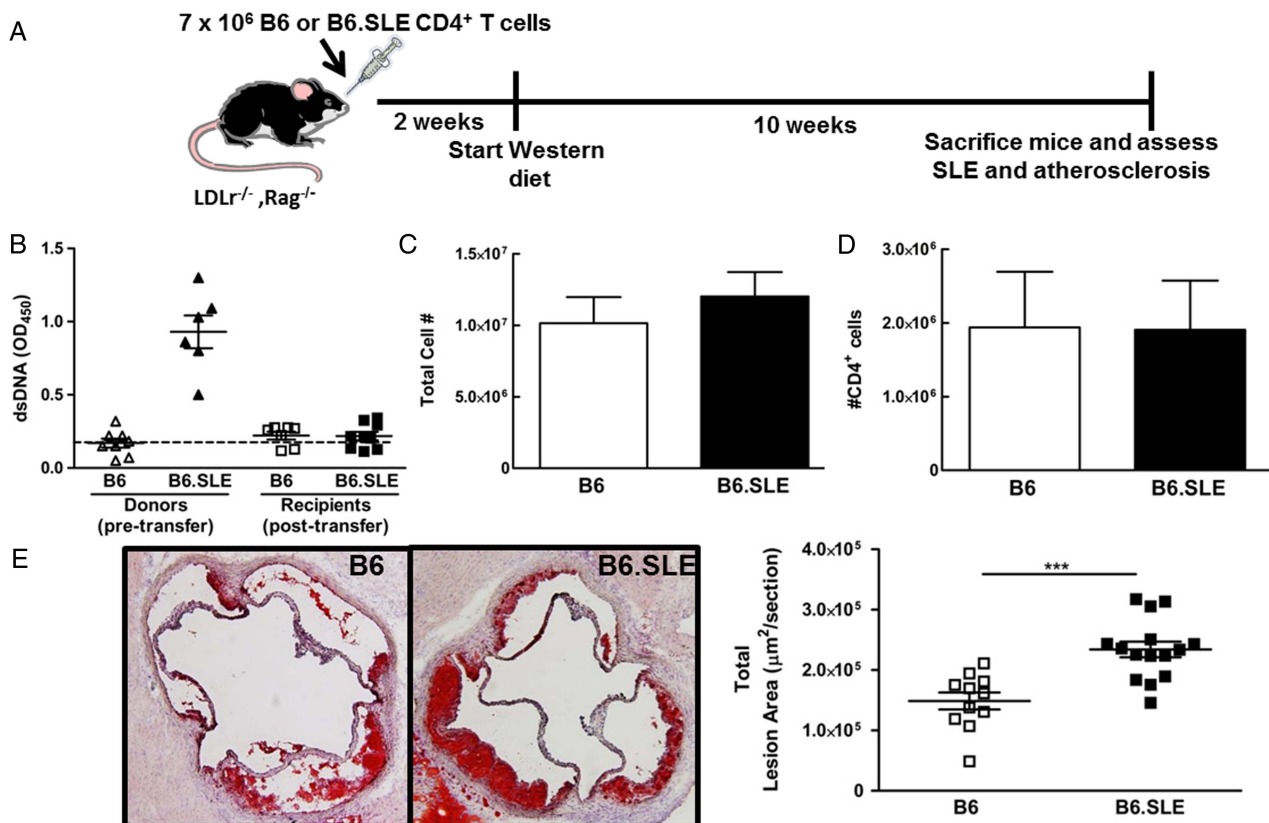

Figure 1 B6.SLE CD4+ T cells are sufficient to accelerate atherosclerosis. (A) Study design. (B) dsDNA titres from donor mice (pretransfer) and recipient mice at the time of sacrifice. (C) Total number of cells in the spleens of recipient mice. (D) The total number of CD4 T cells recovered from the spleens 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-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-10$^+$ cells at baseline. However, after stimulation, B6.SLE $CD4^+FoxP3^-$ cells had a twofold increase in IL-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 $CD4^+FoxP3^-IL-10R^+$ cells was similar between B6 and B6. SLE (figure 3A,B). However, there was a 1.5-fold reduction of IL-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-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-17$^+$ cells in the $CD4^+FoxP3^-$ (left) and $CD4^+FoxP3^+$ (right) populations. (C) The percent of IL-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.
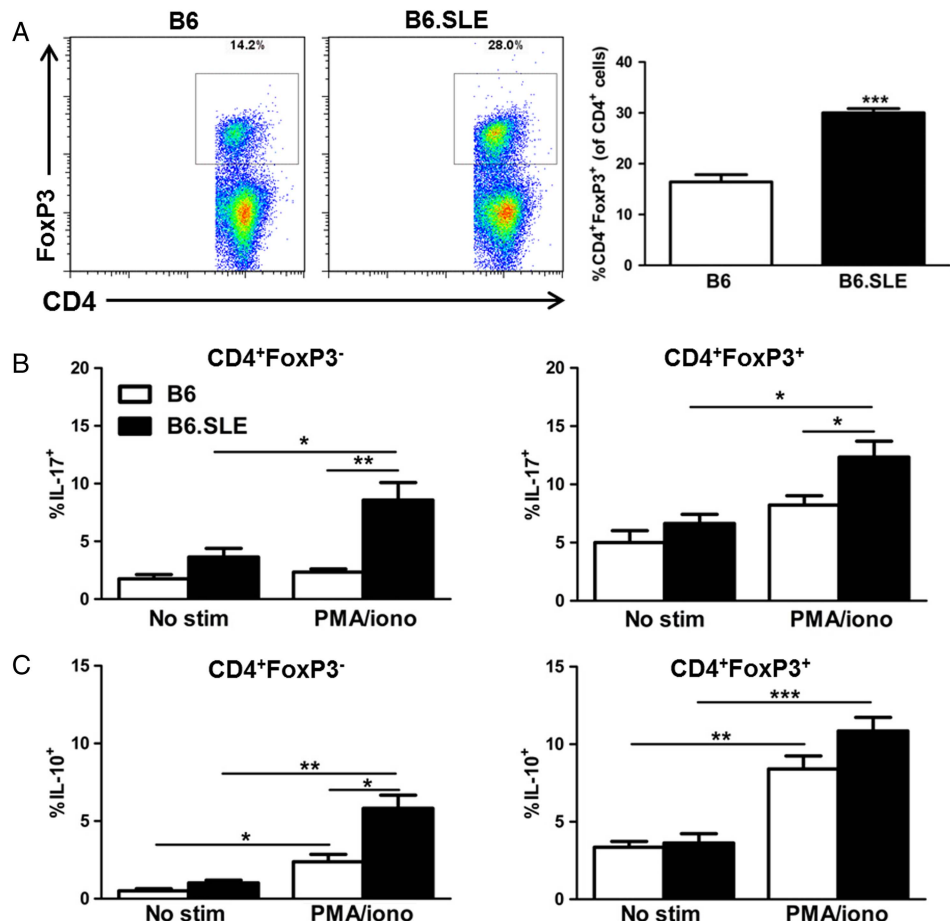
Figure 3 Reduced IL-10 receptor expression in B6.SLE T$_{reg}$ and T$_{eff}$ populations. Whole splenocytes were obtained from 6-month-old mice and examined for surface expression of the IL-10R by flow cytometry. Samples were gated on CD4$^+$ cells, and then the cell populations of interest were examined. (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.
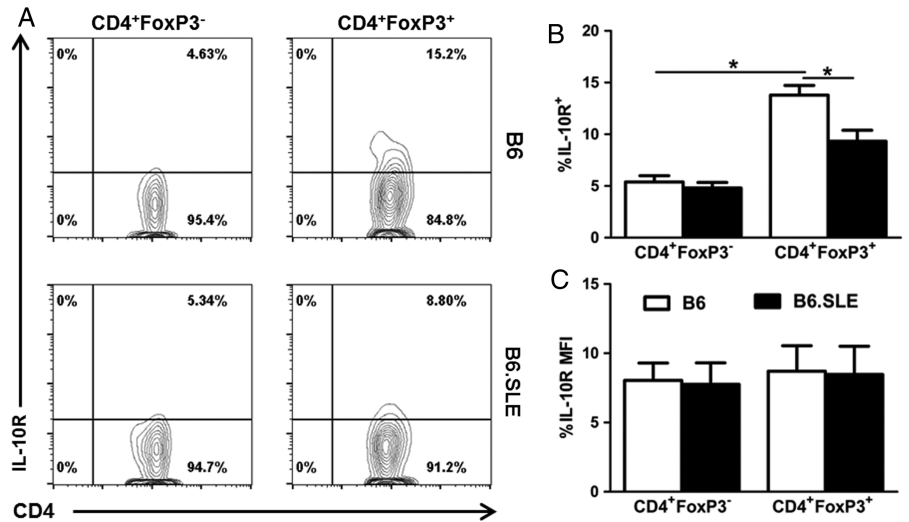
Surprisingly, when challenged with B6 T$_{eff}$, B6.SLE T$_{reg}$ were as suppressive as B6 T$_{reg}$, suggesting that B6.SLE T$_{eff}$ are resistant to suppression. To further confirm this, we incubated both B6 and B6.SLE T$_{reg}$ with B6.SLE T$_{eff}$. The ability of T$_{reg}$ from both strains to 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.
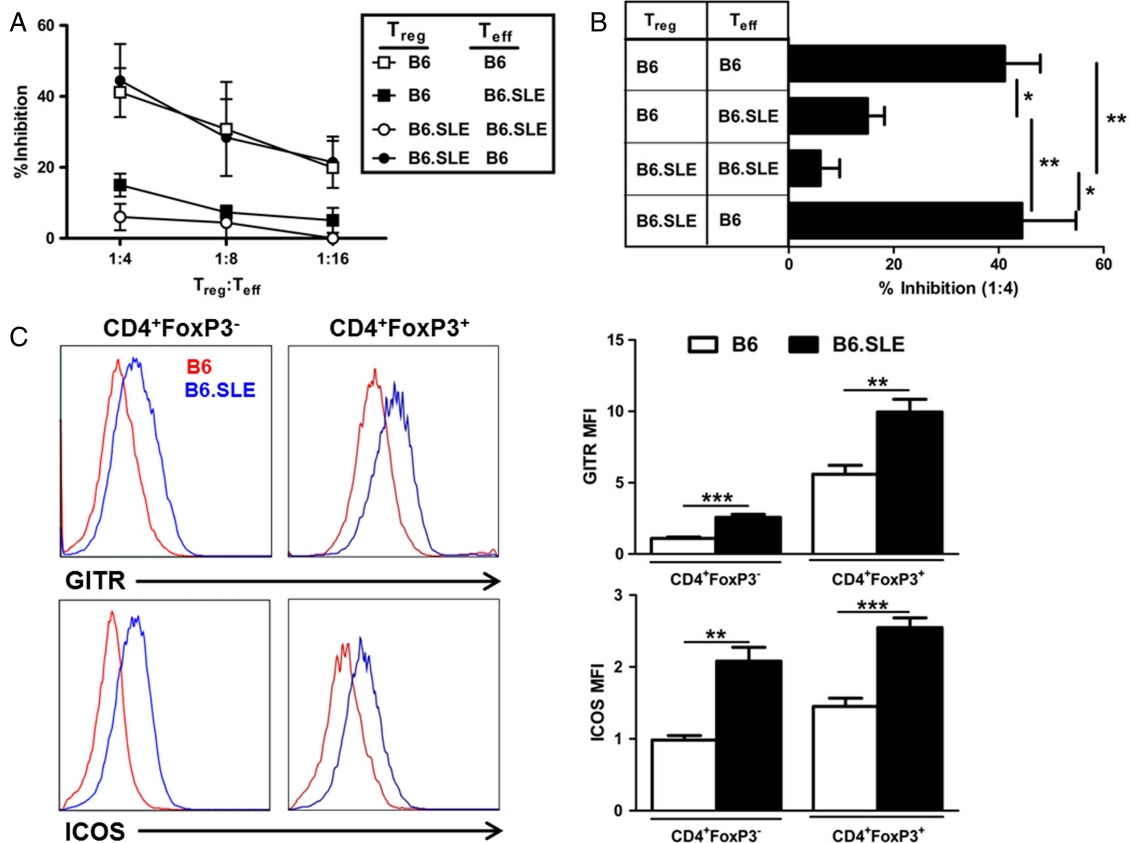

Figure 4 B6.SLE Teff are resistant to T$_{reg}$-mediated suppression. Suppression assays were performed from purified CD4$^+$CD25$^+$ (T$_{reg}$) and CD4$^+$CD25$^-$ (T$_{eff}$) cells. (A) Percent inhibition of proliferation by B6 and B6.SLE T$_{reg}$ challenged with B6 or B6.SLE T$_{eff}$. (B) Comparison 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-17$^+$ cells was a significant reduction in the percentage of IL-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.
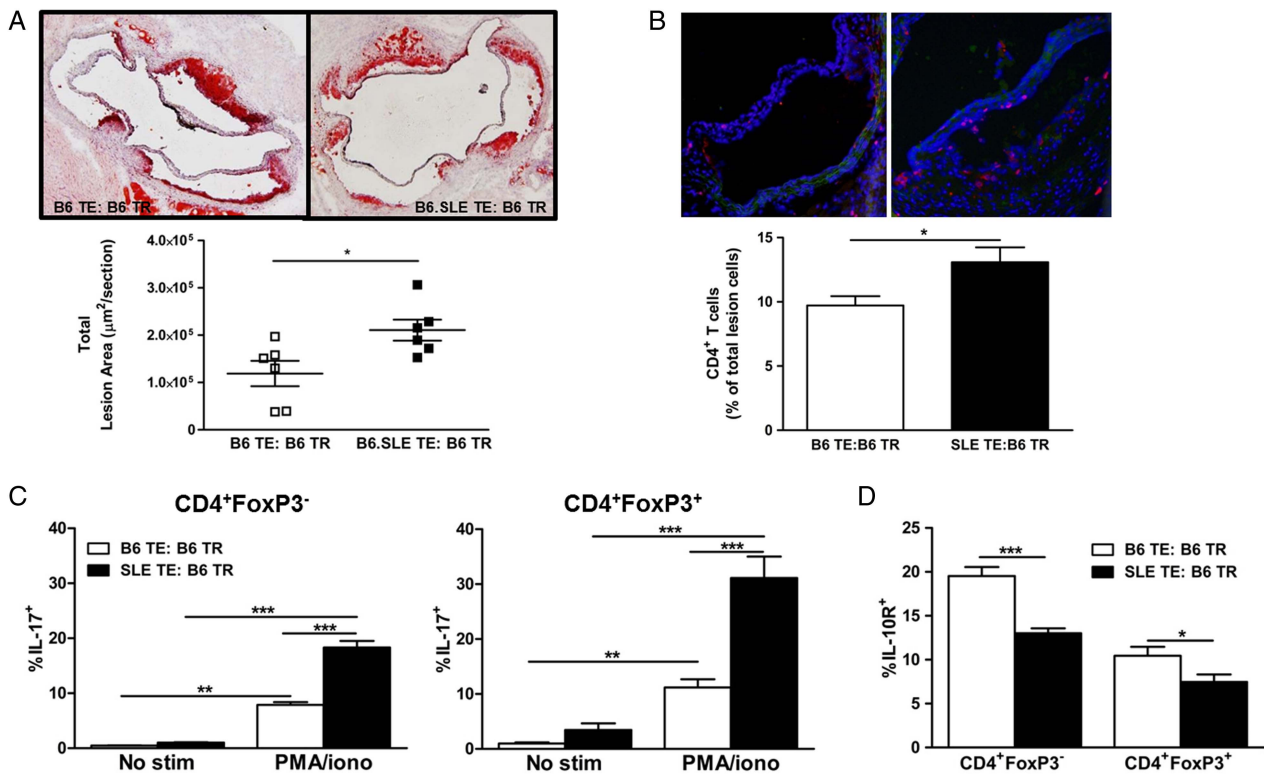

Figure 5 B6.SLE T_{eff} accelerate atherosclerosis independent of the source of T_{reg}. (A) Representative images of Oil Red O stained sections of the aortic root (top) and quantification of the total lesion area based on the Oil Red O staining (bottom). (B) Representative images of immunofluorescent 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-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-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$_{reg}$.

REFERENCES

1 Manzi S, Selzer F, Sutton-Tyrrell K, et al. Prevalence and risk factors of carotid plaque in women with systemic lupus erythematosus. Arth Rheum 1999;42:51–60.
2 Roman MJ, Shanker B-A, Davis A, et al. Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus. NEJM 2003;349:2399–406.
3 Asanuma Y, Oeser A, Shintani AK, et al. Premature coronary-artery atherosclerosis in systemic lupus erythematosus. NEJM 2003;349:2407–15.
4 Lutgens E, Daemen MJAP. CD40-CD40L interactions in atherosclerosis. Trends Cardiovasc Med 2002;12:27–32.
5 Schönbeck U, Sukhova GK, Shimizu K, et al. Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. PNAS 2000;97:7458–63.
6 Mach F, Schönbeck U, Sukhova GK, et al. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. Nature 1998;394:200–3.
7 Zhu J, Liu X, Xie C, et al. T cell hyperactivity in lupus as a consequence of hyperstimulatory antigen-presenting cells. J Clin Invest 2005;115:1869–78.
8 Mallat Z, Gojova A, Marchiol-Fournigault C, et al. Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. Circ Res 2001;89:930–4.
9 Taleb S, Tedgui A, Mallat Z. Adaptive T cell immune responses and atherogenesis. Cur Opin Pharmacol 2010;10:197–202.
10 Dolff S, Bijl M, Huitema MG, et al. Disturbed Th1, Th2, Th17 and T(reg) balance in patients with systemic lupus erythematosus. Clin Immunol 2011;141:197–204.
11 Ait-Oufella H, Salomon BL, Potteaux S, et al. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med 2006;12:178–80.
12 De Boer OJ, Van der Meer JJ, Teeling P, et al. Low numbers of FOXP3 positive regulatory T cells are present in all developmental stages of human atherosclerotic lesions. PloS ONE 2007;2:e779.
13 Gao Q, Jiang Y, Ma T, et al. A critical function of Th17 proinflammatory cells in the development of atherosclerotic plaque in mice. J Immunol 2010;185:5820–7.
14 Xie J, Wang J, Tang T, et al. The Th17/Treg functional imbalance during atherogenesis in ApoE(-/-) mice. Cytokine 2010;49:185–93.
15 Xu Z, Cuda CM, Croker BP, et al. The NZM2410-derived lupus susceptibility locus Sle2c1 increases Th17 polarization and induces nephritis in fas-deficient mice. Arth Rheum 2011;63:764–74.
16 George J, Harats D, Shoenfeld Y. Autoimmunity in atherosclerosis. The role of autoantigens. Clin Rev Allergy Immunol 2000;18:73–86.
17 Zhao Z, Wu Y, Cheng M, et al. Activation of Th17/Th1 and Th1, but not Th17, is associated with the acute cardiac event in patients with acute coronary syndrome. Atherosclerosis 2011;217:518–24.
18 Pejnovic N, Vratimos A, Lee SH, et al. Increased atherosclerotic lesions and Th17 in interleukin-18 deficient apolipoprotein E-knockout mice fed high-fat diet. Mol Immunol 2009;47:37–45.
19 Smith E, Prasad K-MR, Butcher M, et al. Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. Circulation 2010;121:1746–55.
20 Subramanian M, Thorp E, Hansson GK, et al. Treg-mediated suppression of atherosclerosis requires MYD88 signaling in DCs. J Clin Investi 2013;123:179–88.
21 Dietrich T, Hucko T, Schneemann C, et al. Local delivery of IL-2 reduces atherosclerosis via expansion of regulatory T cells. Atherosclerosis 2011;220:329–36.
22 Kelly JA, Griffin ME, Fava RA, et al. Inhibition of arterial lesion progression in CD16-deficient mice: evidence for altered immunity and the role of IL-10. Cardiovasc Res 2010;85:224–31.
23 Meng X, Zhang K, Li J, et al. Statins induce the accumulation of regulatory T cells in the atherosclerotic plaque. Mol Med 2012;18:598–605.
24 Stanic AK, Stein CM, Morgan AC, et al. Immune dysregulation accelerates atherosclerosis and modulates plaque composition in systemic lupus erythematosus. PNAS 2006;103:7018–23.
25 Shah K, Lee W-W, Lee S-H, et al. Dysregulated balance of Th17 and Th1 cells in systemic lupus erythematosus. Arth Res Ther 2010;12:R53.
26 Zhang Z, Kyttaris VC, Tsokos GC. The role of IL-23/IL-17 axis in lupus nephritis. J Immunol 2009;183:3160–9.
27 Morel L, Yu Y, Blenman KR, et al. Production of congenic mouse strains carrying genomic intervals containing SLE-susceptibility genes derived from the SLE-prone NZM2410 strain. Mammal Gen 1996;7:335–9.
28 Sobel ES, Mohan C, Morel L, et al. Genetic dissection of SLE pathogenesis: adoptive transfer of Sle1 mediates the loss of tolerance by bone marrow-derived B cells. J Immunol 1999;162:2415–21.
29 Braun N, Wade NS, Wakeland EK, et al. Accelerated atherosclerosis is independent of feeding high fat diet in systemic lupus erythematosus-susceptible LDLr(-/-) mice. Lupus 2008;17:1070–8.
30 Urowitz MB, Bookman AA, Koehler BE, et al. The bimodal mortality pattern of systemic lupus erythematosus. Ame J Med 1976;60:221–5.
31 Svenungsson E, Jensen-Urstad K, Heimbürger M, et al. Risk factors for cardiovascular disease in systemic lupus erythematosus. Circulation 2001;104:1887–93.
32 Petri MA, Kiani AN, Post W, et al. Lupus Atherosclerosis Prevention Study (LAPS). Ann Rheum Dis 2011;70:760–5.
33 Van Leuven SI, Mendez-Fernandez YV, Wilhelm AJ, et al. Mycophenolate mofetil but not atorvastatin attenuates atherosclerosis in lupus-prone LDLr-/- mice. Ann Rheum Dis 2012;71:408–14.
34 Divekar AA, Dubey S, Gangalum PR, et al. Dicer insufficiency and microRNA-155 overexpression in lupus regulatory T cells: an apparent paradox in the setting of an inflammatory milieu. J Immunol 2011;186:924–30.
35 Lee J-H, Wang L-C, Lin Y-T, et al. Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus. Immunol 2006;117:280–6.
36 Scalapino KJ, Tang Q, Bluestone JA, et al. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. J Immunol 2006;177:1451–9.
37 Venigalla RKC, Tretter T, Krienke S, et al. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus. Arth Rheum 2008;58:2120–30.
38 Mallat Z, Taleb S, Ait-Oufella H, et al. The role of adaptive T cell immunity in atherosclerosis. J Lipid Res 2009;50(Suppl):S364–9.
39 Park YB, Lee SK, Kim DS, et al. Elevated interleukin-10 levels correlated with disease activity in systemic lupus erythematosus. Clin Exp Rheumat 1998;16:283–8.
40 Chaudhry A, Samstein RM, Treuting P, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 2011;34:566–78.
41 Vargas-Rojas MI, Crispín JC, Richaud-Patin Y, et al. Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. Lupus 2008;17:289–94.
42 Yan B, Ye S, Chen G, et al. Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. Arth Rheum 2008;58:801–12.
43 Stephens GL, McHugh RS, Whitters MJ, et al. Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. J Immunol 2004;173:5008–20.
44 Tesciuba AG, Shilling RA, Agarwal MD, et al. ICOS costimulation expands Th2 immunity by augmenting migration of lymphocytes to draining lymph nodes. J Immunol 2008;181:1019–24.
45 Ishikawa S, Akakura S, Abe M, et al. A subset of CD4+ T cells expressing early activation antigen CD69 in murine lupus: possible abnormal regulatory role for cytokine imbalance. J Immunol 1998;161:1267–73.
46 Rankin AL, Guay H, Herber D, et al. IL-21 receptor is required for the systemic accumulation of activated B and T lymphocytes in MRL/MpJ-Fas(lpr/lpr)/J mice. J Immunol 2012;188:1656–67.
47 Kahlenberg JM, Kaplan MJ. Mechanisms of premature atherosclerosis in rheumatoid arthritis and lupus. Ann Rev Med 2013;64:249–63.
48 Zhou X, Nicoletti A, Elhage R, et al. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. Circulation 2000;102:2919–22.
49 Schulte S, Sukhova GK, Libby P. Genetically programmed biases in Th1 and Th2 immune responses modulate atherogenesis. Amer J Pathol 2008;172:1500–8.
50 Wilhelm AJ, Major AS. Accelerated atherosclerosis in systemic lupus erythematosus: mechanisms and prevention approachesNo Title. Int J Clin Rheumatol 2012;7:527–39.
51 Yang H, Zhang W, Zhao L, et al. Are CD4+CD25-Foxp3+ cells in untreated new-onset lupus patients regulatory T cells? Arth Res Ther 2009;11:R153.

52   Klingenberg R, Gerdes N, Badeau RM, *et al*. Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis. *J Clin Investi* 2013;123:1323–34.

53   Chen XQ, Yu YC, Deng HH, *et al*. Plasma IL-17A is increased in new-onset SLE patients and associated with disease activity. *J Clin Immunol* 2010;30:221–5.

54   Xing Q, Wang B, Su H, *et al*. Elevated Th17 cells are accompanied by FoxP3+ Treg cells decrease in patients with lupus nephritis. *Rheumatol Intern* 2012;32:949–58.

55   Taleb S, Romain M, Ramkhelawon B, *et al*. Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *J Exp Med* 2009;206:2067–77.

56   Monk CR, Spachidou M, Rovis F, *et al*. MRL/Mp CD4+,CD25- T cells show reduced sensitivity to suppression by CD4+,CD25+ regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus. *Arth Rheum* 2005;52:1180–4.

57   Mengya Z, Hanyou M, Dong L, *et al*. Th17/Treg imbalance induced by increased incidence of atherosclerosis in patients with systemic lupus erythematosus (SLE). *Clin Rheum* 2013;32:1045–52.

58   Rubtsov YP, Niec RE, Josefowicz S, *et al*. Stability of the regulatory T cell lineage in vivo. *Science* 2010;329:1667–71.

59   Wasserman A, Ben-Shoshan J, Entin-Meer M, *et al*. Interleukin-33 augments Treg cell levels: a flaw mechanism in atherosclerosis. *IMAJ* 2012;14:620–3.

60   Miyara M, Gorochov G, Ehrenstein M, *et al*. Human FoxP3+ regulatory T cells in systemic autoimmune diseases. *Autoimmunity Rev* 2011;10:744–55.