Short-Term Treatment With Anti-CD3 Antibody Reduces the Development and Progression of Atherosclerosis in Mice

Sabine Steffens, PhD; Fabienne Burger, Bsc; Graziano Pelli, Bsc; Yann Dean, PhD; Greg Elson, PhD; Marie Kosco-Vilbois, PhD; Lucienne Chatenoud, MD; François Mach, MD

Background—Atherosclerosis is a chronic inflammatory disease of the large arteries that is the primary cause of heart disease and stroke. Anti-CD3–specific antibodies suppress immune responses by antigenic modulation of the CD3 antibody/T-cell receptor complex. Their unique capacity to restore self-tolerance in a mouse model of diabetes and, importantly, in patients with recent-onset type 1 diabetes involves transforming growth factor-β–dependent mechanisms via expansion and/or activation of regulatory T cells. We hypothesized that treatment with anti-CD3–specific antibodies might inhibit atherosclerosis development and progression in mice.

Methods and Results—Low-density lipoprotein receptor–deficient mice were fed a high-cholesterol diet for 13 or 24 weeks. Anti-CD3 antibody was administered on 5 consecutive days beginning 1 week before or 13 weeks after the high-cholesterol diet was initiated, respectively. Control mice were injected in parallel with phosphate-buffered saline. Anti-CD3 antibody therapy reduced plaque development when administered before a high-cholesterol diet and markedly decreased lesion progression in mice with already established atherosclerosis. We found increased production of the antiinflammatory cytokine transforming growth factor-β in concanavalin A–stimulated lymph node cells and enhanced expression of the regulatory T-cell marker Foxp3 in spleens of anti-CD3 antibody–treated mice. A higher percentage of apoptotic cells within the plaques of anti-CD3 antibody–treated mice was also observed.

Conclusions—Altered disease progression, combined with the emergence of this particular cytokine pattern, indicates that short-term treatment with an anti-CD3 antibody induces a regulatory T-cell phenotype that restores self-tolerance in a mouse model of atherosclerosis. (Circulation. 2006;114:1977-1984.)

Key Words: apoptosis | atherosclerosis | Foxp3 protein, mouse | immunology | inflammation | T-lymphocytes, regulatory | transforming growth factor beta

It is now generally recognized that atherosclerosis is a chronic inflammatory disease that can lead to severe clinical events after plaque rupture and thrombosis. Prevention and current treatments for atherosclerosis are based mainly on drugs that decrease plasma cholesterol concentrations and lower heightened blood pressure. In particular, statins have proved to reduce cardiovascular events significantly, not only by their cholesterol-lowering properties but also by their more recently identified antiinflammatory and immunomodulatory effects. Nevertheless, atherosclerosis remains the primary cause of heart disease and stroke, accounting for up to 50% of deaths in Western countries. Thus, identification and development of promising novel antiinflammatory therapies is of great interest and represents a continued challenge to the medical community. To date, therapies to treat immune disorders are based on potent immunosuppressive agents that block the activation and expansion of antigen-specific T cells.

Editorial p 1901

Clinical Perspective p 1984

Antibodies that target CD3 antibody molecules are potent immunosuppressive agents that act by modulating the CD3 antibody/T-cell receptor (TCR) complex. This property has been exploited for use in patients undergoing a plethora of short-term allograft rejection episodes. Furthermore, short-term administration of an anti-CD3–specific antibody in nonobese diabetic (NOD) mice has proved to induce long-term remission of autoimmune diabetes.

In addition to their potent capacity to alter immune responses via CD3 antibody/TCR modulation, anti-CD3–specific antibodies exhibit unwanted toxic mitogenic properties. These effects manifest as a result of the capacity of the C-terminal domain of the heavy immunoglobulin chain (Fc) of the anti-CD3 antibodies to interact with Fc receptors or Clq molecules on other leukocytes, thereby enhancing cross-linking. To circumvent the
induction of this unwanted property, nonmitogenic antibody variants with mutations or deletion of the Fc portion have been generated that are consequently well tolerated yet remain therapeutically effective.9,10

Experiments using overtly diabetic NOD mice have provided important insights into the molecular mechanisms underlying the induction of tolerance induced by anti-CD3 antibody treatment. Nonmitogenic anti-CD3–specific antibodies induce transforming growth factor (TGF)-β–producing CD4+CD25+ regulatory T cells.11 Furthermore, restoration of self-tolerance in NOD mice was abolished by coadministration of a neutralizing TGF-β–specific antibody. These studies have been translated into the clinical setting in that short-term treatment with 2 different anti-CD3 antibodies preserves residual β-cell function in patients with recent-onset type 1 diabetes.12,13 Thus, because anti-CD3 antibody treatment can provide significant clinical benefit in an immunoinflammatory-related disorder, broader testing is warranted.

In the present study, we tested whether treatment with anti-CD3–specific antibodies inhibits atherosclerosis development and progression in low-density lipoprotein receptor–deficient (LDLR−/−) mice. We chose to use LDLR−/− mice because this model does not spontaneously develop hypercholesterolemia and atherogenesis, but these conditions can be induced by a high-cholesterol diet.14 Our study provides for the first time evidence that short-term treatment with a nonmitogenic anti-CD3–specific antibody modulates inflammatory processes pivotal for the development of atherosclerosis and consequently limits atherosclerotic plaque formation.

**Methods**

**Animals**

We used 10-week-old male LDLR−/− C57BL/6 mice (Charles River Laboratories, Lyon, France). For histological and atherosclerotic plaque development and for proliferation and cytokine analyses, littermate mice were fed a high-cholesterol diet (1.25% cholesterol; Research Diets, New Brunswick, NJ) for 13 or 24 weeks. Anti-CD3 antibody F(ab′)2 (50 μg per mouse per day) was administered intravenously as previously described11 on 5 consecutive days beginning 1 week before (n=6) or 13 weeks after (n=9) the high-cholesterol diet was initiated. For production and purification methods of anti-CD3 antibody F(ab′)2, refer to the online Data Supplement. Control mice (13-week diet, n=6; 24-week diet, n=9) were injected in parallel with phosphate-buffered saline (PBS). All animal studies were approved by the local ethics committee.

**Atherosclerotic Lesion Analysis**

Atherosclerotic lesions within the thoracoabdominal aorta and aortic sinus were analyzed by Sudan IV staining for lipid deposition.15 Immunostaining was performed on acetone-fixed 5-μm cryosections of mouse aortic roots using monoclonal antibodies for mouse CD4 and Mac-3 (BD Biosciences, San Jose, Calif) and mouse smooth muscle myosin (Biomedical Technologies, Stroughton, Mass). Quantifications were performed by computer image analysis with the MetaMorph6 software (Carl Zeiss Meditec, Inc; Feldbach, Switzerland).

**In Situ DNA Nick End Labeling Assay**

Nuclear DNA fragmentation in situ was detected with terminal deoxynucleotidyl transferase incorporation of biotinylated deoxyuridine at the sites of DNA breaks in apoptotic cells. Cryosections (5 μm) of mouse aortic roots and arch were stained with an in situ DNA nick end labeling assay kit according to the manufacturer’s guidelines (Promega, Madison, Wis).

**Blood Analysis**

For measurements of differential blood cell counts and cholesterol content, blood samples were collected at the beginning and end of the diet. High-density lipoprotein and very-low-density lipoprotein cholesterol fractions of sera were measured by fast protein liquid chromatography.

**Flow Cytometry**

For flow cytometric analysis of peripheral blood, anti-CD3 antibody was injected on 5 consecutive days into mice fed a high-cholesterol diet for 13 weeks, and mice were bled on days 5, 8, 12, 17, 24, and 31 after injection. For fluorescent-activated cell sorter analysis of lymphoid organs, lymph node cells and splenocytes were isolated after 1, 3, 6, and 11 weeks of anti-CD3 antibody treatment. Cells were Fc blocked by treatment with anti-mouse CD16/CD32 (BD Biosciences). FITC-, PE-, or allophycocyanine-labeled antibodies to mouse CD3 antibody, CD4, CD25, CD62L, and TCR and corresponding isotype controls were used (BD Biosciences). CellQuest software was used for acquisition and analysis on a FACSCalibur (BD Biosciences).

**Cytokine Analysis**

Lymph node cells were cultured in 96-well plates at a concentration of 5×10⁶ cells/mL and stimulated in duplicates with 2 μg/mL concanavalin A (Con A; Sigma Chemical Co, St Louis, Mo). Murine TGF-β 1, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-4, IL-10, and IL-12 (p70) in cell culture supernatants were assayed by ELISA using paired antibodies according to the manufacturer’s instructions (R&D Systems, Minneapolis, Minn).

**Real-Time Reverse-Transcription Polymerase Chain Reaction**

Total RNA from mouse blood and spleens was extracted with TRI Reagent (MRC Inc, Cincinnati, Ohio), and real-time reverse-transcription polymerase chain reaction was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif).15 We used Foxp3 and eukaryotic 18S primers and probes as previously described.16

**Statistical Analysis**

All results are expressed as mean±SEM. Differences between the values of P<0.05 were considered significant with 2-tailed Student t test or the Mann-Whitney rank sum test.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Pretreatment With an Anti-CD3 Antibody Reduces Atherosclerosis Development**

To analyze whether treatment with anti-CD3 antibodies at the time of disease onset would protect mice from atherosclerosis development, we treated LDLR−/− mice with anti-CD3 antibody for 5 consecutive days 1 week before initiating the high-cholesterol diet. After 13 weeks of diet, atherosclerotic lesions were clearly detectable within the thoracoabdominal aorta (Figure 1A) and in the aortic roots of control mice (Figure 1B), whereas mice treated with the anti-CD3 antibody exhibited markedly reduced vascular lesion development. This significantly beneficial effect on atherogenesis, achieved with treatment given before disease induction, was even more noteworthy in that the timing was different from that previously observed in studies with overtly diabetic NOD mice,10 arthritis,17 or experi-
mental autoimmune encephalitis. Could animals in our study already have elevated plasma lipid levels even when fed a normal chow diet? To address this question, we analyzed plasma lipid profiles from blood samples collected before anti-CD3 antibody treatment. We found significantly elevated cholesterol levels in LDLR−/− mice compared with wild-type animals (Table 1). After 13 weeks on the high-cholesterol diet, the average total plasma values of LDLR−/− mice were 4.5-fold increased compared with the initial measurement, with no significant difference between control and anti-CD3 antibody–treated mice (Table 1). Similarly, no statistical differences in body weight or blood leukocyte, lymphocyte, platelet, or erythrocyte counts were detected between the 2 groups (Table 1 and data not shown), and none of the anti-CD3 antibody–treated mice died during treatment or showed unhealthy behavior.

**Anti-CD3 Antibody Therapy Inhibits Atherosclerosis Progression via Induction of TGF-β**

To assess the therapeutic potential of anti-CD3 antibody treatment on established atherosclerosis, LDLR−/− mice were fed a high-cholesterol diet for 13 weeks before antibody administration. The anti-CD3 antibody was administered for 5 consecutive days, and control mice were injected in parallel with PBS. After an additional 11 weeks of diet (for a total of 24 weeks), the extent of atherosclerotic lesions within aortic roots was significantly increased in the PBS-injected group compared with LDLR−/− controls killed after the first 13 weeks of diet (Figure 2A). In mice treated with the anti-CD3 antibody, progression of atherosclerotic lesions within the aortic roots was significantly inhibited (lesion area reduced 4.3%) and anti-CD3–treated mice revealed no differences in the relative macrophage or smooth muscle cell content between treated and control mice (Figure 2B and 2C). We found, however, a significantly increased percentage of CD4+ cells per lesion area in the anti-CD3 antibody–treated mice (Figure 2D). We next investigated whether the observed antiatherosclerotic effect of an anti-CD3 antibody therapy was mediated by a modulation of proinflammatory or antiinflammatory cytokine secretion. Therefore, lymph node cells from untreated mice or mice treated with the anti-CD3 antibody were collected after 24 weeks of high-cholesterol diet and analyzed for their capacity to produce various cytokines in response to Con A. Treatment with the anti-CD3 antibody did not change secretion profiles of T-helper (Th) type 1 (TNF-α, IFN-γ, IL-2) or Th2 cytokines (IL-10, IL-4, IL-6) compared with control mice (Table 2 and data not shown), and no IL-12 was significantly increased in the PBS-injected group

**Figure 1.** Pretreatment with anti-CD3 antibody inhibits atherosclerotic plaque development in LDLR−/− mice. Anti-CD3 antibody was administered 1 week before a high-cholesterol diet was initiated for 13 weeks (n=6 mice per group). A, Representative section of mouse thoracoabdominal aortas stained for lipid deposition by Sudan IV and quantification of staining. B, Representative cryosections of mouse aortic roots stained for lipid deposition by Sudan IV and quantification of atherosclerotic lesions. *P<0.05 vs PBS-injected controls.

**TABLE 1. Characteristics of Wild-Type and LDLR−/− Mice Before and After Treatment**

<table>
<thead>
<tr>
<th></th>
<th>10-Week Chow Diet</th>
<th>13-Week High-Cholesterol Diet (LDLR−/−)</th>
<th>24-Week High-Cholesterol Diet (LDLR−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=5)</td>
<td>C03 (n=5)</td>
<td>CD3 (n=9)</td>
</tr>
<tr>
<td></td>
<td>LDLR−/− (n=5)</td>
<td>PBS (n=6)</td>
<td>PBS (n=9)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>52.3±22.9</td>
<td>757.8±131.3</td>
<td>962.1±151.5</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>168.7±23.1*</td>
<td>764.5±142.4</td>
<td>915.6±158.2</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>380.4±22</td>
<td>426.4±43.6</td>
<td>538.8±126.4</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>...</td>
<td>140.8±2.4</td>
<td>609.6±180</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>23.8±1.5</td>
<td>28.5±0.8</td>
<td>136.8±17.2</td>
</tr>
<tr>
<td>Peripheral leukocytes, n/μL</td>
<td>...</td>
<td>28.7±1.1</td>
<td>152.4±28.8</td>
</tr>
<tr>
<td>Peripheral lymphocytes, n/μL</td>
<td>...</td>
<td>28.8±1.5</td>
<td>152.4±28.8</td>
</tr>
</tbody>
</table>

WT indicates wild type; PBS, control; CD3, anti-CD3 antibody; LDL, low-density lipoprotein; and HDL, high-density lipoprotein. Data are expressed as mean±SEM. *P<0.05 vs wild type.
secretion was detectable in either group. However, a significant
increase in TGF-β was observed on anti-CD3 antibody treat-
ment (Table 2), suggesting a contribution of this cytokine to the
therapeutic effect. We also analyzed the cytokine expression
profiles in mice treated with anti-CD3 antibody before beginning
the diet (prevention study). We found significantly increased
TGF-β secretion in Con A–stimulated lymph node cell cultures
of anti-CD3-treated mice (274 ± 39 pg/mL) compared with
controls (176 ± 22 pg/mL) after 13 weeks of diet. These findings
correlate very well with previous results obtained in overtly
diabetic NOD mice, demonstrating that treatment with nonmi-
togenic anti-CD3 antibody restores self-tolerance via TGF-β–
dependent mechanisms.11

**Anti-CD3 Antibody Modulates CD3/TCR Expression**

Immunosuppression by nonmitogenic anti-CD3 antibody
therapy has been shown to involve T-cell depletion and
downregulation of the TCR complex on nondepleted T cells.3

To investigate the underlying mechanisms in our atheroscle-
rotic mouse model, we analyzed CD3 and TCR expression in

<table>
<thead>
<tr>
<th>TABLE 2. Cytokine Production of Con A–Stimulated Lymph Node Cells After 3 or 11 Weeks of Anti-CD3 Antibody Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Weeks After Injection (16 Weeks of High-Cholesterol Diet)</td>
</tr>
<tr>
<td>PBS (n=5)</td>
</tr>
<tr>
<td>IFN-γ, ng/mL</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
</tr>
<tr>
<td>IL-10, pg/mL</td>
</tr>
<tr>
<td>TGF-β, pg/mL</td>
</tr>
</tbody>
</table>

PBS indicates control; CD3, anti-CD3 antibody. Data are expressed as mean ± SEM.
*P < 0.05 vs PBS 3 weeks after injection.
†P < 0.05 vs PBS 11 weeks after injection.
the blood of anti-CD3 antibody–treated hypercholesterolemic mice. After 5 days of anti-CD3 antibody treatment, the percentage of CD3-expressing cells was dramatically reduced and recovered to normal levels at day 31 after the first injection (Figure 3A). The same pattern was observed for TCR expression. Analysis of CD3-expressing cells in lymph nodes of treated mice at 1, 3, or 11 weeks after injection (n=4 mice per group at each time point), *P<0.0005, **P<0.05 vs PBS-injected controls.

Figure 3. Time course of T-cell depletion and TCR modulation in anti-CD3 antibody–treated hypercholesterolemic LDLR−/− mice. A, Anti-CD3 antibody was administered on 5 consecutive days, and peripheral blood cells were analyzed by flow cytometry at the indicated time points (day 0=before treatment, day 1=first injection day; n=4 mice per group at each time point). B, Lymph node cells of anti-CD3 antibody– or PBS-treated mice were analyzed after 1, 3, or 11 weeks after injection (n=4 mice per group at each time point). *P<0.0005, **P<0.05 vs PBS-injected controls.

Because anti-CD3 antibody treatment has been reported to increase the proportion of CD4+CD25+ regulatory T cells,11,18 we attempted to assess the distribution of this T-cell subset after 3, 6, or 11 weeks of anti-CD3 antibody treatment in LDLR−/− mice on a high-cholesterol diet. Neither pooled lymph nodes nor isolated para-aortic lymph nodes, spleens, or peripheral blood showed an increase of this population (data not shown). Similarly, we failed to detect an increase in the population of CD4+CD25+ cells expressing the regulatory T-cell marker CD62L. In addition, no difference in the total number of CD4+ cells was found after 11 weeks of anti-CD3 antibody treatment.

Induction of Foxp3 and Apoptosis
Various studies have demonstrated that the fork-head/winged helix transcription factor Foxp3 is specifically expressed in T cells when a phenotype of immunoregulation is observed (regulatory T cells).19–21 We analyzed Foxp3 mRNA levels in splenocytes and peripheral blood cells of anti-CD3 antibody–treated mice after 24 weeks of diet. We found a 2-fold induction of Foxp3 expression in splenocytes and a 2.8-fold induction in peripheral blood cells obtained from anti-CD3 antibody–treated mice compared with control mice (Figure 4).

Recently published data provide evidence for a functional involvement of granzyme B and apoptosis in the immunosuppressor function of T cells with potential regulatory function.22 Granzyme B, a member of the granzyme family of serine proteases, is known to be essential for DNA fragmentation and apoptosis in cytotoxic T lymphocyte and natural killer cell cytotoxicity.23,24 To assess whether rates of apoptosis contribute to the antiatherosclerotic effect observed with anti-CD3 antibody treatment, we analyzed the percentage of DNA fragmentation within lesions of treated and untreated mice. Using the in situ DNA nick end labeling method,25 we observed a significantly increased percentage of apoptotic cells within atherosclerotic lesions of anti-CD3 antibody–treated mice compared with control mice (Figure 5).
Discussion

Anti-CD3 antibodies are potent immunosuppressive agents that have been used in clinical practice for >20 years to prevent and reverse rejection of various organ allografts. More recently, treatment with anti-CD3–specific antibodies in NOD mice has shown long-term remission of autoimmune diabetes. This unique capacity of anti-CD3 antibodies to restore self-tolerance is mediated by a subset of immunoregulatory T cells, which are selectively expanded and/or activated by anti-CD3–specific antibody treatment. Importantly, short-term administration of an anti-CD3 antibody has recently been shown to preserve pancreatic cells for at least 12 to 18 months in patients with recent-onset type 1 diabetes. In the present study, we report that short-term treatment with anti-CD3–specific antibodies modulates inflammatory processes pivotal for the development of atherosclerosis, thus limiting atherosclerotic plaque formation in LDLR−/− mice. Interestingly, this effect was achieved when anti-CD3 therapy was administered before the onset of clinical symptoms and after the manifestation of advanced atherosclerosis. This first observation is different from previous studies in overt diabetic NOD mice, arthritis, or experimental autoimmune encephalitis in which the efficacy of anti-CD3 antibody treatment was tested after disease onset. A possible explanation to the perceived discrepancy is provided by our finding that 10-week-old LDLR−/− mice on normal chow diet already exhibited moderately elevated levels of cholesterol before the conversion to the high-cholesterol diet. This indicates that anti-CD3 antibody treatment, even when administered in the presence of low plasma concentrations of antigen and already having background inflammation, efficiently modulates T-cell function, thus inhibiting atherosclerotic development.

In support of our findings, a previous study has shown that quantitative differences in TCR stimulation might result in distinct differentiation signals, thus promoting a regulatory rather than effector T-cell development. Those authors demonstrated that prolonged subcutaneous infusion of low doses of peptide by means of osmotic pumps transforms mature T cells into CD4+CD25+ suppressor cells that can persist for long periods of time in the absence of antigen and confer specific immunological tolerance on challenge with antigen. Interestingly, in the experimental autoimmune encephalitis model, anti-CD3 antibody therapy failed to inhibit the clinical disease course when administered in parallel to T-cell priming but significantly protected from disease progression when administered after disease onset. As shown by previous reports, the presence of costimulatory signals present at the time of anti-CD3 antibody therapy may influence treatment efficacy.

More important in view of a possible therapeutic application in patients was our second observation that anti-CD3 antibody therapy efficiently inhibited disease progression in mice with already established atherosclerosis. This was accompanied by an increased relative amount of CD4+ cells within atherosclerotic lesions of anti-CD3 antibody–treated mice. One might speculate that this T-cell population has a protective function, thus modulating inflammatory processes ongoing at sites of atherosclerotic lesions. The analysis of cytokine expression in atherosclerotic lesions has demonstrated the presence of a predominantly proinflammatory, Th1-type T-cell response in atherosclerosis. Consistent with this, different studies have provided evidence for a protective role of the immunosuppressive cytokines IL-10 and TGF-β in animal models of atherosclerosis. It has been reported that treatment with nonmitogenic anti-CD3 antibodies may preferentially suppress Th1 cells and promote a Th2 phenotype, whereas others failed to demonstrate a shift from Th1 to Th2. The discrepant findings on Th2 induction might be explained by the different genetic backgrounds in the mouse models used. In our experiments, we did not observe a shift from Th1 to a Th2 phenotype within lymphoid organs of anti-CD3 antibody–treated mice. A transient Th2 polarization has been described in the overtly diabetic NOD mouse model as demonstrated by a temporary increase in IL-4 production. However, this modulation proved irrelevant for the long-term protective effect because anti-CD3 antibody–induced long-term remission also was present in IL-4–deficient NOD mice. Interestingly, we observed downregulation of both Th1 and Th2 cytokines after 3 weeks of antibody injection, indicating a transient immunosuppressive action of anti-CD3 antibody. This might be explained by the observed temporary downregulation of the CD3/TCR complex, thus inhibiting effector T-cell function. The immunosuppressive action might contribute in part to the observed antiatherosclerotic effect of anti-CD3 antibody treatment.

In addition, TGF-β seems to play a central role in the antiatherogenic effect; we detected a significant increase in anti-CD3 antibody–treated mice 11 weeks after antibody administration. This corresponds well with the findings in overtly diabetic NOD mice, demonstrating that anti-CD3 antibody–induced long-term remission can be TGF-β dependent. Interestingly, a significant increase in TGF-β also was found after 13 weeks of diet in mice receiving anti-CD3 antibody treatment before initiation of diet, indicating similar underlying mechanisms in both the prevention and the progression study.

Anti-CD3 antibody–induced immunosuppression has been associated with mechanisms such as clearance of T cells from the lymphoid organs.
the circulation, depletion of T cells from lymphoid organs, and modulation of the TCR on remnant T cells. However, the effect is less pronounced when nonmitogenic anti-CD3 antibody fragments are used compared with whole anti-CD3 antibodies and does not induce long-term anergy of CD4+ and CD8+ T cells. Consistent with previous studies, we observed a temporary downregulation of CD3 and TCR expression in peripheral blood and lymphoid organs of anti-CD3 antibody–treated mice. The effect was less pronounced in the lymphoid organs. The disappearance of CD3+ cells might be explained either by their transient depletion or, more likely, by downregulation of CD3 expression on the cell surface. In addition, it has been suggested in a previous study that the depletion of T cells after anti-CD3 antibody treatment may be, in part, a consequence of sequestration rather than destruction of T cells. This might explain the rapid disappearance of T cells from the blood, the delay in T-cell depletion in secondary lymphoid organs, and the rapid recovery after treatment.

The percentage of CD3/TCR expression recovered to normal levels after ∼4 weeks after treatment. Thus, it is unlikely that immunosuppression via T-cell depletion and/or downregulation of CD3/TCR expression is the major cause of the observed long-term atheroprotective effect.

Recent advances in understanding the underlying mechanisms that control immune tolerance have highlighted the role of T cells with a regulatory phenotype that exhibit immunosuppressive properties. Adoptive transfer experiments with CD4+CD25+ and CD4+CD62L+ T cells have demonstrated their implication in autoimmune disease. Moreover, natural CD4+CD25+ regulatory T cells have recently been shown to play an important role in the pathogenesis of atherosclerosis. These “regulatory” T cells inhibit the antigen-induced activation of other T cells by producing suppressive cytokines such as IL-10 or TGF-β. Accordingly, our findings of elevated TGF-β production suggest that anti-CD3 antibody therapy induces TGF-β–producing T cells. However, we did not find an increased portion of CD4+CD25+ cells after anti-CD3 antibody therapy, which is in contrast to what has been observed in overtly diabetic NOD mice and the experimental autoimmune encephalitis model. The implication of CD4+CD25+ T cells in the encephalitis model is not fully clear. The percentage of CD4+ cells expressing high levels of CD25 and CD62L was increased in lymph nodes but not in spleens of anti-CD3 antibody–treated mice, whereas antigen-specific proliferation and cytokine secretion were suppressed only in spleens. In addition, depletion of CD4+CD25+ cells failed to reverse the protective effect of anti-CD3 therapy. Similarly, cotreatment with a blocking anti–TGF-β antibody in parallel with anti-CD3 antibody administration did not reverse inhibition of the disease. The lack of a systemic increase in CD4+ or CD4+CD25+ cells in our model is in contrast to the increased accumulation of CD4+ cells within the lesions. Our findings therefore suggest that anti-CD3 antibody treatment induces a selective recruitment of rather than a systemic increase in a protective T-cell population.

Apart from the lack of increase in the CD4+CD25+ cell population, the induction of Foxp3 expression provides further evidence for the activation of regulatory T cells contributing to the antiatherogenic effect. Granzyme B–mediated apoptosis has recently been identified as a key mechanism for the suppressive function of a regulatory subset. In support of these findings, we found an accelerated apoptosis rate in atherosclerotic lesions of anti-CD3 antibody–treated mice. Regarding the question of which cells undergo apoptosis, we can only speculate that regulatory T cells might induce apoptosis of effector T cells.

**Conclusions**

The antiatherosclerotic effect of short-term anti-CD3 antibody treatment was associated with increased TGF-β secretion and Foxp3 induction and with enhanced infiltration of CD4+ cells and apoptosis rate within atherosclerotic lesions. Hence, we hypothesize that anti-CD3 therapy triggers the activation and infiltration of TGF-β–secreting T cells into atherosclerotic lesions, thus modulating inflammatory processes ongoing during atherogenesis.

**Acknowledgments**

We would like to thank W. Ferlin for helpful discussion, Y. Poitevin and A. Topol for antibody purification, and R.W. James for fast protein liquid chromatography analysis of cholesterol fractions.

**Sources of Funding**

This work was supported by grants from the Swiss National Science Foundation and the Novartis Research Foundation to Dr Mach, F. Burger, G. Pelli, and Drs Steffens and Mach belong to the European Vascular Genomics Network (http://www.evgn.org), a Network of Excellence supported by the European Community.

**Disclosures**

None.

**References**


It is now generally recognized that atherosclerosis is a chronic immunoinflammatory disease that can lead to acute clinical events after plaque rupture and thrombosis. Anti-CD3 antibodies are potent immunosuppressive agents that act via modulating the CD3/T-cell receptor complex. Treatment with CD3-specific antibody in nonobese diabetic mice has shown to induce long-term remission of autoimmune diabetes by restoring self-tolerance. Recently, short-term administration of anti-CD3 antibody has been shown to preserve pancreatic cells for at least 18 months in patients with recent-onset type 1 diabetes. This unique capacity of anti-CD3 antibodies to restore self-tolerance, not only in animal models but also in humans, is mediated by a subset of T cells called regulatory T cells, which are selectively expanded and/or activated by anti-CD3-specific antibody treatment. Here, we report that a short-term treatment with anti-CD3-specific antibodies modulates inflammatory processes pivotal for development of atherosclerosis and thus limits atherosclerotic plaque formation in a mouse model. Interestingly, this effect was achieved when anti-CD3 therapy was administered before the onset of clinical symptoms and after manifestation of advanced atherosclerosis.

We document the pleiotropic immunomodulatory effects of anti-CD3 therapy on inflammatory cells by demonstrating anti-CD3 increased production of the proinflammatory cytokine transforming growth factor-β and enhanced expression of the regulatory T-cell marker Foxp3. Thus, our results demonstrate an antitherapeutic potential of a short-term anti-CD3 antibody treatment, suggesting a novel use of these antibodies as a therapeutic agent to treat cardiovascular diseases.
Short-Term Treatment With Anti-CD3 Antibody Reduces the Development and Progression of Atherosclerosis in Mice
Sabine Steffens, Fabienne Burger, Graziano Pelli, Yann Dean, Greg Elson, Marie Kosco-Vilbois, Lucienne Chatenoud and François Mach

Circulation. 2006;114:1977-1984; originally published online October 16, 2006; doi: 10.1161/CIRCULATIONAHA.106.627430
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/114/18/1977

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2006/10/16/CIRCULATIONAHA.106.627430.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/