Mycophenolate Mofetil Decreases Atherosclerotic Lesion Size by Depression of Aortic T-Lymphocyte and Interleukin-17–Mediated Macrophage Accumulation

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Objectives	This study tested whether immunosuppression with mycophenolate mofetil (MMF) inhibits atherosclerosis development in apolipoprotein-E–deficient ($Apoe^{-/-}$) mice and investigated the mechanism.
Background	Chronic vascular inflammation involving both innate and adaptive immunity is central in the development of atherosclerosis, but immunosuppressive treatment is not uniformly beneficial. The immunosuppressive MMF targets lymphocyte proliferation by inhibiting inosine-monophosphate dehydrogenase.
Methods	Young and aged $Apoe^{-/-}$ mice were treated with 30 mg/kg daily MMF during 12 and 3 weeks of a high-fat diet, respectively. Aortic lesion size and composition was investigated by histology and flow cytometry; soluble inflammatory mediators were investigated by enzyme-linked immunosorbent assay.
Results	Macroscopic and histologic aortic atherosclerotic lesions were significantly decreased in both MMF-treated groups. While systemic immunoglobulin G directed against low-density lipoproteins was not significantly altered, the T-cell cytokine interleukin (IL)-17 was significantly reduced in plasma of MMF-treated mice and supernatants from their aortas after T-cell stimulation. The MMF treatment decreased aortic $\alpha\beta$ T-cell receptor$^+$ lymphocyte proliferation and cell numbers. Also, aortic contents of CD11b$^+$CD11c$^+$ cells and their proliferation were reduced in MMF-treated $Apoe^{-/-}$ mice. The IL-17 supplementation restored the number of proliferating aortic CD11b$^+$CD11c$^+$ cells in MMF-treated mice. The IL-17 receptor A was highly expressed on circulating monocytes that are macrophage progenitors. Genetic deletion of IL-17 receptor A or IL-17A reduced inflammatory peritoneal CD11b$^+$CD11c$^+$ macrophage accumulation.
Conclusions	The lymphocyte-directed immunosuppressant MMF that curbs IL-17 production was a successful antiatherosclerotic treatment. Our data delineate a role for IL-17 in CD11b$^+$CD11c$^+$ cell accumulation. (J Am Coll Cardiol 2011;57:2194–204) © 2011 by the American College of Cardiology Foundation

Inflammatory leukocyte immigration into the vessel wall, proliferation, and differentiation is central during atherosclerotic plaque formation (1), the major cause of death worldwide (2). While foam cell forming macrophages are the most prominent cells, lymphocytes are present in the atherosclerotic intima, media, and adventitia in humans (3) and mice (4), and proliferate in human atherosclerotic arteries (5). Dissection of adaptive immune components in genetic deletion models provides evidence that B cells protect against atherosclerosis development by production of antibodies directed against oxidized low-density lipoprotein (LDL) (6–8). The effect of T cells on atherosclerosis formation depends on the T-cell subtype. The T_{H1} cells and the T_{H1} marker interferon-γ promoted atherosclerotic disease in a large number of models, the role of T_{H2} cells is not clear (9,10), and anti-inflammatory regulatory T cells and their key signaling molecules limited atherosclerotic lesion formation (11,12). The role of T_{H17} cells that produce interleukin (IL)-17 (also called IL-17A [13]) in atherosclerosis is currently under investigation. Transplantation of LDL-receptor deficient ($Ldlr^{-/-}$) mice with IL-17 receptor A (IL-17ra) deficient ($Il17ra^{-/-}$) bone marrow decreased lesion size (14) as did blockade of IL-17 receptor signaling (15) or IL-17A in apolipoprotein E (Apoe) deficient

($Apoe^{-/-}$) mice (16), but IL-17 injection into $Ldlr^{-/-}$ mice decreased atherosclerotic lesion size according to another report (17).

Pharmacologic intervention during atherosclerotic lesion formation with systemic immunosuppression yielded diverse and rarely beneficial effects. Cyclosporin A at a dose that affected T-cell function, but not B cell function, enhanced atherosclerotic lesion development in cholesterol-fed rabbits (18). FTY720 reduced atherosclerosis in $Apoe^{-/-}$ mice (19) and $Ldlr^{-/-}$ mice (20) on a high-fat diet, but also increased circulating lipid levels in $Apoe^{-/-}$ mice on a chow diet (21). Mycophenolic acid (MPA) is a purine antagonist that acts by inhibition of inosine-monophosphate dehydrogenase and thereby blocks de novo generation of guanosine nucleotides required by proliferating cells, while other cells use a salvage pathway (22). MPA preferentially targets the inosine-monophosphate dehydrogenase-II isoform that is mainly expressed in activated B and T lymphocytes (22,23). Mycophenolic acid effects on vascular disease in transplanted organ allografts were favorable compared to those of calcineurin inhibitors, but also compared to 6-mercaptopurine, another purine antagonist (24–26). Beneficial effects of MPA on native vessel atherosclerosis have been proposed (27,28) but experimental data are limited to 2 short reports describing decreased intimal thickness in rabbits that were given either 30 mg/kg of the pro-drug mycophenolate mofetil (MMF) per gavage (29) or 80 mg/kg subcutaneously (30) during 12 weeks of a high-fat diet. No mechanism was investigated.

This study tested the impact of MMF, an immunosuppressant approved for use in humans that is comparatively well tolerated, on atherosclerotic lesion formation in $Apoe^{-/-}$ mice. We characterized the aortic leukocyte infiltrate and cytokine production to determine mechanisms of MMF's atheroprotective effects.

Methods

Animals. Wild-type C57Bl/6 mice, $Apoe^{-/-}$ mice (Jackson Labs, Bar Harbor, Maine), mice lacking IL-17A ($Il17a^{-/-}$ [Dr. Iwakura, Tokyo, Japan]), or IL-17-receptor-A ($Il17ra^{-/-}$ [Dr. Peschon, Amgen, Thousand Oaks, California], both 96% C57Bl/6 background, males and females) were genotyped by polymerase chain reaction and used in age- and sex-matched groups. Animal numbers for each specific analysis are given in the figure legends. Animal experiments were approved by the Animal Care Committee at La Jolla Institute for Allergy and Immunology.

Mycophenolate as the pro-drug MMF (Roche Pharma AG, Grenzach-Wyhlen, Germany) was incorporated into a high-fat diet without cholate (405 kcal from fat, 1.5% cholesterol [Research Diets, New Brunswick, New Jersey]) to reach an oral dose of 30 mg/kg body weight daily at a chow consumption of 0.1 g/g body weight. Monitored chow consumption was 2.9 ± 0.09 g (n = 33 daily measurements at a body weight of 21.7 ± 1 g [n = 8 mice]), resulting in an average consumption of 0.13 g/g daily. Addition of 10%

plasma from treated mice inhibited T-cell proliferation to 59.9 ± 8% of control $Apoe^{-/-}$ mice on a high-fat diet plasma (n = 6), representing a plasma concentration of 1.6 ± 1.4 µg/ml compared to a standard curve, as described (31).

Blood for leukocyte counts was analyzed by an automatic analyzer (Hemavet 950FS, DREW Scientific, Oxford, Connecticut). Total plasma cholesterol was analyzed using resorufin fluorescence according to the manufacturer's instructions (Cayman, Ann Arbor, Michigan). Recombinant IL-17A was from Peprotech (Rocky Hill, New Jersey).

Quantification of atherosclerosis and histologic analysis. Aortas were excised, fixed, and stained with Sudan IV (Sigma-Aldrich, St. Louis, Missouri) (counterstain fast green/hematoxylin) (32). Digital images were obtained using Moticam 1000 (Motic Instruments, Richmond, British Columbia, Canada) on an Olympus S267 dissection scope (Olympus, Center Valley, Pennsylvania). Five-millimeter sections of aortic roots starting at the aortic valve plane and covering 300 µm in 50-µm intervals were used for histologic lesion size quantification (photomicrographs taken with a 4× objective/Nikon eclipse 80i microscope, Sudan IV/hematoxylin/light-green stain). Lesion size was determined using National Institutes of Health (NIH) ImageJ software and averaged over all sections per mouse. For immunofluorescence, purified rat-anti-CD3ε (17A2, eBioscience, San Diego, California) was used with goat anti-rat-Alexa488 (Invitrogen, Carlsbad, California). Images were acquired on a Leica DM6000 upright microscope with DIC optics using a HCX PLAPO 20× oil-immersion objective at 488 nm excitation wavelength (Leica, Wetzlar, Germany). The NIH Image J software was employed to adjust brightness and for 1-step smoothing.

Enzymatic digestion of tissues and flow cytometry. After sacrifice and perfusion (phosphate-buffered saline with 20 U/ml heparin), complete thoracic and abdominal aortas were freed of all visible adventitial fat at 4× magnification and digested as described (33). The T-cell stimulation was conducted with plate-bound purified anti-CD28 and anti-CD3 (Biolegend, San Diego, California) in full RPMI medium. Flow cytometry analysis was performed on a Becton Dickinson LSR II (Franklin Lakes, New Jersey), and data were analyzed using FlowJo software (Tree Star, Ashland, Oregon). For aorta analysis, all events were acquired; 500,000 events were read for all other organs. Gating was performed for live, CD45$^+$ events constituting the total leukocyte population (leukocyte viability 35% to 60%). Antibody clone numbers are available from the authors. Aqua LIVE/DEAD fixable dead cell stain kit (Invitrogen), BD-Fix-Perm (Becton Dickinson), and APC bromodeoxyuridine (BrdU) flow kit (BD Pharmingen, San Jose,

Table 1	Characteristics of *Apoe*$^{-/-}$ Mice After 12 Weeks of a High-Fat Diet		
	Control	**MMF**	**p Value**
Body weight, g	27.6 ± 1.8 (14)	24.8 ± 1.7 (14)	0.003*
Spleen weight, g	0.13 ± 0.01 (14)	0.11 ± 0.01 (14)	0.47
Plasma cholesterol, mg/dl	543 ± 52 (6)	603 ± 61 (6)	0.16
Lymphocytes/spleen	145 ± 9.3 · 10^6 (12)	121 ± 6.5 · 10^6 (12)	0.05†
Peripheral blood lymphocytes, 10^3/ml	4.77 ± 0.48 (10)	3.44 ± 0.28 (10)	0.03†
Peripheral blood monocytes, 10^3/ml	0.45 ± 0.06 (10)	0.32 ± 0.03 (10)	0.06

Values given as mean ± SEM (n). The p value was calculated using Student *t* test. *p < 0.05. †p < 0.01.
Apoe = apolipoprotein E; MMF = mycophenolate mofetil.

California) were used according to the manufacturer's instructions. The gate for BrdU$^+$ cells was set by cells from non-BrdU–injected animals after identical preparation and antibody treatment.

Thioglycolate-induced peritonitis. One milliliter BBL fluid thioglycolate medium (Becton Dickinson, Sparks, Maryland) was injected intraperitoneally, and cells were recovered after 3 days by washing twice with 5-ml phosphate-buffered saline.

Enzyme-linked immunosorbent assay. DuoSet enzyme-linked immunosorbent assay development kit for IL-17A (R&D Systems, Minneapolis, Minnesota) and BD cytometric beads array mouse Th1/Th2/Th17 cytokine kits (BD Biosciences, Sparks, Maryland) were used according to the manufacturers' instructions.

Quantification of anti-native LDL, anti-malondialdehyde LDL, and anti-oxidized LDL antibody titers. Antibody titers were determined by chemiluminescent enzyme-linked immunosorbent assay, as described (34).

Statistical analysis. Two-tailed Student *t* test or Wilcoxon test was used to compare treated with untreated conditions for normally and non-normally distributed parameters (if indicated by d'Agostino-Pearson test), respectively. One-way analysis of variance was used if >2 conditions were compared. p values <0.05 were considered significant. Data are expressed as mean ± SEM. (All p values are indicated as p < 0.05 and p < 0.01.)

Results

MMF treatment is well tolerated and decreases aortic lesion size in *Apoe*$^{-/-}$ mice. *Apoe*$^{-/-}$ mice were maintained on a high-fat diet for 12 weeks. MMF at a typical clinical dose of 30 mg/kg daily was well tolerated, and no diarrhea, a typical side effect, occurred. Although treated mice gained slightly less weight than controls, plasma cholesterol levels were not significantly different (Table 1). The MMF treatment decreased total lymphocyte counts in spleen and peripheral blood (Table 1).

Atherosclerotic lesion size was determined by Sudan IV stain of whole aortas and aortic roots after 12 weeks on a high-fat diet. MMF significantly decreased en face atherosclerotic lesion size (Figs. 1A and 1B). Histology of the aortic roots also demonstrated a significant decrease in

lesion area (Figs. 1C and 1D). While the total lesion size was decreased, the proportions of lipid or collagen staining (Sudan IV and Picrosirius red; data not shown) were not significantly altered.

MMF decreases inflammatory cytokine concentration in plasma of atherosclerotic mice. To test the impact of MMF treatment on mediators of systemic adaptive immunity, plasma antibody and cytokine concentrations were studied after 12 weeks of a high-fat diet. Total circulating
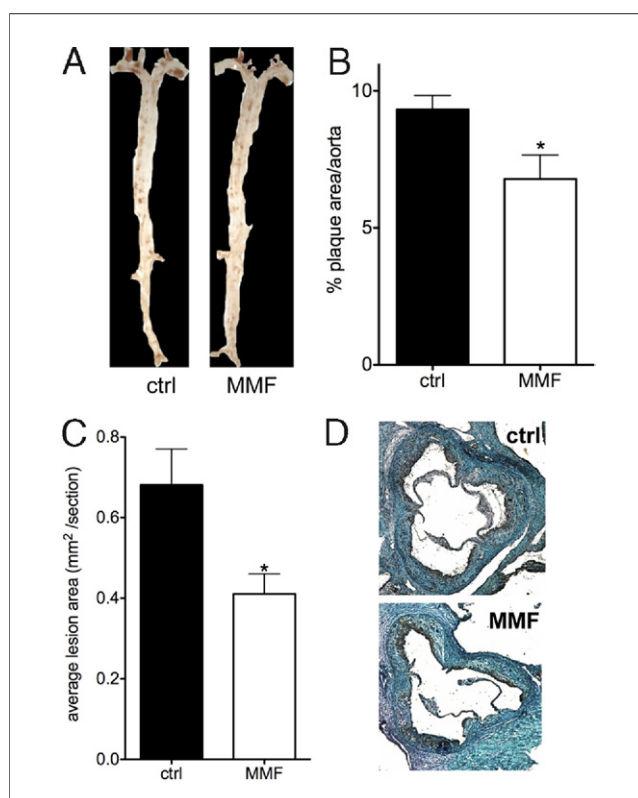

Figure 1 **MMF Reduces Aortic Atherosclerotic Lesion Size**

Seven-week-old apolipoprotein-E–deficient (*Apoe*$^{-/-}$) mice were treated with a high-fat diet for 12 weeks. **(A, B)** Aortic lesion size was assessed by Sudan IV staining percent of aortic surface area (control [ctrl], n = 5, and mycophenolate mofetil [MMF], n = 7, from 2 independent experiments). **(C)** Aortic root lesions (300 mm following the aortic valve in 50-mm intervals) were significantly reduced by MMF (n = 4). **(D)** Examples of aortic root lesions are shown (Sudan IV/light green); *p < 0.05 by Student *t* test.

immunoglobulin (Ig) M and IgG2c levels were unchanged, but IgG1 that is considered T_{H2} associated was mildly but significantly lower (Fig. 2A). The IgG1 and IgG2c directed against native LDL, oxidized LDL, and MDA-LDL were not significantly decreased by MMF treatment (Figs. 2B to 2D). The pro-inflammatory cytokine tumor necrosis factor-α and IL-6 plasma concentrations were significantly lower in MMF-treated mice (Fig. 2E). Among T-cell cytokines in plasma, the T_{H2} marker IL-4 was below detection limit in both groups (data not shown). Interferon-γ as a T_{H1} marker was not significantly reduced. Interleukin-17A as a marker of T_{H17} lineage was significantly reduced (Fig. 2E).

Aortic T-cell IL-17 production and leukocyte infiltration in atherosclerosis is decreased by MMF treatment. To address cytokine changes in the atherosclerotic vessel, we examined culture supernatants of aortic explants. Single-cell

suspensions from whole aortas were subjected to 48-h T-cell stimulation with anti-CD3 and anti-CD28. Subsequently, T-helper subtype marker cytokine secretion into the supernatant was analyzed. Figure 3A shows that aortic IL-17, but not interferon-γ or IL-4, production was significantly decreased in aortas harvested from MMF-treated animals.

We examined the effects of oral MMF treatment on aortic leukocytes in *Apoe*$^{-/-}$ mice at 3 weeks on a high-fat diet. At this time point, atherosclerotic lesions became visible as fatty streaks, mainly in the innominate artery. Splenic and circulating lymphocyte counts were very similar in MMF-treated and control mice (data not shown). Leukocyte quantification was performed by flow cytometry. Figures 3B and 3C show a significant decrease in all leukocytes and $\alpha\beta$ T-cell receptor (TCR)$^+$ cells in the aortas of MMF-treated mice. We also tested whether MMF
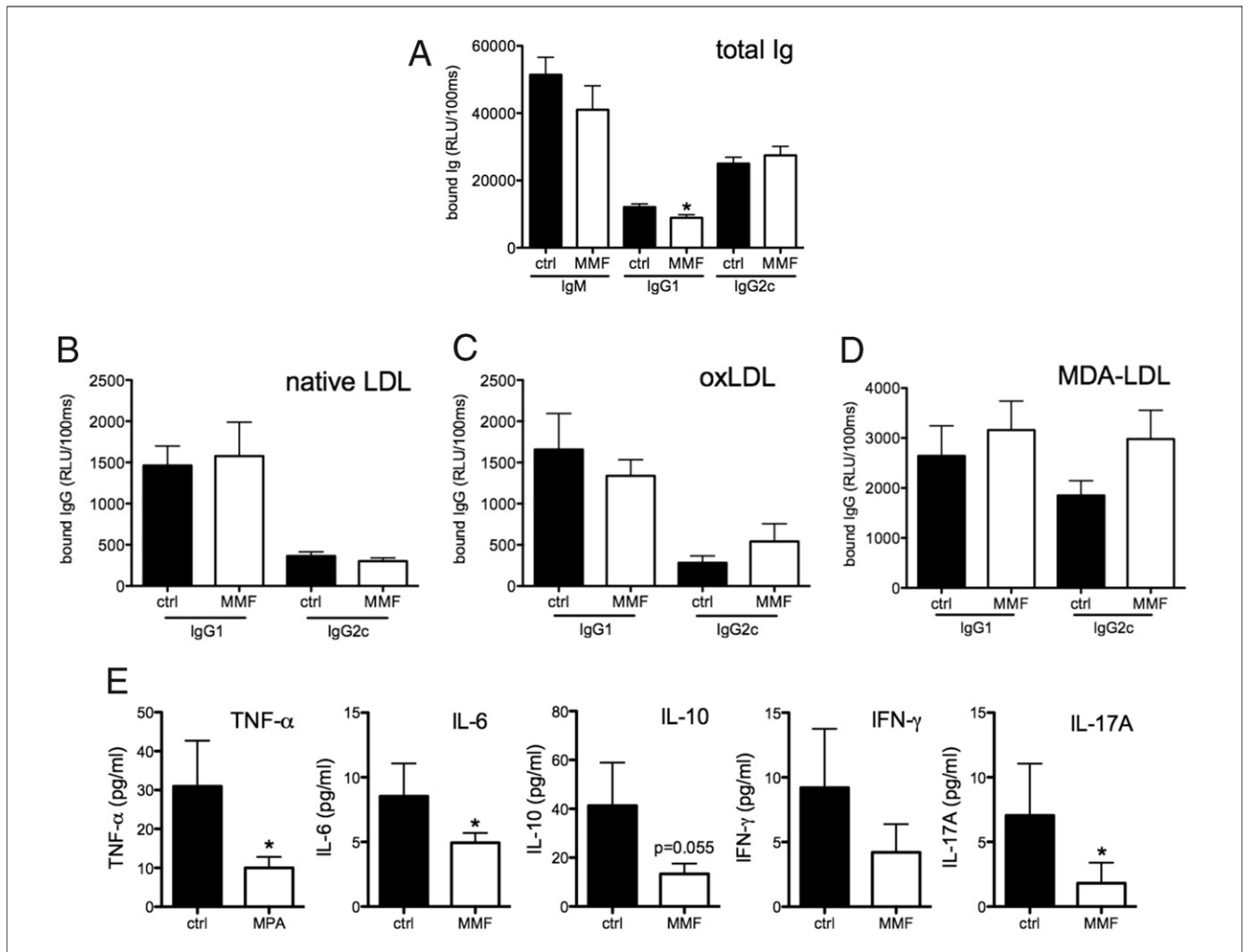

Figure 2   Plasma Ig and Cytokine Levels in Control and MMF-Treated *Apoe*$^{-/-}$ Mice

Plasma immunoglobulin (Ig) levels after 12 weeks of a high-fat diet (starting age 7 weeks) were assessed by enzyme-linked immunosorbent assay. **(A)** Total IgM and IgG2c were unchanged; IgG1 slightly decreased in MMF-treated mice. **(B to D)** No significant changes were observed in IgG1 or IgG2c directed against either low-density lipoprotein (LDL). **(E)** Plasma cytokine levels were determined by cytometric beads assay (n = 10 per group from 3 independent experiments); *p < 0.05 by Student *t* test. *Apoe*$^{-/-}$ = apolipoprotein-E–deficient; IFN = interferon; IL = interleukin; MDA = malondialdehyde; MPA = mycophenolic acid; oxLDL = oxidized low-density lipoprotein; TNF = tumor necrosis factor; other abbreviations as in Figure 1.
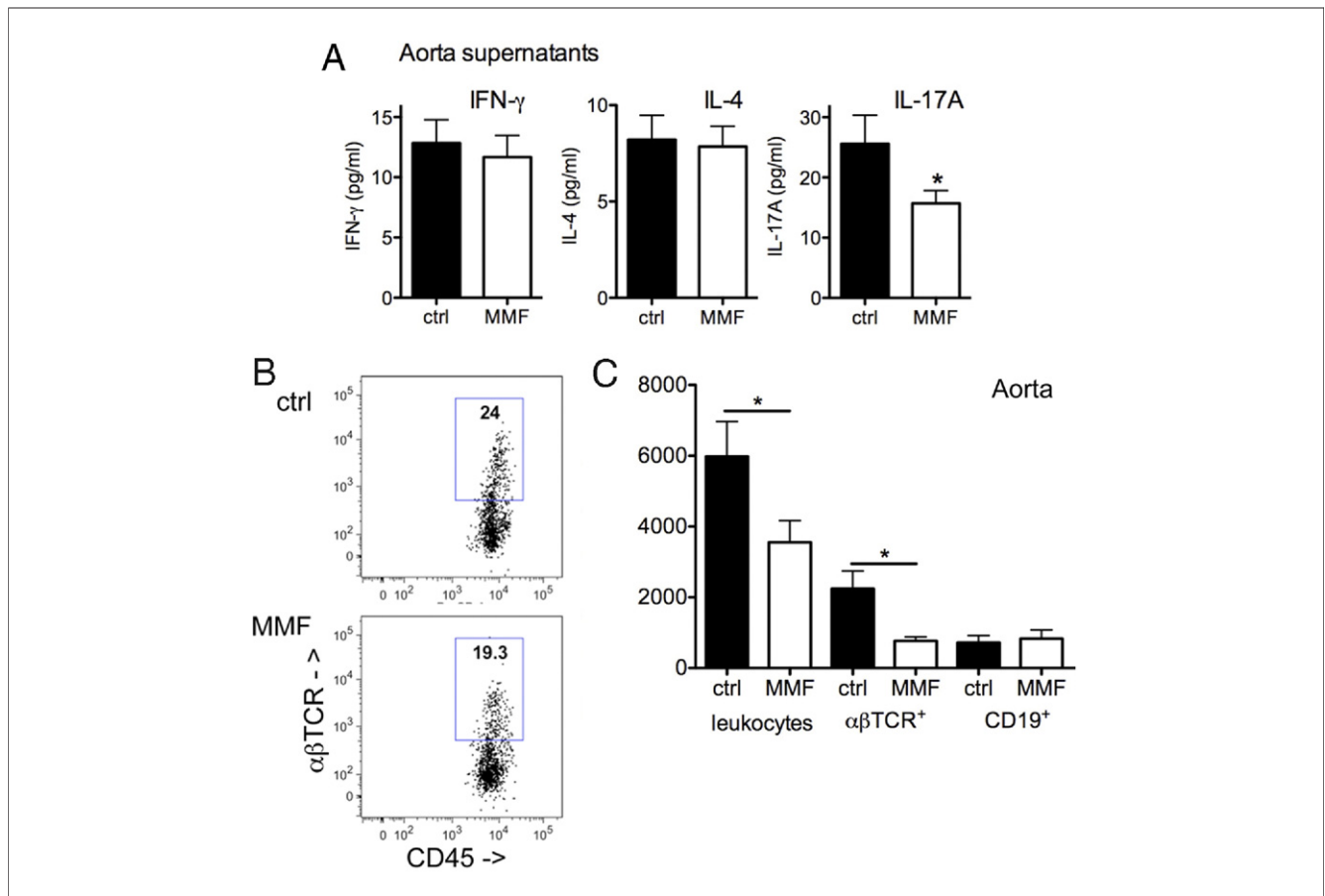
Figure 3   MMF Treatment Reduces Aortic IL-17 Production and T-Cell Accumulation in Aorta

T cells from aortas of apolipoprotein-E–deficient (*Apoe$^{-/-}$*) mice after 3 weeks of a high-fat diet with and without mycophenolate mofetil (MMF [starting age, 7 weeks]) were stimulated with anti-CD3 and anti-CD28 for 48 h. **(A)** Cytokines in the supernatants were analyzed by cytometric bead assay (control [ctrl], n = 5, and MMF, n = 6). **(B)** Aortic leukocytes were assessed by flow cytometry. **(C)** Total cell counts and $\alpha\beta$ T-cell receptor (TCR)$^+$ cells were significantly reduced by MMF treatment (control, n = 8, and MMF, n = 10, from 3 independent experiments); *p < 0.05 by Student *t* test. IFN = interferon; IL = interleukin.

treatment was effective in aged mice with pre-existing disease. Twenty-week-old to 22-week-old *Apoe$^{-/-}$* mice were given a high-fat diet with or without MMF for 3 weeks. Again, T-cell infiltration into the aorta quantified by flow cytometry was significantly smaller in the MMF treatment group (Fig. 4A). Both macroscopic aortic arch (Fig. 4B) and histologic lesions (Figs. 4C and 4D) were smaller in MMF-treated mice.

MMF treatment decreases the number of proliferating T lymphocytes in the aorta during development of atherosclerosis. Treatment with MMF inhibits lymphocyte proliferation. Aortic leukocyte proliferation is highest early in atherosclerosis development (35). Cell proliferation was assessed 24 h after intraperitoneal BrdU injection after 3 weeks of a high-fat diet with and without MMF (Fig. 5). Proliferation in $\alpha\beta$TCR$^+$ cells recovered from the aorta was markedly higher than in spleen, where <1% of $\alpha\beta$TCR$^+$ cells proliferated within 24 h (Figs. 5A and 5B). The low level of proliferation in spleen was not significantly altered by MMF at 30 mg/kg as employed in our trial. The amount of $\alpha\beta$ TCR$^+$ cells in the aorta that proliferated was significantly decreased by MMF (Figs. 5C and 5D).

Fewer aortic T cells in MMF-treated mice with established atherosclerosis after 12 weeks of a high-fat diet. Leukocytes from aortas of mice with established atherosclerosis after 12 weeks of a high-fat diet were analyzed. Similar to the early time point, both the percentage among all leukocytes and the absolute number of infiltrating $\alpha\beta$TCR$^+$ cells were significantly decreased by MMF (Figs. 6A and 6B). The proportions of CD4$^+$CD25$^+$ cells, which include regulatory T cells, in aorta and spleen were unaltered (data not shown), arguing against a major impact of MMF on this cell type in this model. Immunofluorescence was used for localization of T cells within the aortic wall. The CD3$^+$ T cells were found in clusters in the adventitia, and also in the plaque. In aortas of MMF-treated mice, the CD3$^+$ T-cell numbers were decreased and adventitial clusters dispersed (Fig. 6C).

MMF treatment alters aortic macrophages indirectly via depression of IL-17. We further investigated aortic macrophages, which are central in atherosclerosis development. Although MMF did not significantly decrease the total number of CD11b$^+$ macrophages, approximately one-half
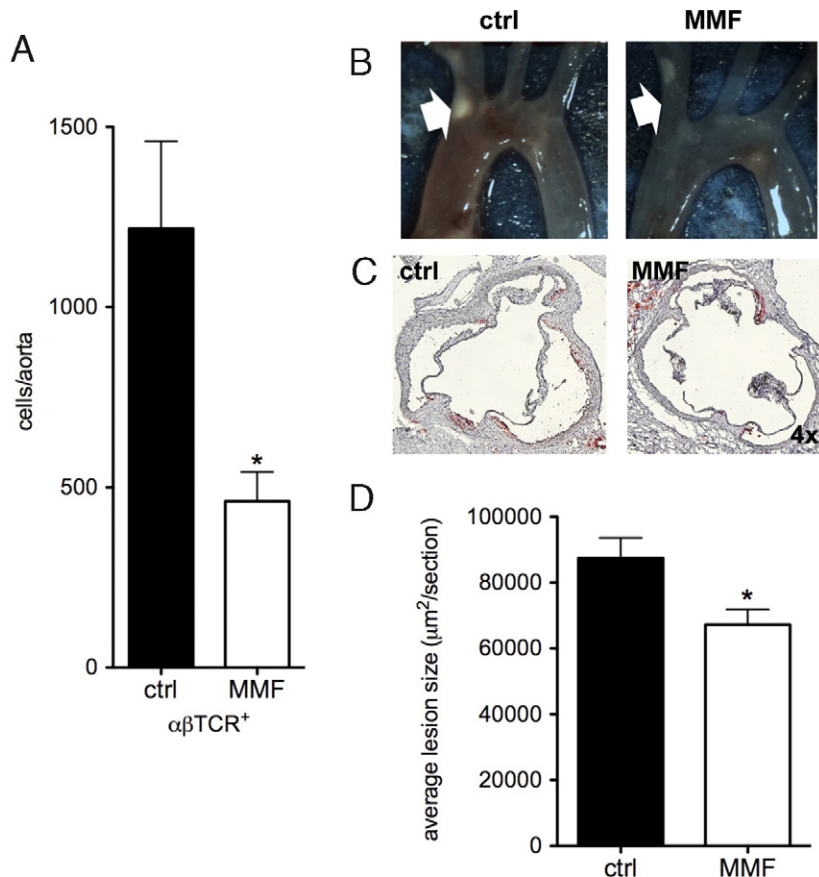
Figure 4   MMF Reduces Atherosclerotic Lesion Size and Aortic T-Cell Infiltration in Aged *Apoe*$^{-/-}$ Mice

Twenty- to 22-week-old apolipoprotein-E–deficient (*Apoe*$^{-/-}$) mice were given 3 weeks of a high-fat diet with and without mycophenolate mofetil (MMF). **(A)** Flow cytometry analysis was performed for quantification of aortic $\alpha\beta$ T-cell receptor (TCR)$^+$ cells. **(B)** Macroscopic aortic arch lesion size **(arrows)** was smaller in MMF-treated mice than in control mice (typical examples). **(C)** Histologic assessment of aortic roots revealed a significantly decreased lesion size (typical examples). **(D)** Statistical analysis (control [ctrl], n = 4, and MMF, n = 5, from 2 independent experiments); *p < 0.05 by Student *t* test.

of aortic CD11b$^+$CD45$^+$ leukocytes also expressed the b2 integrin subunit CD11c after 12 weeks of a high-fat diet in control *Apoe*$^{-/-}$ mice. In MMF-treated animals, there were fewer CD11b$^+$CD11c$^+$ than CD11b$^+$CD11c$^-$ cells among all CD11b$^+$ cells (Fig. 7A). The mean fluorescence intensity reflecting the number of CD11b and CD11c molecules on the surface of each individual cell in the aortic CD11b$^+$CD11c$^+$ population remained unaffected by MMF treatment (data not shown), suggesting that the observed effect was a decrease in a cell population rather than relative integrin surface expression. The CD11c is expressed on CD11b$^+$ cells in atherosclerotic plaques and up-regulated by oxidized LDL (35,36). A CD11c deficiency decreased atherosclerotic lesion size (37). We assessed proliferation of aortic CD11b$^+$CD11c$^+$ cells after 3 weeks of a high-fat diet. It was significantly decreased by treatment with MMF (Fig. 7B).

To test whether the decreased proportion of CD11b$^+$CD11c$^+$ macrophages in MMF-treated mice aortas was a direct or a downstream effect due to decreased IL-17 production,

Apoe$^{-/-}$ mice were injected with either recombinant IL-17 or phosphate-buffered saline control on days 5, 10, and 15 of a 3-week course of a high-fat diet and MMF. That completely restored CD11b$^+$CD11c$^+$ proliferation in the aorta (Fig. 7C). At this point, atherosclerotic lesions were minimal in the aortic root (data not shown), but macroscopically visible in the branching innominate artery. These were reduced in MMF-treated mice, and this was reverted by IL-17 treatment (Fig. 7D). Indeed, IL-17ra was highly expressed on circulating CD11b$^+$CD115$^+$ monocytes (Fig. 7E) that are precursors of aortic macrophages (35,36). To address whether IL-17 had a role in inflammatory macrophage accumulation in circumstances other than MMF treatment, we employed IL-17A (*Il17a*$^{-/-}$)-deficient mice and *Il17ra*$^{-/-}$ mice. Baseline circulating monocyte counts were not significantly different from wild-type mice (data not shown). Thioglycolate-induced peritoneal macrophages were harvested after 3 days. Fewer CD11b$^+$CD11c$^+$ cells were recovered from the peritoneal cavity of both *Il17ra*$^{-/-}$ and *Il17a*$^{-/-}$ mice than from wild-type controls (Fig. 7F).
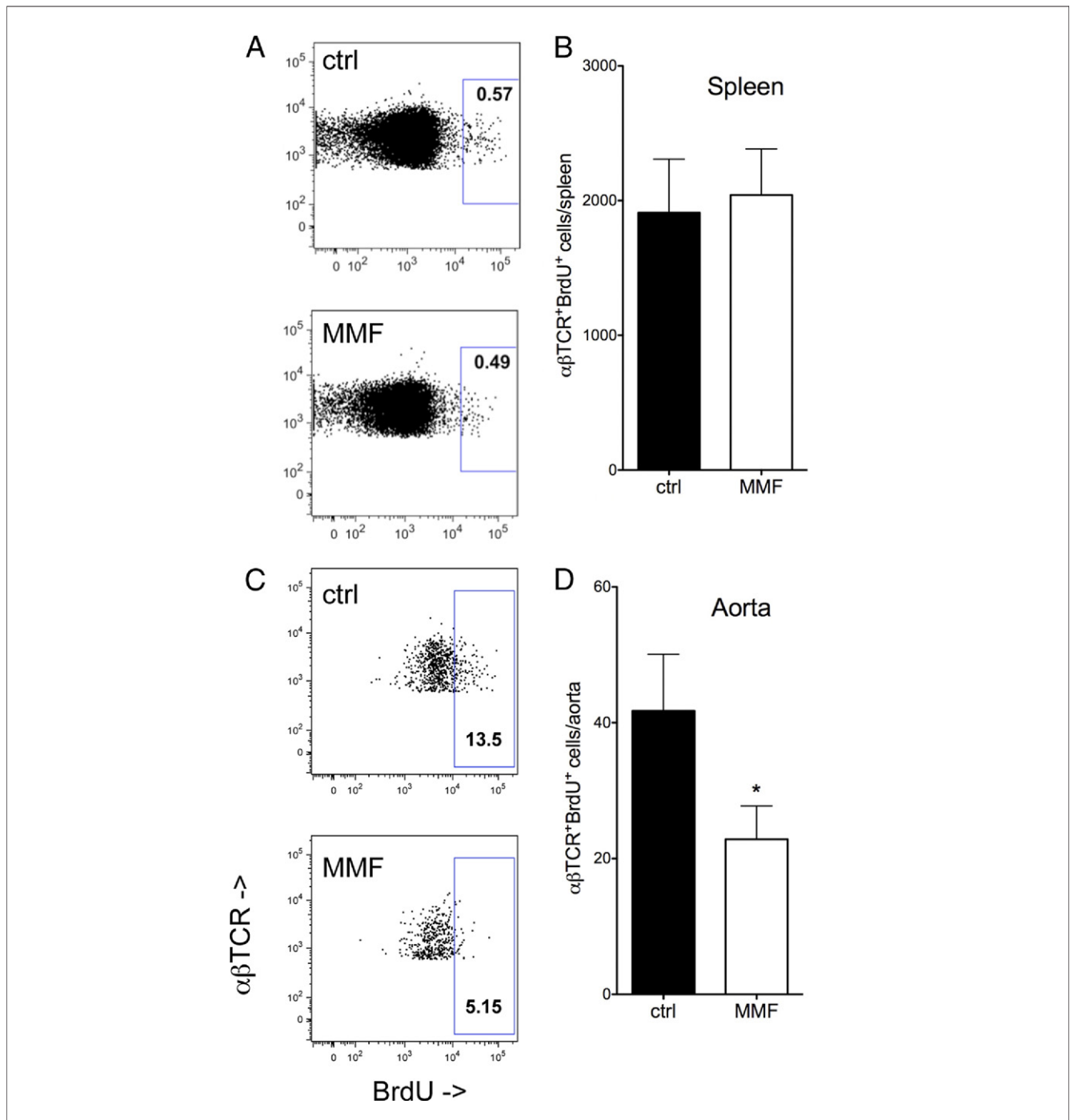
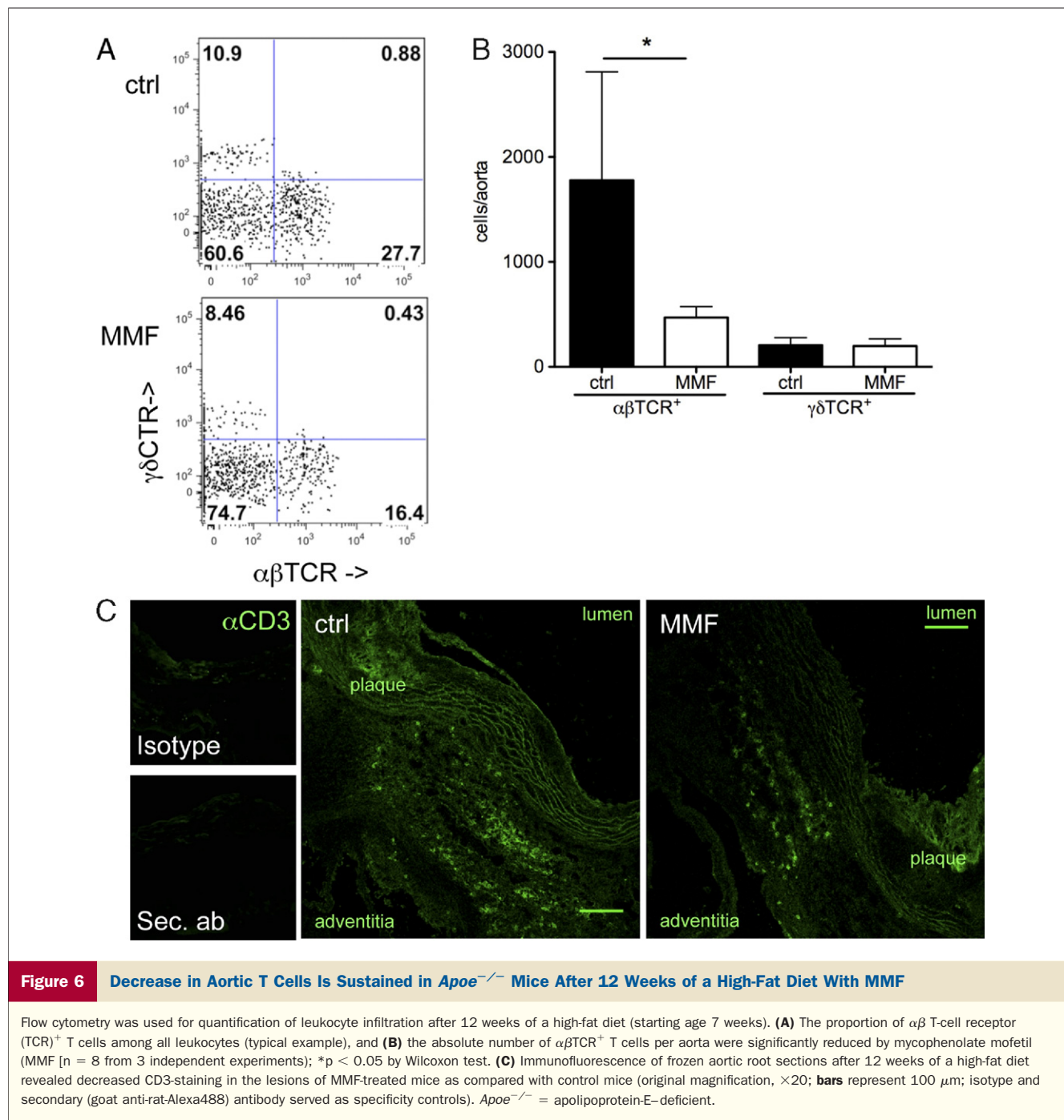**Figure 5   MMF Treatment Reduces Aortic Lymphocyte Proliferation During Development of Atherosclerosis**

After 3 weeks of a high-fat diet (starting age, 7 weeks) with and without mycophenolate mofetil (MMF), apolipoprotein-E–deficient ($Apoe^{-/-}$) mice were injected with bromodeoxyuridine (BrdU) 24 h before sacrifice. The BrdU incorporation was detected by subsequent intracellular staining of single cell suspensions from **(A, B)** spleen and **(C, D)** aorta. Gates were set by identically prepared cells from the same organ of mice not given BrdU. **(D)** Aortic T-cell proliferation was significantly decreased by MMF treatment (control [ctrl], n = 8, and MMF, n = 10, from 3 independent experiments); *p < 0.05 by Student *t* test. TCR = T-cell receptor.

Discussion

Targeting lymphocyte proliferation by inhibition of inosine-monophosphate dehydrogenase by MMF reduced atherosclerotic lesion formation in $Apoe^{-/-}$ mice. This was achieved both during 12 weeks of a high-fat diet in young or during 3 weeks in aged mice.

MMF effects on B cells and T cells and T-cell cytokine production during development of atherosclerosis. The MMF targets proliferating lymphocytes (22). We observed

Figure 6 Decrease in Aortic T Cells Is Sustained in *Apoe*$^{-/-}$ Mice After 12 Weeks of a High-Fat Diet With MMF

Flow cytometry was used for quantification of leukocyte infiltration after 12 weeks of a high-fat diet (starting age 7 weeks). **(A)** The proportion of $\alpha\beta$ T-cell receptor (TCR)$^+$ T cells among all leukocytes (typical example), and **(B)** the absolute number of $\alpha\beta$TCR$^+$ T cells per aorta were significantly reduced by mycophenolate mofetil (MMF [n = 8 from 3 independent experiments]; *p < 0.05 by Wilcoxon test. **(C)** Immunofluorescence of frozen aortic root sections after 12 weeks of a high-fat diet revealed decreased CD3-staining in the lesions of MMF-treated mice as compared with control mice (original magnification, ×20; **bars** represent 100 μm; isotype and secondary (goat anti-rat-Alexa488) antibody served as specificity controls). *Apoe*$^{-/-}$ = apolipoprotein-E–deficient.

no significant change in IgG, which is considered to mediate atheroprotection by B cells (6–8). Concerning T lymphocytes, MMF treatment predominantly reduced aortic $\alpha\beta$TCR$^+$ cell accumulation. The T-cell numbers were reduced in both plaque and adventitia. The proportion of proliferating T cells was much higher in aortas with nascent atherosclerotic lesions than in the spleen of the same animal. Aortic T-cell proliferation was preferentially affected by MMF. Subsequently, after both 3 and 12 weeks of a high-fat diet, MMF treatment had a stronger effect on aortic lymphocyte populations than in spleen or peripheral

blood. A very recent report suggests that similar effects can be observed in humans: T lymphocytes in carotid endarterectomy specimens from patients treated with MMF for 2 weeks before surgery were decreased (38).

Certain T-cell populations, especially T$_{H1}$ and possibly T$_{H17}$ cells, have a pro-atherogenic role (1,11,39). Inhibition of T-cell proliferation during an inflammatory event such as atherosclerotic lesion formation can reduce T-cell cytokines by decreasing the number of cytokine producers. Indeed, systemic and aortic IL-17 cytokine levels were lower in MMF-treated mice than in control mice. Our present
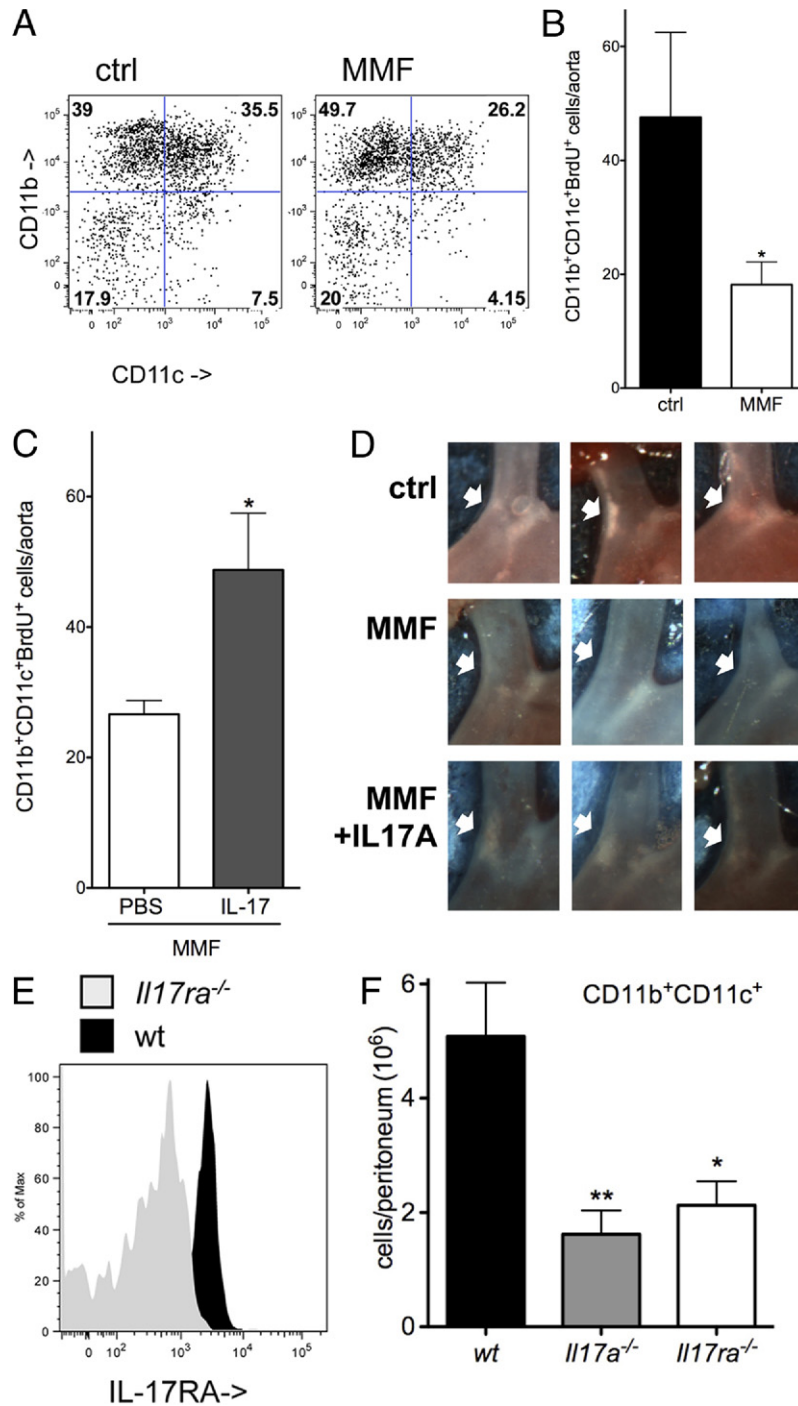
Figure 7   **MMF-Mediated Decrease of Aortic CD11b$^+$CD11c$^+$ Macrophage Proliferation Is Rescued by IL-17, and IL-17 Promotes CD11b$^+$CD11c$^+$ Cell Accumulation In Vivo**

(A) After 12 weeks of a high-fat diet, CD11c$^+$ among CD11b$^+$ leukocytes were reduced in aortas of mycophenolate mofetil (MMF)-treated mice compared with control apolipoprotein-E–deficient (*Apoe*$^{-/-}$) mice. **(B)** Aortic CD11b$^+$CD11c$^+$ cell proliferation measured by bromodeoxyuridine (BrdU) incorporation was reduced after 3 weeks of a high-fat diet (control, n = 8, and MMF, n = 10, from 3 independent experiments, starting age 7 weeks); *p < 0.05 by Student *t* test. To test whether the decrease in CD11b$^+$CD11c$^+$ cell proliferation was due to inhibition of interleukin (IL)-17 production by MMF, recombinant IL-17 (1 mg/mouse) or phosphate-buffered saline (PBS) control was injected intraperitoneally on days 5, 10, and 15 of a high-fat diet with MMF. **(C)** Aortic CD11b$^+$CD11c$^+$ cell proliferation is depicted (control, n = 9, and MMF, n = 10, from 2 independent experiments, starting age 7 weeks); *p < 0.05 by Student *t* test. **(D)** Innominate artery lesions **(arrows)** at 3 weeks of Western diet were reduced by MMF but restored in IL-17–substituted MMF-treated *Apoe*$^{-/-}$ mice (typical examples). **(E)** Interleukin-17–receptor expression on peripheral blood CD11b$^+$CD115$^+$ monocytes was analyzed by flow cytometry; monocytes from a *Il17ra*$^{-/-}$ mice served as control. Peritoneal macrophages from wild-type (wt) mice, *Il17a*$^{-/-}$ mice, and *Il17ra*$^{-/-}$ mice were harvested on day 3 after thioglycolate injection. **Gray area** indicates *Il17ra*$^{-/-}$; **black area** indicates wild type. **(F)** The number of CD11b$^+$CD11c$^+$ macrophages per peritoneal cavity was lower in *Il17a*$^{-/-}$ mice and *Il17ra*$^{-/-}$ mice than in wild-type mice (n = 4, from independent experiments, Dunnett's test after 1-way analysis of variance); *p < 0.05; **p < 0.01. Apoe = apolipoprotein E; IL-17ra = interleukin-17 receptor A.

experiments do not explore why IL-17–producing T cells were preferentially affected by MMF treatment. However, from the mechanism of action of MMF, it is conceivable that these cells were most affected because they or their progenitors were proliferating most actively, making them most vulnerable to guanosine nucleotide depletion, similar to the setting of bone marrow regeneration, as we recently explored (31).

The role of IL-17 in atherosclerosis development. Most, although not all, current literature suggests a pro-atherogenic role of IL-17 (14–17,40) by a yet undefined mechanism. Our data do not exclude that additional MMF effects beyond IL-17 suppression have contributed to its beneficial effects. However, we investigated whether IL-17 was involved in the effects of MMF-mediated T-cell suppression on atherosclerosis by substituting IL-17 into MMF-treated mice. This reverted the MMF effect on nascent lesion formation and on a cellular level on aortic $CD11b^+CD11c^+$ macrophages. These cells are expanded in atherosclerosis (35,36), present antigens to $CD4^+$ T cells, and phagocytose lipids (39,40). The IL-17 receptor was highly expressed on circulating monocytes that are progenitors of aortic macrophages. We tested for a direct link between IL-17 and $CD11b^+CD11c^+$ cells in acute peritoneal inflammation in mice lacking IL-17A or IL-17ra. The number of $CD11b^+CD11c^+$ cells was reduced in both genotypes. Our data suggest IL-17 as a mediator of $CD11b^+CD11c^+$ cell accumulation. Also, aortic $CD11c^+$ number was lower in $Il17ra^{-/-}$ mice on a high-fat diet compared with wild-type controls (unpublished observation), suggesting relevance in vascular inflammation. The IL-17 increased monocyte adhesion to the atherosclerotic aortic wall ex vivo (15), but it remains to be determined at which step IL-17 promotes $CD11b^+CD11c^+$ cells. The MMF-induced decrease in $CD11b^+CD11c^+$ cells may have contributed to systemically lower tumor necrosis factor-α and IL-6 inflammatory cytokine levels (Fig. 2) and decreased numbers of neutrophils in early atherosclerotic aortas (data not shown). The IL-6 can again induce T-cell IL-17 production (41), a pro-inflammatory loop that was tempered in MMF-treated mice.

Immunosuppression in atherosclerosis management. Systemic inhibition of T-cell immunity in native vessel atherosclerosis with other agents was pro-atherogenic for cyclosporine (18) or only partially successful for FTY720 (19–21). Our finding that MMF conferred protection not only to young mice, but also to aged mice is promising. Further studies are necessary to delineate whether, in addition to inhibiting new plaque formation, MMF also decreased pre-existing disease in aged mice. Systemic immunosuppression with any of the currently available agents, including MMF, despite a relatively favorable side effects profile (22,42), confers major risks to patients. These preclude their chronic use for atherosclerosis as the sole indication; however, beneficial vascular side effects might influence the choice of immunosuppressant prescribed for other conditions in patients with high cardiovascular risk. Also, our finding that the alteration in $CD11b^+CD11c^+$ cells in aortas of MMF-treated atherosclerotic mice depended on IL-17 suggests IL-17 as a mediator, and possibly a more specific target, for immunosuppressive therapy of atherosclerosis.

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