Cytokine Therapy With Interleukin-2/Anti–Interleukin-2 Monoclonal Antibody Complexes Expands CD4+CD25+Foxp3+ Regulatory T Cells and Attenuates Development and Progression of Atherosclerosis

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Background—CD4+CD25+Foxp3+ regulatory T cells (Tregs) attenuate atherosclerosis, but their therapeutic application by adoptive transfer is limited by the need for their expansion in vitro and limited purity. Recently, an interleukin (IL)-2/anti–IL-2 neutralizing monoclonal antibody (IL-2/anti–IL-2 mAb) complex has been shown to expand these Tregs. We examined the capacity of a modified IL-2/anti–IL-2 mAb treatment to expand Tregs and inhibit both the progression and development of atherosclerosis.

Methods and Results—Six-week old apolipoprotein E–deficient mice fed a high-fat diet for 8 weeks were administered IL-2/anti–IL-2 mAb commencing 2 weeks after starting the diet. Tregs in the spleen, lymph node, and liver were selectively expanded without affecting CD4+, CD8+, or natural killer cells. Tregs were increased in lesions and lesion size reduced. CD4+ T-cells, macrophages, mature dendritic cells, proliferating cell nuclear antigen+ cells, and monocyte chemoattractant protein-1 and vascular cell adhesion molecule-1 were reduced. In anti-CD3–stimulated splenocytes, proliferation and secretion of Th1, Th2, and Th17 (IL-17) cytokines and IL-1β were reduced. To determine whether treatment attenuated progression of atherosclerosis, 6-week-old apolipoprotein E–deficient mice were fed a high-fat diet for 6 weeks, followed by IL-2/anti–IL-2 mAb treatment for 6 weeks while continuing the high-fat diet. Treatment also increased Tregs without affecting CD4+, CD8+, or natural killer cells, suppressed inflammation, and greatly attenuated progression of atherosclerosis.

Conclusions—IL-2/anti–IL-2 mAb treatment in vivo attenuates atherosclerosis via selective Tregs expansion. The findings suggest that cytokine-based IL-2/anti–IL-2 mAb complex therapy could represent an attractive approach for treating atherosclerosis, because it markedly attenuates progression as well as development, by modulating its immunoinflammatory component. (Circulation. 2012;126:1256-1266.)

Key Words: atherosclerosis ■ cytokines ■ inflammation

Atherosclerosis is a multifactorial vascular disease involving interactions between cholesterol-rich lipoproteins, endothelial and smooth muscle cells, macrophages, dendritic cells, and lymphocytes.1 It leads to severe clinical events caused by plaque rupture and thrombotic occlusion of arteries and is the main cause of acute coronary syndrome and stroke. Studies of atherosclerotic lesions indicate that CD4+ lymphocytes are present in human and mouse lesions early in the development of disease,2,3 and adoptive transfer of these cells is proatherogenic.4 CD4+ T-cells produce a variety of cytokines including interferon-γ, tumor necrosis factor-α, interleukin (IL)-1, and IL-10, which can influence the development and characteristics of atherosclerotic plaques.

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More recently, other CD4+ T cell subtypes have been identified in lesions, including Th17 cells4 and CD4+CD25+ Foxp3+ regulatory T cells (Tregs).5 Deletion of Tregs accelerates development of atherosclerosis,6 and their adoptive transfer suppresses atherosclerosis.7 Although the mechanisms by which they suppress atherosclerosis are yet to be defined, these cells produce a number of immunosuppressive

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Figure 1. Expansion of CD4+CD25+Foxp3+ Tregs in lymphoid organs after IL-2/anti–IL-2 mAb treatment and dose-dependent suppression of CD4+ T cells. A, Representative results from individual mice of CD25 and Foxp3 expression in spleen, liver, and inguinal lymph nodes assessed by flow cytometry, 7 and 14 days after commencing treatment with IL-2/JES6–1 mAb complexes or vehicle showing expansion of Tregs; for day 7, mice were treated with complex or vehicle from day 1 to day 5 and examined on day 7, whereas for day 14, mice were treated from day 1 to 5 and then days 10 to 12 and examined on day 14. FACS plots represent the percentage of CD25+ and Foxp3+ cells within the CD4+ population. B, CD4+CD25+Foxp3+ cells within the CD4+ population in spleen, liver, and inguinal lymph nodes. C, Spleen cells x10⁶ for CD4+CD25+Foxp3+ cells. D, LAG-3, ICOS, and CD62L expression in CD4+CD25+Foxp3+ cells. E, CFSE dilution in CD4+ and CD8+ T cells after IL-2/JES6–1 mAb treatment.
molecules, including transforming growth factor (TGF)-β, and IL-10. In vitro, Tregs inhibit the proinflammatory effects of macrophages by steering macrophage differentiation toward an anti-inflammatory phenotype and reducing lipid accumulation.

Because of their ability to attenuate atherosclerosis, it has been suggested that Tregs may be suitable for treating atherosclerosis. Adoptive transfer of naturally occurring Tregs as well as antigen-specific Tregs attenuate development of atherosclerosis. Also, immunization with an apolipoprotein B-100 fusion protein has been associated with increases in antigen-specific regulatory T cells and reductions in atherosclerosis. Despite such studies, therapy using anti-CD3 treatment of mice induces CD4+CD25+ Tregs and attenuates development of atherosclerosis but not progression of already developed atherosclerosis. Other approaches, including inducing tolerance to antigens such as oxidized low-density lipoprotein, also appear restricted to preventing development rather than progression of developed disease. Given these limitations, an alternative approach to suppress the immunoinflammatory component of atherosclerosis might be to expand Tregs in vivo using specific cytokine therapy.

IL-2 is a cytokine mainly produced by activated T cells and is a potent growth factor for immune cells, including T cells and natural killer (NK) cells. IL-2 has 2 major clinical uses: as antitumor therapy for renal carcinoma and melanoma and for treating HIV disease. The magnitude and duration of effects of such cytokines can be markedly improved by complexing with antibodies, and more recently selective targeting of immune cells has also been achieved. By injecting low doses of IL-2 complexed with the anti–IL-2 neutralizing monoclonal antibody (mAb) JES6–1, Webster recently reported selective expansion of Tregs. The increase in Tregs is specific to this IL-2/anti–IL-2 mAb complex as recently reported selective expansion of Tregs. The increase in Tregs is specific to this IL-2/anti–IL-2 mAb complex as complexing IL-2 with other anti–IL-2 neutralizing antibody clones (eg, S4B6) drives expansion of CD8+ T cells and NK cells. Short-term treatment with IL-2/anti–IL-2 mAb (JES6–1) complex increases Tregs up to 6-fold.

Here we investigated whether treatment with IL-2/anti–IL-2 mAb (JES6–1) complex inhibits atherosclerosis development and progression of developed atherosclerosis in apolipoprotein E–deficient (ApoE–/–) mice. We modified earlier short-term treatment protocols making the treatment suitable for inducing long-term elevations in Tregs. We demonstrate that IL-2/anti–IL-2 mAb (JES6–1) complex therapy induces sustained increases in Tregs, attenuates immunoinflammatory processes that promote atherosclerosis, thereby inhibiting progression of already developed atherosclerosis as well as development of atherosclerosis.

Methods

Animals and Experimental Protocol
Six-week-old male ApoE–/– mice were commenced on a high-fat diet, then 2 weeks later they were administered a recombinant mouse (rm) IL-2/anti–IL-2 mAb (JES6–1) complex (1 μg rmIL-2 plus 5 μg anti–IL-2 mAb). During the first week of treatment the mice received 5 consecutive daily doses i.p., after which they received 3 doses weekly for the duration of the study. Control mice received PBS or 5 μg anti–IL-2 mAb. Mice were euthanized at 14 weeks of age and atherosclerotic lesions assessed. To evaluate the effects of rmIL-2/anti–IL-2 mAb complex treatment on progression of atherosclerosis, 6-week-old ApoE–/– mice were fed a high-fat diet for 6 weeks, then the mice were treated with the cytokine–antibody complex as above for 6 weeks while continuing the high-fat diet.

An expanded Materials and Methods section is available in the online-only Data Supplement.

Statistical Analyses
Results are expressed as mean±SEM. Comparisons between groups were carried out using Student t test or Mann–Whitney U test, depending on whether the data were normally distributed, as assessed by using the Kolmogorov–Smirnov test. For multiple comparisons, results were analyzed using 2-way (2-factor) ANOVA (after confirming normality of distribution using the Kolmogorov–Smirnov test), followed by Bonferroni post test. A value of P<0.05 was considered as statistically significant.

Results

Characteristics of CD4+CD25+Foxp3+ Regulatory Cells Expanded In Vivo by IL-2/Anti-IL-2 mAb Complex Treatment
Previous studies on Treg expansion after IL-2/anti–IL-2 mAb (JES6–1) complex injection have been limited to 3 days. Consequently, we designed a dosage regimen that could be used for stimulating prolonged increases in Tregs numbers during development of atherosclerosis. We administered a total dose of 6 μg (5 μg anti–IL-2 mAb [JES6–1] plus 1 μg rmIL-2) per i.p. injection on 5 consecutive days, measured Tregs on day 7, then administered the IL-2/anti–IL-2 mAb complex on days 9 to 11 and again measured Tregs on day 14. Tregs within the CD4+ T cell population increased markedly in spleen, liver, and inguinal lymph nodes after 5 days of treatment, 4-fold in the spleen (P=0.0032) and liver (P=0.0394) and 3-fold in the inguinal lymph nodes (P=0.0129; Figure 1A and 1B); total Treg cell numbers were also increased (P=0.0057; Figure 1C). Fourteen days after commencing the treatment, Tregs remained elevated (P=0.0002, P=0.0043, and

Figure 1 (Continued). Liver, and inguinal lymph nodes of ApoE–/– mice 7 and 14 days after commencing treatment (see A) with vehicle (control) or IL-2/JES6–1 mAb complexes; results are means±SEM of each treatment group, n=7 each group. C, Total CD4+CD25+Foxp3+ T cells, CD4+ T cells, and CD8+ T cells in spleens of ApoE–/– mice 7 and 14 days after commencing treatment (see A) with vehicle (control) or IL-2/JES6–1 mAb complexes; results are means±SEM of each treatment group, n=7 each group. D, LAG-3, ICOS, and CD62L expressing Tregs as % of total CD4+CD25+Foxp3+ T cells in the spleens of ApoE–/– mice treated with vehicle (control) or IL-2/JES6–1 mAb; n=3 each group. E, Dose-dependent suppression of stimulated spleen CFSE+CD4+ T cell proliferation by Tregs isolated from ApoE–/– treated with IL-2/JES6–1 mAb complexes or vehicle; ratios represent CFSE+CD4+ T cells; Tregs. Results are representative of two similar experiments. *P<0.05 from control. Treg indicates regulatory T cell; IL, interleukin; mAb, monoclonal antibody; LAG, lymphocyte-activating gene-3; and ICOS, inducible costimulator.
In the spleen, the liver, and the inguinal lymph nodes, respectively, although those in the liver were reduced in number compared with day 7 (Figure 1A through 1C). Because IL-2 is essential for Tregs homeostasis, playing a role in their maintenance and peripheral expansion,20 we examined whether sustained elevated levels of IL-2 in plasma could contribute to the increase in Tregs. Administration of IL-2 alone (5 μg) resulted in plasma concentrations of 171 ± 58 and 16 ± 9 pg/mL (n = 3) 6 and 24 hours later; in contrast, the same dose of IL-2 complexed with the antibody resulted in plasma concentrations in excess of 1000 pg/mL (n = 3), both 6 and 24 hours later (P < 0.0001; ie, up to 60-fold increases compared with IL-2 alone). Consequently we used the IL-2/anti-IL-2 mAb protocol to increase and maintain elevated Tregs numbers during the course of our atherosclerosis studies, administering doses of IL-2/anti-IL-2 mAb complex 3 times weekly (see below). To determine whether the IL-2/anti-IL-2 mAb treatment altered the phenotypic characteristics of the Tregs we compared the proportion of Tregs that expressed lymphocyte-activating gene-3 (LAG-3), inducible costimulator (ICOS), and L-selection (CD62L). LAG-3 is a CD4-related molecule that binds major histocompatibility complex class II, is expressed on activated Tregs,21 and

Figure 2. Selective expansion of CD4+CD25+Foxp3+ T cells in spleens and their increased numbers in atherosclerotic lesions of ApoE−/− mice fed a high-fat diet and treated for 6 weeks with IL-2/anti-IL-2 mAb complex. A, Expanded Tregs as % of the spleen CD4+ T cell population in ApoE−/− mice treated with vehicle or IL-2/anti-IL-2 mAb complex; representative FACS profiles (left) and means (bar graphs, right); n = 10 each group. B, Bar graphs showing unchanged mean numbers of CD4+, CD8+, and NK cells in spleens of ApoE−/− mice treated with vehicle (control) and IL-2/anti-IL-2 mAb complexes for 6 weeks; n = 10 each group. C, Immunohistochemical staining demonstrating Foxp3+ cells (arrows) in lesions of vehicle (Control; top left) and IL-2/anti-IL-2 mAb complex (IL-2/JES6-1; right) treated mice (top middle); top right panel represents immunostaining with a control IgG. Bar graphs show the increases in Foxp3+ cells (n = 7–9) and Foxp3 mRNA (n = 5) in lesions of IL-2/anti-IL-2 mAb–treated mice. *P < 0.05; Scale bar = 50 μm. Treg indicates regulatory T cell; IL, interleukin; mAb, monoclonal antibody; ApoE−/−, apolipoprotein E–deficient; FACS, fluorescence-activated cell sorting; and NK, natural killer cells.
ICOS is a T cell receptor-inducible receptor that enhances T cell responses; ICOS+ Tregs use interleukin-10 to suppress dendritic cell function and TGF-β to suppress T cell function, whereas ICOS-Tregs use only TGF-β.21,22 CD62L is an important T-cell homing receptor.23 The expression of LAG-3, ICOS, and CD62L was unaltered on the IL-2/anti–IL-2 mAb complex expanded Tregs population (P > 0.05; Figure 1D). Tregs expanded using the IL-2/anti–IL-2 mAb complex effectively attenuated CD4+ T cell proliferation in vitro (Figure 1E).

IL-2/anti–IL-2 mAb Complexes Initiate Sustained Selective Expansion of Tregs and Increase Tregs in Atherosclerotic Lesions

After 6 weeks of IL-2/anti–IL-2 mAb complex therapy, Tregs were selectively elevated 2.2-fold in the spleen (P = 0.0001; Figure 2A), whereas CD4+ T cells, CD8+ T cells, and NK cell numbers were unaffected (P > 0.05; Figure 2B). Tregs were also elevated in the liver (0.67 ± 0.15 × 10^5 [n = 6] versus 0.27 ± 0.08 × 10^5 [n = 4; control], P = 0.0480) and inguinal lymph nodes (0.97 ± 0.14 × 10^5 [n = 6] versus 0.50 ± 0.10 × 10^5 [n = 4; control], P = 0.0401). In lesions the Tregs were elevated 4.3-fold (P = 0.0003), and mRNA encoding Foxp3 nearly 3-fold (P = 0.0349; Figure 2C).

IL-2/anti–IL-2 mAb Therapy Inhibits Atherosclerotic Plaque Development and Reduces Plaque Inflammatory Cells

To determine whether cytokine therapy with IL-2/anti–IL-2 mAb complexes attenuates atherosclerosis, we treated 6-week-old ApoE−/− mice with IL-2/anti–IL-2 mAb complexes while they were fed a high-fat diet for 8 weeks, commencing treatment 2 weeks after starting the high-fat diet. No adverse effects frequently associated with high dose IL-2 treatment were observed and body weights and plasma lipid profiles were unaffected (Table); plasma aspartate aminotransferase and alanine transaminase were also unaffected (not shown). Mice treated with the IL-2/anti–IL-2 mAb complex exhibited ≈50% reduction in aortic sinus atherosclerotic lesion size compared with control mice (mean aortic sinus plaque area 88 889 ± 10 755 μm^2 versus 172 375 ± 16 410 μm^2 in IL-2/anti–IL-2 mAb complex–treated mice and control mice, respectively, P = 0.0060; Figure 3A). To confirm that this was a result of the IL-2/anti–IL-2 mAb complex rather than the anti–IL-2 mAb, the same dose of anti–IL-2 mAb without IL-2 was administered; an additional control group received vehicle. In the anti–IL-2 mAb–treated mice lesion size was similar to control, averaging 158 950 ± 19 200 μm^2 (anti–IL-2 mAb group) and 169 800 ± 20 400 μm^2 in the control group (n = 6; P > 0.05). To determine whether treatment affected immune cells in the lesions, we performed immunohistochemistry. IL-2/anti–IL-2 mAb complex treatment reduced CD4+ T cell numbers by 73% (P = 0.0108; Figure 3C), macrophage accumulation by 65% (P = 0.0061; Figure 3B), and cell proliferation (proliferating cell nuclear antigen) by 30% (P = 0.0320; Figure 3D). CD83, a marker of mature dendritic cells, which is required for T cell activation,24 was reduced by 67% (P = 0.0473; Figure 3E); mRNAs encoding CD4, proliferating cell nuclear antigen, and CD83 was also markedly reduced (P = 0.0489, P = 0.0482, and P = 0.0495, respectively; Figure 4B).

Expression of Inflammatory Markers in Atherosclerotic Lesions

To determine the mechanisms by which IL-2/anti–IL-2 mAb complex reduced lesion development, we examined effects on markers of inflammation. Expression of monocyte chemoattractant protein-1 (MCP-1) was markedly reduced in lesions of IL-2/anti–IL-2 mAb complex–treated mice (P = 0.0028; Figure 4A); vascular cell adhesion molecule-1 (VCAM-1) was also reduced by ≈65% (P = 0.0008; Figure 4A). mRNA encoding MCP-1 and VCAM-1 were also reduced, by 75% (P = 0.0410) and 72% (P = 0.0081), respectively (Figure 4B). TGF-β1 mRNA tended to increase but was not significant (P > 0.05; not shown).

IL-2/anti–IL-2 mAb Complexes Attenuate Splenocyte Proliferation and Cytokine Secretion

To determine whether induction of Tregs by IL-2/anti–IL-2 mAb complex treatment affected the proliferative ability of splenocytes, we examined their proliferation in response to anti-CD3 antibodies. After 3 days culture with anti-CD3 antibodies, the proliferation of splenocytes from the IL-2/anti–IL-2 mAb complex–treated mice was attenuated by up to 35% compared with control mice (P = 0.0100, P = 0.0010, and P = 0.0010 for 0.25 μg, 0.5 μg, and 1.0 μg anti-CD3 antibodies used, respectively; Figure 5A).

Table. Plasma Lipid Profiles and Body Weights of Control and IL-2–mAb–Treated Mice After 8 Weeks (Development Study) or 12 Weeks (Progression Study) on a High-Fat Diet

<table>
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<th>8 Weeks HFD</th>
<th>12 Weeks HFD</th>
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<tr>
<td></td>
<td>Control (Mean ± SEM) (n)</td>
<td>IL-2–mAb (Mean ± SEM) (n)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>12.8 ± 0.2 (10)</td>
<td>13.4 ± 0.4 (10)</td>
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<tr>
<td>VLDL/LDL cholesterol, mmol/L</td>
<td>10.2 ± 0.4 (10)</td>
<td>9.4 ± 0.7 (10)</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td>2.0 ± 0.2 (10)</td>
<td>1.9 ± 0.2 (10)</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.4 ± 0.1 (10)</td>
<td>1.4 ± 0.4 (10)</td>
</tr>
<tr>
<td>Body weights, g</td>
<td>30 ± 0.5 (10)</td>
<td>29 ± 1.0 (10)</td>
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The number of mice in each group is indicated by n. HFD indicates high-fat diet; IL, interleukin; mAb, monoclonal antibody; VLDL/LDL, very low–density/low-density lipoprotein; and HDL, high-density lipoprotein.
To determine whether secretion of cytokines was also affected, splenocytes isolated from the IL-2/anti–IL-2 mAb–treated and control mice were stimulated with anti-CD3 antibodies and cytokines in the medium measured. Stimulated splenocytes from IL-2/anti–IL-2 mAb complex–treated mice secreted markedly lower amounts of Th1, Th2, and Th17 cell–derived cytokines compared with splenocytes from control mice. IL-1β was reduced by >90%, IL-4 by 50%, IL-10 by 70%, IL-13 by 80%, IL-17 by 55%, interferon-γ by 85%, and tumor necrosis factor-α by 90% (Figure 5B and Figure I in the online-only Data Supplement); cell viability was similar, averaging 80%.

**IL-2/Anti–IL-2 mAb Complex Therapy Attenuates Progression of Developed Atherosclerosis**

Previous studies using anti-CD3 antibodies to elevate CD4+LAP+ Tregs suppressed only development of atherosclerosis. To determine whether the IL-2 antibody treatment suppressed already developed atherosclerosis, 6-week-old ApoE−/− mice were fed a high-fat diet for 6 weeks after which they were treated with the IL-2/anti–IL-2 mAb complex for the ensuing 6 weeks while continuing the high-fat diet. The treatment markedly attenuated further progression of atherosclerosis, by 67% when compared with lesion size at the commencement of treatment (*P*=0.0015; Figure 6A). Immunohistochemical comparison of lesions showed marked reductions in macrophage accumulation (*P*=0.0064), CD4+ T cell numbers (*P*=0.0189), CD83+ dendritic cells (*P*=0.0213), proliferating cell nuclear antigen–positive cells (*P*=0.0260) (Figure II in the online-only Data Supplement). These reductions were associated with a doubling in Tregs in the spleen (*P*=0.0001; Figure 6B) and no changes in CD4+, CD8+ T cells, or NK cells (*P*>0.05; Figure 6C and 6D).
Recent studies have provided evidence of a protective role for Tregs in the development of atherosclerosis.\textsuperscript{14,15} Adoptively transferred naturally occurring Tregs, defined by high expression of CD25 on their surface and the transcription factor forkhead box P3 (Foxp3), attenuate development of atherosclerosis.\textsuperscript{7} However, adoptive transfer therapy using Tregs is limited by inadequate purity of isolated Tregs cell population, insufficient numbers for transfer, and the potential instability of these cells.\textsuperscript{11} In this study we demonstrate an alternative to adoptive transfer by selective in vivo expansion of Tregs numbers that are normally present in low numbers in atherosclerotic lesions.\textsuperscript{6} Low doses of IL-2 complexed with an anti–IL-2 neutralizing antibody (JES6–1) induced a sustained increase in systemic and lesional Tregs, which attenuates not only development of atherosclerosis but also progression of developed atherosclerosis.\textsuperscript{28} Our data demonstrate that low-dose IL-2 cytokine/anti–IL-2 neutralizing mAb complex therapy can be used to attenuate both progression and development of atherosclerosis, by selectively expanding in vivo the natural Treg population; the action of the IL-2/anti–IL-2 (JES6–1) mAb complexes bind selectively to the relatively small population of CD25-expressing cells, mostly CD4\textsuperscript{+}CD25\textsuperscript{+} T cells with activity highly dependent on the neonatal IgG receptor FcRn.\textsuperscript{27} IL-2/anti-IL2 mAb interaction with Tregs is highly dependent on this mAb because complexing IL-2 with another mAb, S4B6, stimulates expansion of NK and CD8 T cells.\textsuperscript{19} In our study IL-2/anti–IL-2 (JES6–1) mAb complexes selectively increased Tregs numbers in spleens, lymph nodes, and atherosclerotic lesions of hyperlipidemic mice without affecting CD4\textsuperscript{+}, CD8\textsuperscript{+} T cells, or NK cells. Also, the pattern of Lag-3, ICOS, and CD62L expression by Tregs was unaltered by IL-2 mAb complex treatment.

The sustained selective expansion of Tregs in lymphoid compartments and their increased numbers in atherosclerotic lesions after treatment with IL-2/anti–IL-2 mAb complexes suppressed atherosclerosis development and exerted multiple effects on immune cells within developing lesions. CD4\textsuperscript{+} T cells are a population with demonstrated proatherogenic activity,\textsuperscript{4} and reductions in CD4\textsuperscript{+} T cells are associated with reductions in lesion size.\textsuperscript{28} We found reduced CD4\textsuperscript{+} T cells in lesions. This is likely a result of local inhibition of CD4\textsuperscript{+}...
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Figure 5. Suppression of proliferation and cytokine secretion by anti-CD3-stimulated splenocytes after IL-2/anti–IL-2 mAb complex treatment of ApoE<sup>−/−</sup> mice fed a high-fat diet. A, Proliferation of anti-CD3 stimulated splenocytes isolated from ApoE<sup>−/−</sup> fed a high-fat diet and treated with vehicle (control) or IL-2/anti–IL-2 mAb complex (IL-2/JES6–1) for 6 weeks, showing suppression of proliferation; n = 7 to 8. B, Bar graphs showing mean cytokine levels relative to positive controls in conditioned media of splenocytes isolated from ApoE<sup>−/−</sup> fed a high-fat diet and treated with vehicle (control) or IL-2/anti–IL-2 mAb complex (IL-2/JES6–1), after stimulation for 3 days with anti-CD3 antibodies; cytokines in conditioned media were analyzed using a cytokine protein array kit. Results are the average of 3 independent experiments. IL indicates interleukin; MAb, monoclonal antibody; and ApoE<sup>−/−</sup>, apolipoprotein E–deficient.

T cell proliferation by the increased Tregs within atherosclerotic lesions This is supported by our observation of reduced numbers of proliferating cell nuclear antigen–expressing proliferating cells in lesions, by reduced splenocyte proliferation, and dose-dependent inhibition of CD4<sup>+</sup> T cell proliferation in vitro by the expanded Treg population. Expansion of Tregs in lymphoid compartments including lymph nodes also raises the possibility of concomitant suppression of atherogenic CD4<sup>+</sup> T cells in lymph nodes. Although our study focused on CD4 T cells, it is possible that the expanded Treg population also inhibited other proatherogenic T cells, namely CD8<sup>+</sup> T cells and NKT cells, which also accumulate in lesions and are inhibited by Tregs.29,30 These cells also contribute to the splenocyte population whose proliferation was attenuated in mice treated with IL-2/anti–IL-2 (JES6–1) mAb.

Both CD4<sup>+</sup> Th1- and Th2-derived cytokines, including interferon-γ, IL-12, and IL-4, contribute to development of atherosclerosis.31,32 In this study IL-2/anti–IL-2 mAb complex therapy effectively prevented their secretion in stimulated splenocytes, indicating that the Tregs expanded by IL-2/anti–IL-2 mAb complex, like naturally occurring Tregs, suppress the ability of CD4<sup>+</sup> Th1, Th2, and Th17 cells to secrete cytokines.33 Our finding that splenocytes from control mice express significant quantities of Th2 cytokines—IL-4, IL-10, and IL-13 as well as the Th1 cytokine interferon-γ is consistent with studies indicating that a high-fat diet initiates Th1 and Th2 responses in hyperlipidemic mice.34

At present it is unclear as to the mechanisms used by the expanded Tregs to inhibit T cell responses and atherosclerosis. Given that a number of different subtypes of Tregs are present, including LAG-3, CD62L, and ICOS<sup>+</sup> ve and -ve Tregs, it is likely that a number of mechanisms are involved. ICOS<sup>+</sup> Tregs use IL-10 to suppress dendritic cell function and TGF-β to suppress T cell function whereas ICOS<sup>-</sup> Tregs use predominantly IL-10.9 Tregs can also use cytokines or enzymes expressed on their cell surface to inhibit immune responses. Contact-dependent inhibition by Tregs is dependent on cell surface expression of TGF-β or galactin-1,35 whereas their ability to generate the immunosuppressive agent adenosine is dependent on cell surface expression of CD39 and CD73.36 Tregs may also attenuate CD4<sup>+</sup> T cell activation by secreting phospholipase A2-IIId.37 Because we dosed the mice with IL-2/anti–IL-2 mAb complexes, it is unlikely that inhibition is the consequence of Tregs depriving other immune cells of IL-2.38

In addition to suppressive effects on CD4 T cells, macrophage accumulation in lesions was also reduced in developing lesions. This is associated with similar reductions in expression of MCP-1 and VCAM-1, which very likely contribute to the reduction in macrophage accumulation. MCP-1 is produced by both macrophages and vascular smooth muscle cells, is chemotactic for monocytes/macrophages, and preventing its expression attenuates atherosclerosis.39 Similarly, VCAM-1 contributes to macrophage accumulation. The reduction in VCAM-1 expression is most likely the consequence of Tregs attenuating both macrophage and CD4<sup>+</sup> T cell activation; inhibiting CD4<sup>+</sup> T cell activation by Tregs reduces IL-1β expression, a powerful inducer of VCAM-1 expression in lesions. Our finding of reductions in expression of CD83 suggests that IL-2–mAb treatment also attenuates maturation of dendritic cells. Tregs are known to inhibit dendritic cell maturation and can modulate dendritic cell phenotype, reducing expression of CD83.40 As dendritic cells have a key role as antigen-presenting cells in initiating immunogenic CD4 T cell responses, their reduction may contribute to the suppression of atherogenic CD4 T cells.

Our data clearly demonstrate that IL-2/anti–IL-2 mAb complex treatment inhibits both atherosclerosis development and also progression of developed atherosclerotic lesions. Our studies also indicate that the expanded Tregs effectively target CD4<sup>+</sup> T cells, macrophages, and dendritic cells in lesions to attenuate atherosclerosis. As only small numbers of Foxp3-expressing Tregs are present in human atherosclerotic lesions,6 this approach of administering IL-2 complexed with an IL-2 neutralizing antibody that selectively targets Tregs expansion could be a promising therapeutic approach for attenuating immunoinflammation and progression of preexisting atherosclerotic vascular disorders. Recently, neutralizing the chemokine CCL17 has also been shown to increase Treg numbers and attenuate development of atherosclerosis.41 However, unlike IL-2/anti–IL-2 mAb treatment, which expands natural Tregs,18 neutralizing CCL17 appears to accelerate the conversion of CD4<sup>+</sup>CD25<sup>+</sup>T cells to inducible Tregs in the periphery, their proliferation, and also the proliferation of CD4<sup>+</sup>CD25<sup>+</sup>T cells. Furthermore, in vitro
Figure 6. IL-2/anti–IL-2 mAb complex treatment suppresses progression of developed atherosclerosis in ApoE−/− mice. A, Photomicrographs of aortic sinus atherosclerotic lesions stained with Oil Red O after feeding mice a high-fat diet for 6 weeks before commencing therapy with vehicle (control) or IL-2/anti–IL-2 mAb complex (IL-2/JES6–1) for 6 weeks while continuing the high-fat diet. Bar graphs show means ± SEM for the 3 groups at the end of the study; n = 10 to 13. B, CD4+CD25+Foxp3+ T cells in spleen at the end of the study; n = 10 to 13. C, Representative results of CD4+ and CD8+ T cells, CD4+CD25+Foxp3+ T cells, and NK cells in spleen of mice treated with vehicle (control) or IL-2/JES6–1 mAb complex at the end of the study. D, Bar graphs showing the percentages of CD4+, CD8+, and NK cells in spleens of ApoE−/− mice with developed atherosclerosis after treatment with vehicle (control, n = 13) and IL-2/anti–IL-2 mAb complexes (n = 13) for 6 weeks. *P < 0.05 from control. IL indicates interleukin; MAb, monoclonal antibody; ApoE−/−, apolipoprotein E−deficient; and NK, natural killer cells.
CCL17 attenuated expansion of in vitro polarized Tregs, stimulated with anti-CD3/IL-2. Whether this also applies to natural Tregs remains to be elucidated but raises the interesting possibility that IL-2/anti–IL-2 mAb treatment plus CCL17 neutralization may further elevate Tregs and augment the antiatherosclerotic effects of IL-2/anti–IL-2 mAb treatment.

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CLINICAL PERSPECTIVE

Atherosclerosis is a multifactorial inflammatory disease characterized by the accumulation of lipids and innate and adaptive immune cells, leukocytes, and lymphocytes. Activated lymphocytes are believed to be essential for both the development and exacerbation of atherosclerosis. One way to prevent their activation is to increase regulatory T cells, cells that can control both innate and adaptive immune responses. Regulatory T cells, defined by expression of CD4, CD25, and a transcription factor Foxp3, are mostly thymus derived and account for 5% to 10% of the circulating CD4+ T cell population. The cytokine interleukin-2 can stimulate their expansion but is not selective. In this article we present a novel way to chronically expand this T cell population, by complexing the cytokine interleukin-2 with an interleukin-2–neutralizing antibody, which prolongs its duration of action and specifically targets regulatory T cells, markedly increasing their numbers. Other interleukin-2–responsive lymphocytes that can accelerate atherosclerosis do not respond to this cytokine–antibody complex. Treatment with this cytokine–antibody complex inhibits the activation and proliferation of proatherogenic T cells and markedly attenuates development and progression of already developed atherosclerosis. From a clinical perspective, this finding is important because it demonstrates that by specifically targeting cells that can protect against atherosclerosis, in this case pharmacologically expanding regulatory T cell numbers, it is possible to suppress inflammation associated with atherosclerosis as well as attenuate progression of disease.
Cytokine Therapy With Interleukin-2/Anti–Interleukin-2 Monoclonal Antibody Complexes Expands CD4+CD25+Foxp3+ Regulatory T Cells and Attenuates Development and Progression of Atherosclerosis
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Supplementary Material

Cytokine Therapy with IL-2/anti-IL-2 mAb Complexes Expands CD4+CD25+Foxp3+ Regulatory T Cells and Attenuates Development and Progression of Developed Atherosclerosis

Expanded Methods

Animals

Male ApoE/- mice backcrossed 10 times onto the C57Bl6 background were used for the study and were fed a high fat diet containing 21% fat and 0.15% cholesterol obtained from Speciality Foods (Glen Forrest, Western Australia; Diet Number SF00-219). In addition to containing fat, protein and fibre it also contained essential fatty acids-alpha-linolenic acid 18:3 n3 (0.20%) and linoleic acid 18:2 n6 (0.40%); water was provided ad libitum. Mice were maintained on a 12:12-h light-dark cycle in the AMREP Animal Precinct. All experiments conducted on the mice were approved by the AMREP Animal Ethics Committee.

Experimental Design

Six week old ApoE/- mice were commenced on a high fat diet, then 2 weeks later they were administered a recombinant mouse (rm) IL-2/anti-IL-2 mAb (JES6-1) complex (1µg rmIL-2 plus 5µg anti-IL-2 mAb). During the first week of treatment the mice received 5 consecutive daily doses i.p., after which they received 3 doses weekly for the duration of the study. Control mice received PBS. Mice were killed at 14 weeks of age and atherosclerotic lesions assessed. To evaluate the effects of rmIL-2/anti-IL-2 mAb complex treatment on progression of atherosclerosis six week old ApoE/- mice were fed a high fat diet for 6 weeks, then the mice were treated with the cytokine-antibody complex as above for 6 weeks whilst continuing the high fat diet. To determine the effects of complexing IL-2 with anti-IL-2 mAb (JES6-1) on plasma IL-2 levels, recombinant IL-2 (5µg) was administered alone or complexed with anti-IL-2 mAb and plasma IL-2 concentrations determined 6 and 24 hours later using a mouse IL-2 ELISA kit (BioLegend).
Blood analyses

Blood was collected by cardiac puncture whilst the mice were under pentobarbitone sodium (80mg/kg i.p.) induced anaesthesia. Plasma was obtained after centrifugation and stored at -20°C. Plasma concentrations of total cholesterol, very low/low and high density lipoprotein (VLDL/LDL and HDL)-cholesterol and triglycerides were determined enzymatically using a cholesterol assay kit (Roche/Hitachi) and automated chemistry analyzer. Plasma alanine transaminase and aspartate aminotransferase were measured using commercially available mouse ELISA kits (Uscn Life Science Inc).

Atherosclerotic Lesion Assessment

Atherosclerotic lesion area was quantified on cross-sections of the aorta beginning at the level of the aortic sinus. Specifically, five sections taken at 80-μm intervals, spanning 320μm of the aortic bulb, from the commissures of the aortic leaflets and upward, were stained with Oil Red O and evaluated microscopically. Plaque area was measured using computer assisted image analysis (Leica microscope coupled to a JVC digital camera and Optimus 6.2 VideoPro-32 software). The amount of atherosclerosis in the aortic sinus was expressed as mean plaque size of the 5 sections for each mouse.

Immunohistochemistry

Sections of the aortic sinus (see above) were subjected to immunohistochemistry as previously described. Briefly, sections were fixed in cold (-20°C) acetone for 20 min. Then the sections were sequentially incubated in 3% hydrogen peroxide in PBS, 10% normal goat serum/PBS and biotin/avidin blocking reagents (Vector Laboratories). Thereafter, the sections were incubated (1h) with primary antibodies in normal goat serum (NGS)/PBS, rat anti-mouse CD68 (1-100, Serotec: cat#MCA1957), rat anti-mouse CD4 (1-20; BD Pharmingen: Cat#550280), rat anti-mouse CD83 (1-50; eBiosciences: cat#14-
Sections were washed and incubated (40min) with the appropriate secondary antibodies (in NGS/PBS), followed by incubation with streptavidin horseradish peroxidase complex. Antigens were visualised using DAB (3,3-diaminobenzidine) and sections counterstained with hematoxylin. Expression of antigens was quantified using Optimus 6.2 VideoPro-32 and stained areas expressed as percentage of the total plaque area. In other instances individually stained cells were counted and expressed as cell numbers per unit area of lesion.

**Flow Cytometry Analysis**

For fluorescent-activated cell sorter (FACS) analyses of immune cells, lymph nodes, liver and spleen were isolated after administering IL-2/anti-IL-2 mAb antibody complex or vehicle. Lymphocytes from spleen were isolated using Ficoll-Paque (GE Healthcare) whereas liver lymphocytes were isolated by centrifugation through a 33% isotonic Percoll density gradient (GE Healthcare). Then single cell suspensions of lymph node, liver and spleen lymphocytes were pre-incubated with anti-mouse CD16/32 (clone 2.4G2, cat#553141, BD Biosciences) antibody to prevent non-specific binding of antibodies to FcRγ, followed by staining with relevant fluorochrome-conjugated primary antibodies for 30 minutes at 4°C. Antibodies used were fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone RM4-5, cat#100509, BioLegend), phycoerythrin (PE)- conjugated anti-NK1.1 (clone PK136, cat#IM2904, Beckman Coulter), allophycocyanin (APC)-conjugated anti-TCRβ (clone H57-597, Cat#553170, BD Biosciences), PE conjugated anti-CD 8 (clone 53-6.7, cat#12-0081-82, eBioscience), FITC conjugated anti-CD25 (clone 7D4, Cat#553071, BD Biosciences), FITC conjugated anti-ICOS (clone C398.4A, cat#11-9949-
80, eBioscience), RPE conjugated anti-Lag3 (clone C9B7W, cat#MCA2386PET, Serotec) and PE and APC conjugated anti-Foxp3 (clone FJK-165, cat#12-5773-80 and Cat#17-5773-80, eBioscience). For Foxp3 the cells were permeabilized using a permeabilization buffer (Miltenyi Biotec), followed by FcR blocking reagent and anti-Foxp3 antibody. Flow cytometry was performed using a FACSCalibur and CellQuest software.³

**In vitro suppression assay**

CD4+CD25+ regulatory and CD4+CD25- T cells were isolated from the spleens of mice 36 hours after receiving 3 doses of IL-2/anti-IL-2 mAb complex or vehicle using a mouse CD4+CD25+ regulatory T cell isolation kit from Miltenyi Biotec. T-cell depleted spleen cells were also isolated magnetically by AutoMACS using Pan-T cell isolation kit (Miltenyi Biotec). For the in vitro suppression assay, purified CD4+ CD25- T-eff cells were suspended in complete RPMI containing 10% fetal calf serum (FCS) at 2 x 10⁶ cells/ml and incubated with 10µM CFSE (Molecular Probe) for 5 minutes at 37°C. CFSE-stained cells were washed three times with RPMI containing FCS with 5 minutes incubation at 37°C between washes. After the final wash, cells were counted and suspended in RPMI containing FCS. CD4+CD25+ regulatory T cells were co-cultured with CD4+ CD25- T-eff cells in the following ratios, 1:0, 1:0.0125, 1:0.5 and 1:2 (where 1 = 2 x 10⁴ cells) in RPMI containing FCS. T cells were stimulated with Concanavalin A (2µg/ml; Sigma) in the presence of 5 x 10⁴ irradiated (3300 rads) T cell-depleted splenocytes in triplicate. After 5 days in culture cells were stained using anti-CD4 antibodies (Pacific blue) and anti-CD25 (APC-Cy7) antibodies (BD Pharmigen) and analysed using a Canto II FACS (BD Bioscience) and FACSDiva software (BD Bioscience).
Cell Proliferation

To assess effects on cell proliferation, splenocytes were isolated using Ficoll-Paque (GE Healthcare) and cultured at 0.2 x 10^6 cells/well in flat-bottomed 96-well plates and stimulated with purified soluble anti-CD3 antibody (1 µg/ml, R & D Systems) at 37°C with 5% CO₂ for 72 h. The cells were pulsed with 1 µCi of [³H] thymidine for the last 16 hours and harvested with an automatic cell harvester (Pharmacia). Thymidine incorporation was assessed using a liquid scintillation counter (LKB Wallac, Perkin Elmer).

Cytokine Analyses

Splenocytes were isolated from spleen using Ficoll-Paque (GE Healthcare). After washing with PBS containing 2% FCS, the cells were plated in culture dishes in RPMI medium for 2 hours at 37°C. The non-adhesive cells were collected and cultured (1 x 10^6 cells/ml) in RPMI in U-bottom 96-well plates and stimulated with soluble anti-CD3 antibody (1 µg/ml, R & D Systems) at 37°C with 5% CO₂. Culture supernatants were collected at 72h and analysed for cytokines using a Mouse Cytokine Array kit (Proteome Profiler™ Array) according to the manufacture’s instructions (R & D Systems). Cell viability at the end of the experiment was determined by trypan blue exclusion. Comparisons between vehicle and IL-2-anti-IL-2 antibody treated mice were made after standardization using positive controls.

Real-Time RT-PCR Analyses

RNA was extracted from the Aortic Arch of control and IL-2/JES6-1 treated mice using a RNeasy Fibrous Tissue Minikit (cat# 74704, QIAGEN). Briefly, tissues were first homogenized and the homogenate diluted before being treated with Proteinase K, to remove contractile proteins, as directed by the manufacturer. Residual debris was pelleted by centrifugation and the supernatant collected. The supernatant was then mixed with ethanol and centrifuged through a spin column, binding the RNA to the silica membrane.
Contaminating DNA was removed by DNase treatment of the silica membrane and after washing, total RNA was eluted using RNase-free water. The quality and quantity of the RNA was assessed using a MultiNA Electrophoresis System (Shimadzu Biotech). Reverse transcription was performed using a Quantifast SYBR Green One-step RT-PCR kit (cat# 204154, QIAGEN) and quantitative gene expression analysis performed on a AB 7500 Fast Real-time PCR Machine (Applied Biosystems). Oligonucleotide primers were designed using PerlPrimer software package. The sequences of oligonucleotides used were, Foxp3: sense, 5’-TGGACTACTTCAAATATGGC-3’ and antisense, 5’- GCGAAGATGCGAGTAAACCAAT-3’; MCP-1: sense, 5’-GCATCCACGTTGGCTCA-3’ and antisense, 5’-CTCCAGCCTACTCATTGGGATCA-3’; VCAM-1: sense, 5’-AGAACCAGACAGACGTCC-3’ and antisense, 5’-GGATCTTCAGGGAATGAGTAGAC-3’; CD4: sense, 5’-GTGGTGATGAAAGTGGCTCAG-3’ and antisense, 5’- TTTCTGCTCAGAGACGTCC-3’; CD83: sense, 5’-TTTCACCTGCAAATTTGCAC-3’ and antisense, 5’- GGTGAGAGGACTTCAAGAAAGG-3’; PCNA: sense, 5’- CTGGGAATCCAGACAGGTCC-3’ and antisense, 5’- AACAGGCTTCATCTCTGAGG-3’; 18S: sense: 5’- CGGCTACCACATCCAGAAGGAAGGCA-3’ and antisense, 5’- GCTGGAATTACCAGCAGGCTGCTG-3’. Expression of genes was calculated as fold increases using the $2^{-\Delta\Delta CT}$ method.8
Legends for Supplementary Figures

**Figure I.** Suppression of cytokine secretion by anti-CD3 stimulated splenocytes after IL-2/anti-IL-2 mAb complex treatment of ApoE−/− fed a high fat diet. Cytokine array of conditioned media from splenocytes isolated from vehicle (control) and IL-2/anti-IL-2 mAb complex (IL-2/JES6-1) treated mice stimulated for 3 days with anti-CD3 antibodies. Results are typical of three similar experiments.
Figure II. IL-2/anti-IL-2 mAb complex treatment attenuates accumulation of immune and proliferating cells and proinflammatory mediators during progression of developed atherosclerosis. (A) Immunohistochemical staining using anti-CD68 (macrophage) antibody and mean areas of staining (bar graphs) in mice treated with vehicle (control) and IL-2/anti-IL-2 mAb (IL-2/JES6-1). (B) Cross sections stained with anti-CD4 antibodies, detecting CD4+ T cells in plaques of ApoE−/− mice treated with vehicle (control) and IL-2/anti-IL-2 mAb complex (IL-2/JES6-1) and mean cell numbers (bar graphs). (C) anti-CD83 stained atherosclerotic cross sections from vehicle (control) and IL-2/anti-IL-2 mAb treated ApoE−/− mice detecting mature dendritic cells and mean numbers of CD83+ cells in lesions (bar graphs). (D) PCNA stained cross sections detecting proliferating cells in lesions of vehicle treated (control; left) and IL-2/anti-IL-2 mAb treated mice (right) and mean number of proliferating cells/unit lesion area (bar graphs). (E) VCAM-1 staining in lesions of control and IL-2/anti-IL-2 mAb treated mice. (F) MCP-1 staining in lesions of control and IL-2/anti-IL-2 mAb treated mice; n = 10-12 mice. *P < 0.05 from control. Size bars represent 100μm.
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