

Natural regulatory T cells control the development of atherosclerosis in mice

Hafid Ait-Oufella¹, Benoît L Salomon², Stéphane Potteaux¹, Anna-Karin L Robertson³, Pierre Gourdy⁴, Joffrey Zoll¹, Régine Merval¹, Bruno Esposito¹, José L Cohen², Sylvain Fisson², Richard A Flavell⁵, Göran K Hansson³, David Klatzmann², Alain Tedgui¹ & Ziad Mallat¹

Atherosclerosis is an immunoinflammatory disease elicited by accumulation of lipids in the artery wall and leads to myocardial infarction and stroke^{1,2}. Here, we show that naturally arising CD4⁺CD25⁺ regulatory T cells, which actively maintain immunological tolerance to self and nonself antigens^{3,4}, are powerful inhibitors of atherosclerosis in several mouse models. These results provide new insights into the immunopathogenesis of atherosclerosis and could lead to new therapeutic approaches that involve immune modulation using regulatory T cells.

Atherosclerosis is a chronic disease of the arterial wall. Traditional risk factors include hypercholesterolemia, smoking, male gender, hypertension, diabetes and age. But an increasing body of evidence suggests that the immune system is a major factor modulating the atherogenic process^{1,2}. There is solid evidence from several independent groups that T helper type 1 (T_H1)-driven responses are detrimental to the atherosclerotic process^{1,2}. A current paradigm in atherosclerosis stipulates that the opposing forces of T_H1 and T_H2 responses control the disease process^{1,2,5}. This idea is based on findings by our group and others that interleukin (IL)-10, a T_H2-related cytokine, exerts major antiatherogenic effects⁶. In addition, immunization of mice susceptible to atherosclerosis with oxidized low-density lipoproteins is associated with IL-5-dependent atheroprotection, suggesting a T_H2-related effect⁷. However, deficiency in IL-4, the prototypic T_H2-related cytokine, decreases the formation of atherosclerotic lesions⁸, and T_H2 responses may promote progression of atherosclerotic lesions⁹.

A specific component of the immune system, known as the CD4⁺CD25⁺ regulatory T cell (T_{reg}) repertoire, is specialized for the suppression of both T_H1 and T_H2 pathogenic immune responses against self or foreign antigens, and controls T-cell homeostasis (reviewed in refs. 3,4). Recently, we showed that administration of a clone of type 1 regulatory T cells producing high levels of IL-10 downregulated the pathogenic immune response and led to a decrease

in development of atherosclerotic plaques and inflammation in the apolipoprotein E-deficient (*ApoE*^{-/-}) model¹⁰. But the role of the natural CD4⁺CD25⁺ T_{reg} cell repertoire in the control of atherosclerotic plaque development remains unknown. Here, we tested the hypothesis that in atherosclerosis, an imbalance between regulatory and pathogenic immunity substantially contributes both to plaque inflammation and plaque development.

We first examined the effect of deficiency of CD4⁺CD25⁺ T_{reg} cells on the development of atherosclerosis. Because CD25 (IL-2 receptor alpha chain)-deficient (*Il2ra*^{-/-}) mice die prematurely from severe autoimmune disease with cachexia and malabsorption¹¹, they are not suitable for the study of the effect of T_{reg} cell deficiency on atherosclerosis. Similarly, reconstitution of irradiated mice with *Il2ra*^{-/-} bone marrow¹¹ or transfer of *Il2ra*^{-/-} T cells to immunodeficient *ApoE*^{-/-} mice (H.A.-O., B.L.S., P.G., A.T. & Z.M., unpublished data) induces severe autoimmune disease leading to death. Interactions between the costimulatory molecules CD80, CD86 and CD28 are required for the generation and homeostasis of CD4⁺CD25⁺ T_{reg} cells, and these interactions control the development of autoimmune diabetes in nonobese diabetic mice^{12,13}. Therefore, we examined the effect of combined deficiency of *Cd80* and *Cd86* (which encode CD80 and CD86, respectively) on the development of atherosclerosis in C57Bl/6 low-density lipoprotein receptor-deficient (*Ldlr*^{-/-}) mice, known to be susceptible to development of disease when fed a high-fat, high-cholesterol diet. We used the irradiation–bone marrow transplantation model in order to be less dependent on costimulatory activation of T cells and to permit the study of the role of T_{reg} cell deficiency. Development of atherosclerosis was assessed by investigators blinded to the study conditions (**Supplementary Methods** online). After 20 weeks of atherogenic diet, spleen T_{reg} cell number (CD25^{very high} T_{reg}) assessed by flow cytometry was significantly higher in mice reconstituted with bone marrow from *Cd80*^{+/+}*Cd86*^{+/+} mice (2.91 ± 0.13%) compared to those reconstituted with bone marrow from *Cd80*^{-/-}*Cd86*^{-/-} mice (1.00 ± 0.17%; *P* < 0.01). Despite similar total serum cholesterol levels (12.41 ± 0.44 g/l versus 12.65 ± 0.51 g/l in controls and *Cd80*^{-/-}*Cd86*^{-/-} chimeric mice, respectively), we found a marked twofold increase in lesion size at the aortic root in mice reconstituted with *Cd80*^{-/-}*Cd86*^{-/-} bone marrow compared with controls (**Fig. 1a**), indicating an inverse relationship between the presence of T_{reg} cells and the development of atherosclerosis. Similar results were obtained using *Cd28*^{-/-} bone marrow (**Fig. 1a**). Several factors may explain the apparent discrepancy between our results and other previously reported results¹⁴ showing a reduction in lesion development in young *Cd80*^{-/-}*Cd86*^{-/-}*Ldlr*^{-/-} mice. In our model, *Ldlr*^{-/-} mice expressed CD80 and CD86 before irradiation and most probably expressed these proteins on nonhematopoietic cells

¹Institut National de la Santé et de la Recherche Médicale (Inserm), Unité 689, Centre de Recherche Cardiovasculaire Lariboisière, 41, Bd de la Chapelle, 75010 Paris, France. ²Centre National de la Recherche Scientifique, Unité mixte de Recherche 7087, Groupe Hospitalier Pitié-Salpêtrière, 75013 Paris, France. ³Center for Molecular Medicine, Department of Medicine, Karolinska Hospital, Karolinska Institute, SE-17176 Stockholm, Sweden. ⁴Inserm U589, Institut L. Bugnard, 31432 Toulouse Cedex 4, France. ⁵Section of Immunobiology and Howard Hughes Medical Institute, Yale University School of Medicine, 330 Cedar Street, New Haven, Connecticut 06520, USA. Correspondence should be addressed to Z.M. (mallat@larib.inserm.fr).

Received 25 August 2005; accepted 15 November 2005; published online 5 February 2006; doi:10.1038/nm1343



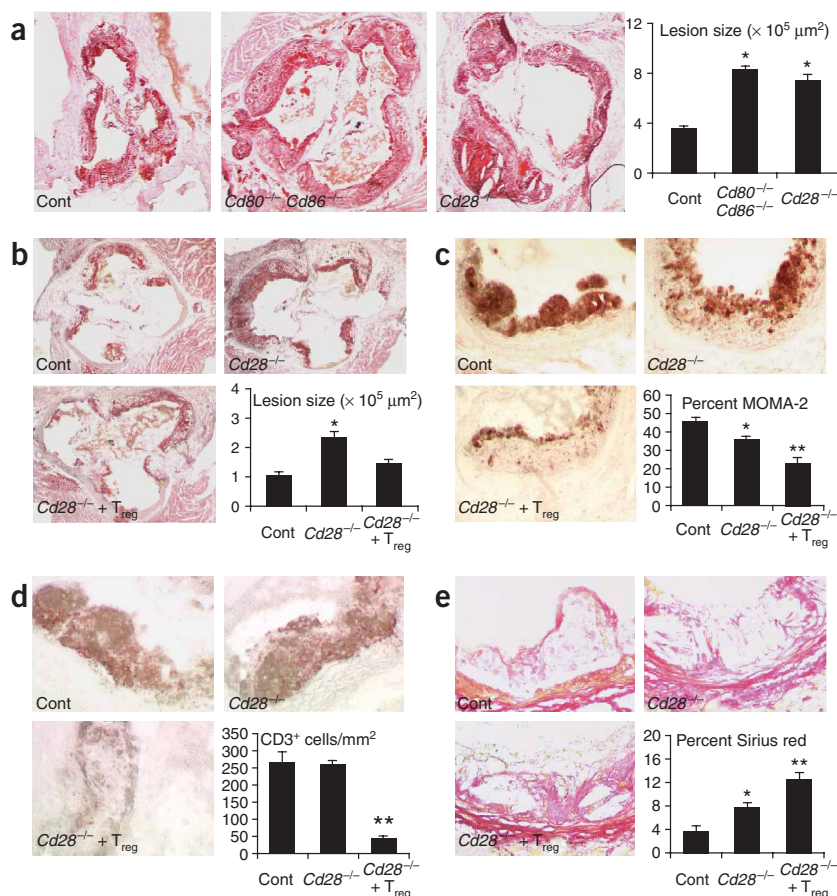


Figure 1 Natural regulatory T cells control the development of atherosclerosis. **(a)** Representative photomicrographs and quantitative analysis of atherosclerotic lesion size in the aortic root of irradiated *Ldlr*^{-/-} mice reconstituted with bone marrow from either wild-type control mice (Cont, *n* = 10), *Cd80*^{-/-}*Cd86*^{-/-} mice (*n* = 9) or *Cd28*^{-/-} mice (*n* = 11), showing increased lesion size in *Cd80*^{-/-}*Cd86*^{-/-} and *Cd28*^{-/-} mice. *Cd80*^{-/-}*Cd86*^{-/-} and *Cd28*^{-/-} mice are deficient in natural regulatory T cells. Values represent mean ± s.e.m. **P* < 0.01. **(b–e)** CD4⁺CD25⁺ T_{reg} cells control the increase in lesion size and inflammation induced by transfer of *Cd28*^{-/-} splenocytes. Eight-week-old female *Apoe*^{-/-}*Rag2*^{-/-} mice were transferred with 10⁷ splenocytes from either wild-type (one group of seven mice) or *Cd28*^{-/-} mice (three groups, seven mice per group). Two of the three groups injected with *Cd28*^{-/-} splenocytes were coinjected with either 10⁶ CD4⁺CD25⁺ or 10⁶ CD4⁺CD25⁻ cells isolated from wild-type mice. Mice were put on a proatherogenic diet for 6 weeks. **(b)** Representative photomicrographs and quantitative analysis of atherosclerotic lesion size in the aortic root. Cont indicates injection with *Cd28*^{+/+} splenocytes. **(c)** Representative photomicrographs and quantitative analysis of lesion macrophage content (MOMA-2). **(d)** Representative photomicrographs and quantitative analysis of lesion T-cell infiltration (number of CD3⁺ cells/mm²). **(e)** Representative photomicrographs and quantitative analysis of lesion collagen (Sirius red) content. Values represent mean ± s.e.m. **P* < 0.05 compared with control, ***P* < 0.05 compared with *Cd28*^{-/-}.

after bone marrow transplantation. Lymphopenia-induced T-cell proliferation in our model is less dependent on costimulation, whereas deficiency of CD80 and CD86 from birth¹⁴ would presumably have affected proliferation and activation of naive T cells. Others¹⁴ have noted a secondary ‘acceleration’ of atherosclerosis in *Cd80*^{-/-}*Cd86*^{-/-} mice, which could be explained by a deficiency of T_{reg} cells.

We next examined the causal relationship between deficiency of CD4⁺CD25⁺ T_{reg} cells and the development of atherosclerotic lesions. First, we examined whether transfer of splenocytes deficient in CD4⁺CD25⁺ T_{reg} cells affected the development of atherosclerosis in *Apoe*^{-/-} mice also deficient in recombinase activating gene-2 (*Rag2*). These mice develop marked atherosclerosis upon transfer of mature T cells. We intravenously administered T_{reg} cell-poor *Cd28*^{-/-} splenocytes to *Apoe*^{-/-}*Rag2*^{-/-} mice. Other *Apoe*^{-/-}*Rag2*^{-/-} mice received wild-type splenocytes and served as controls. No signs of spontaneous autoimmune disease were present in these mice when they were killed (data not shown). Lesion size more than doubled (*P* = 0.012) in *Apoe*^{-/-}*Rag2*^{-/-} mice injected with *Cd28*^{-/-} splenocytes compared to mice injected with wild-type splenocytes (Fig. 1b), despite no difference in serum cholesterol levels (14.5 ± 0.9 g/l versus 13.3 ± 0.7 g/l in mice injected with control or *Cd28*^{-/-} splenocytes, respectively, *P* > 0.5). The lesions of mice injected with the *Cd28*^{-/-} splenocytes showed a more advanced plaque phenotype, as shown by an increase in collagen content (Fig. 1) and a decrease in accumulation of macrophages (Fig. 1c). But substantial T-cell infiltration still occurred in these lesions, suggesting an unabated inflammation (Fig. 1d). Notably, cotransfer of CD4⁺CD25⁺ T_{reg} cells abrogated the induction of atherosclerosis induced by *Cd28*^{-/-} splenocytes (Fig. 1), indicating a major role for natural CD4⁺CD25⁺ T_{reg} cells in the control of atherosclerosis.

In addition, cotransfer of T_{reg} cells resulted in a marked reduction in infiltration of T cells (82% reduction) and macrophages (35% reduction) into plaques and a substantial increase in collagen content (58%), suggesting reduced plaque inflammation and increased plaque healing (Fig. 1). Cotransfer of CD25-depleted CD4⁺ cells with *Cd28*^{-/-} splenocytes into *Apoe*^{-/-}*Rag2*^{-/-} mice led to severe autoimmune disease (colitis, dermatitis, splenomegaly) with a significant decrease in serum cholesterol levels, probably resulting from malabsorption (8.3 ± 0.8 g/l, *P* < 0.0001 compared to the other groups). Nevertheless, these mice still developed inflammatory lipid lesions that were comparable in size to those observed in mice injected with wild-type splenocytes.

We next examined whether the cotransfer of T_{reg} cells resulted in the reconstitution of a regulatory CD4⁺CD25⁺ compartment in *Apoe*^{-/-}*Rag2*^{-/-} mice injected with *Cd28*^{-/-} splenocytes. Absolute cell numbers recovered from spleens were similar between mice injected with *Cd28*^{+/+} or *Cd28*^{-/-} splenocytes with or without T_{reg} cells (data not shown). The percentage of CD4⁺ cells that were also CD25⁺ in mice transferred with *Cd28*^{-/-} splenocytes and coinjected with T_{reg} cells (11.6 ± 0.3%) was similar to that found in mice injected with wild-type splenocytes (9.0 ± 0.6%), and significantly higher than the percentage found in mice injected with *Cd28*^{-/-} splenocytes without cotransfer of T_{reg} cells (4.3 ± 0.9%, *P* < 0.05) or with cotransfer of CD25⁻ cells (3.1 ± 0.5%, *P* < 0.05; Supplementary Fig. 1 online). Using quantitative RT-PCR, we found substantial expression of *Foxp3* mRNA in CD4⁺ cells obtained from *Apoe*^{-/-}*Rag2*^{-/-} mice coinjected with *Cd28*^{-/-} splenocytes and T_{reg} cells, whereas this expression was barely detectable in mice without cotransfer of T_{reg} cells or in mice coinjected with *Cd28*^{-/-} splenocytes and CD4⁺CD25⁻ cells (Supplementary Fig. 1 online).

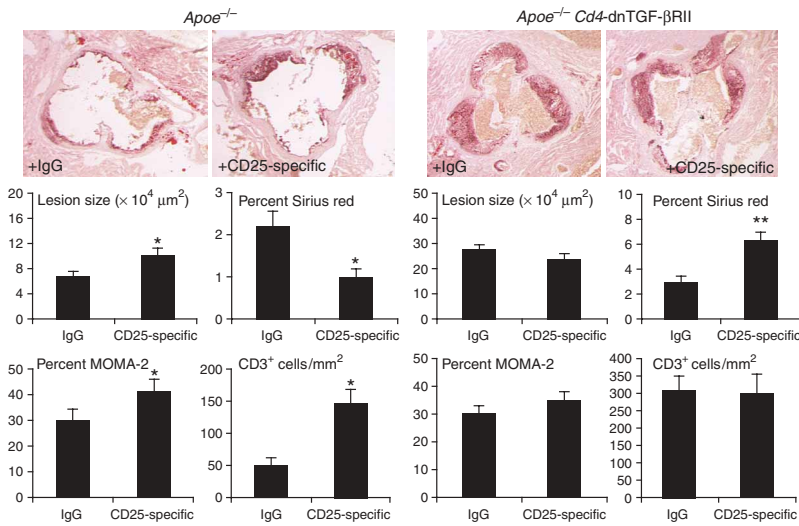


Figure 2 Partial depletion in CD25⁺ cells increases lesion size and inflammation in *Apoe*^{-/-} mice but not in *Apoe*^{-/-} *Cd4*-dnTGF-βRII mice. Representative photomicrographs and quantitative analyses of atherosclerotic lesion size, lesion collagen content (Sirius red) and lesion infiltration by macrophages (MOMA-2) and T cells (CD3) in *Apoe*^{-/-} or *Apoe*^{-/-} *Cd4*-dnTGF-βRII mice treated either with a CD25-specific depleting antibody or isotype-matched control IgG. *n* = 7 for mice treated with IgG and *n* = 8 for mice treated with PC61 antibody. Values represent mean ± s.e.m. **P* < 0.05, ***P* < 0.01.

We also used the CD45.1 congenic marker to track the injected CD4⁺CD25⁺ T_{reg} cells. Six weeks after T_{reg} cell transfer, 6.6% of total lymph node and spleen-derived cells were CD45.1⁺, and 33% of CD45.1⁺ cells showed high expression of CD25 (Supplementary Fig. 1 online). In comparison, only 9.5% of CD45.1⁺ cells showed high expression of CD25 in mice coinjected with CD45.1⁺CD4⁺CD25⁻ cells. CD4⁺CD45.1⁺ cells recovered from the mice with T_{reg} cell cotransfer showed much higher expression of *Foxp3* mRNA as compared with CD4⁺CD45.1⁺ cells from mice coinjected with CD25-depleted cells (Supplementary Fig. 1 online). We next directly assessed the regulatory function of the recovered T_{reg} cells using a coculture experiment. CD45.1⁺ cells isolated from mice coinjected with CD45.1⁺CD4⁺CD25⁺ T_{reg} cells were anergic in culture in response to stimulation with CD3, whereas CD45.1⁺ cells recovered from the mice coinjected with CD45.1⁺CD4⁺CD25⁻ cells proliferated vigorously. This proliferation was substantially inhibited by coculture with CD45.1⁺ cells from T_{reg} cell-coinjected mice in a cell-cell contact-dependent mechanism (Supplementary Fig. 1 online). Neutralization of transforming growth factor (TGF)-β substantially reversed the suppressive effect of T_{reg} cells on the proliferation of effector T cells. In addition, purified CD4⁺ T cells from mice coinjected with T_{reg} cells showed a fourfold increase in production of *Tgfb1* mRNA (Supplementary Fig. 2 online), a threefold increase in CD3-induced production of IL-10 (*P* < 0.001; Supplementary Fig. 3 online) and a 64% reduction in production of interferon (IFN)-γ (*P* < 0.001; Supplementary Fig. 3 online), compared with mice without T_{reg} cell coinjection. Cotransfer of T_{reg} cells did not affect production of IL-4. Our results show that the transferred CD45.1⁺CD4⁺CD25⁺ cells survived *in vivo*, retained a regulatory potential, induced a switch toward an 'antiatherogenic' cytokine profile and halted the development of atherosclerosis.

To further substantiate the role of endogenous T_{reg} cells on the development of atherosclerosis and to confirm their role in a setting with intact co-stimulation, we examined development of atherosclerosis in *Apoe*^{-/-} mice treated with a CD25-specific antibody (PC61) that depletes T_{reg} cells. In pilot experiments using escalating

doses of the antibody (data not shown), we observed a >90% reduction in the percentage of CD25^{high} cells (in lymph nodes) at day 14 and a >75% reduction at day 28 after a single intraperitoneal injection of 100 μg of PC61 antibody. Mice were killed 4 weeks after the second injection. We found a 50% increase in lesion size (*P* = 0.029) in *Apoe*^{-/-} mice treated with the CD25-depleting PC61 antibody (*n* = 8) in comparison with *Apoe*^{-/-} mice treated with the control IgG (*n* = 7; Fig. 2). In addition, lesions of mice treated with the depleting antibody showed less accumulation of collagen and increased accumulation of macrophages and T cells (Fig. 2), indicating enhanced plaque inflammation and reduced healing. Notably, depletion of CD25⁺ cells did not affect lesion development or plaque inflammation in age- and sex-matched littermate *Apoe*^{-/-} mice expressing a dominant negative TGF-β type II receptor under the control of the *Cd4* promoter¹⁵ (Fig. 2), suggesting a requirement for T-cell TGF-β signaling in the T_{reg} cell protective effect on atherosclerosis.

In conclusion, we show that a specific subset of CD4⁺ T cells, termed natural regulatory cells and known to be crucial in the maintenance of peripheral tolerance, has an important role in the development of atherosclerotic lesions. These results identify a new target for the modulation of atherosclerosis and may shed light on potential mechanisms relating autoimmune diseases and atherosclerosis.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by Inserm and by Assistance Publique-Hôpitaux de Paris as a Contrat d'Interface (to Z.M.). Inserm U689 and the Center for Molecular Medicine at Karolinska Institute are partners of the European Vascular Genomics Network, a Network of Excellence granted by the European Commission (contract LSHM-CT-2003-503254). We are indebted to B. Levacher and J. Vilard for technical assistance in Q-PCR experiments, to F. Djelti for her technical support and to J. Nemeth for her assistance in the reading of pathological specimens. We are also indebted to L. Chatenoud for providing us with the 2G7 TGF-β-specific antibody and for discussions. H.A.-O. was supported by Fédération Française de Cardiologie.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at <http://www.nature.com/naturemedicine/>
Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

- Binder, C.J. *et al.* *Nat. Med.* **8**, 1218–1226 (2002).
- Hansson, G.K. *N. Engl. J. Med.* **352**, 1685–1695 (2005).
- Sakaguchi, S. *Nat. Immunol.* **6**, 345–352 (2005).
- von Boehmer, H. *Nat. Immunol.* **6**, 338–344 (2005).
- Daugherty, A. & Rateri, D.L. *Circ. Res.* **90**, 1039–1040 (2002).
- Mallat, Z. *et al.* *Circ. Res.* **85**, e17–e24 (1999).
- Binder, C.J. *et al.* *J. Clin. Invest.* **114**, 427–437 (2004).
- King, V.L., Szilvassy, S.J. & Daugherty, A. *Arterioscler. Thromb. Vasc. Biol.* **22**, 456–461 (2002).
- Davenport, P. & Tipping, P.G. *Am. J. Pathol.* **163**, 1117–1125 (2003).
- Mallat, Z. *et al.* *Circulation* **108**, 1232–1237 (2003).
- Almeida, A.R., Legrand, N., Papiernik, M. & Freitas, A.A. *J. Immunol.* **169**, 4850–4860 (2002).
- Salomon, B. *et al.* *Immunity* **12**, 431–440 (2000).
- Tang, Q. *et al.* *J. Immunol.* **171**, 3348–3352 (2003).
- Buono, C. *et al.* *Circulation* **109**, 2009–2015 (2004).
- Robertson, A.K. *et al.* *J. Clin. Invest.* **112**, 1342–1350 (2003).