Vascular Medicine

Measles Virus Nucleoprotein Induces a Regulatory Immune Response and Reduces Atherosclerosis in Mice

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Background—Recent studies clearly suggest that regulatory T cells play a critical role in the control of the immunoinflammatory response in atherosclerosis and substantially limit lesion development. Measles virus infection or vaccination is associated with immune depression, in part through the induction of an antiinflammatory response by measles virus nucleoprotein. We hypothesized that the antiinflammatory properties of measles virus nucleoprotein may limit the development atherosclerosis.

Methods and Results—Here, we show for the first time that repetitive administration of measles virus nucleoprotein to apolipoprotein E–deficient mice promotes an antiinflammatory T-regulatory-cell type 1–like response and inhibits macrophage and T-cell accumulation within the lesions. Treatment with measles virus nucleoprotein significantly reduces the development of new atherosclerotic plaques and markedly inhibits the progression of established lesions. The antiatherosclerotic potential of nucleoprotein is retained in its short N-terminal segment. The protective effects on lesion size are lost in mice with lymphocyte deficiency.

Conclusions—Our findings identify a novel mechanism of immune modulation by measles virus nucleoprotein through the promotion of a regulatory T-cell response and suggest that this property may be harnessed for treating atherosclerosis, the first cause of heart disease and stroke. (Circulation. 2007;116:1707-1713.)

Key Words: atherosclerosis ■ blood cells ■ immune system ■ inflammation ■ leukocytes ■ viruses

Atherosclerosis develops in response to arterial wall injury caused by several agents, namely modified lipids,1-2 and ultimately leads to tissue ischemia, infarction, and death. Immune pathogenic and regulatory responses control the development and progression of atherosclerosis.1-3 Whereas early lesion development appears to be driven by a T helper cell type 1 (Th1) response,4,5 the progression of advanced lesions also may be promoted under a Th2-biased immune environment.6 This highlights the need to control both Th1- and Th2-driven responses to ensure efficient and sustained protection against atherosclerosis. Recently, we have shown that defined populations of naturally occurring or antigen-induced regulatory T cells, known to suppress both Th1 and Th2 responses,7,8 greatly contribute to inhibition of lesion development in murine models of atherosclerosis,9,10 in part through the production of transforming growth factor (TGF)-β10,11 and interleukin (IL)-10.9 Thus, strategies promoting an endogenous regulatory immune response would be of major interest in developing novel therapies to treat atherosclerosis. Such strategies are in fact part of the armory of some pathogens, the constituents of which are able to induce regulatory immune responses. This is particularly the case with infection with measles virus, which is associated with immunosuppression.12 Measles virus nucleoprotein (NP), in the absence of virus replication, reproduces, at least in part, this systemic immunosuppression in murine models by modulating dendritic cell function and inhibiting antigen-specific T-cell proliferation.13 We hypothesized that a long-term reduction in dendritic cell-dependent T-cell activation after repetitive administration of NP might lead to the generation of a regulatory immune response, of potential interest in the context of atherosclerosis.

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Methods

Production of Recombinant Measles Virus NP
We produced and purified recombinant measles virus NP (525 aa) and its C-terminal part NPe (125 aa) using recombinant baculovirus...
as described. Briefly, we inserted measles virus NP cDNA or NPc cDNA of the Hallé strain into pAcHLT baculovirus transfer vectors containing a 6xHis tag (BD baculogold 6xHis expression and purification kit, BD Biosciences, Le Pont de Claix, France). Recombinants baculoviruses were then generated in the Sf9 insect cell line by homologous recombination with linearized Baculogold, according to manufacturer’s instructions (BD Baculogold 6xHis Expression and purification kit, BD Biosciences). For recombinant protein production, confluent monolayers of High Five insect cells (Invitrogen, Carlsbad, Calif) were infected with the corresponding virus at a multiplicity of 1 plaque-forming unit per cell; infected cells were harvested 4 days after infection, divided into pellets, and stored at −80°C until protein purification. Nucleocapsid formation in infected insect cells allowed the purification of recombinant NP on discontinuous CsCl gradients as described, whereas NPc was purified by affinity chromatography on an Ni-NTA agarose column according to manufacturer’s instructions (BD Baculogold 6xHis expression and purification kit, BD Biosciences). Once purified, each batch of NP or NPc was placed in aliquots in PBS, analyzed by 10% SDS-PAGE and Western blot, and stored at −80°C.

Animals
We used several sets of male apolipoprotein E–deficient (Apoe−/−) mice of 12 or 30 weeks of age (Charles River, Rôle, France). Mice were fed a chow diet and treated, as indicated, with PBS, purified recombinant NP, or purified recombinant NPc. We also used Apoe−Rag2−/− mice at 8 weeks of age. These mice were fed a cholate-free, high-fat diet (1.25% cholesterol, 15% fat) to induce substantial lesion formation, similar to those observed in Apoe−/− mice. These mice also received, as indicated, injections of PBS or purified recombinant NP. Experiments were conducted according to the French community guidelines and those formulated by the European community for experimental animal use (L358–86/609/EEC).

Extent and Composition of Atherosclerotic Lesions
We measured plasma cholesterol using a commercial cholesterol kit (Biomerieux, Marcy l’Etoile, France). The hearts of the mice were removed, and we obtained successive 10-µm transversal sections of aortic sinus. Lipids and collagen were detected using Oil Red O and Sirius Red stainings, respectively. We studied the presence of macrophages, T lymphocytes, and smooth muscle cells using specific antibodies, as previously described. At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used. Thoracic aortas were dissected and stained with Oil Red O. We performed morphometric studies using Histolab software (Microinension Instruments, Evry, France).

Cell Recovery and Purification
We performed negative selection of CD4+ cells using a cocktail of antibody-coated magnetic beads from Miltenyi Biotech (Paris, France) (DX5-, ter 119-, CD8a-, CD11b-, and CD45R-specific antibodies) and purified dendritic cells using CD11c-coated magnetic beads according to manufacturer’s instructions. In some experiments, we purified regulatory and effector T cells with biotin-conjugated anti-CD25 monoclonal antibody (7D4, Pharmingen, San Diego, Calif) and streptavidin microbeads (Miltenyi Biotec), followed by 2 consecutive magnetic cell separations using LS columns (Miltenyi Biotec), giving 80% CD4+CD25+ cells. The CD25+ cells, which did not bind to the beads, were harvested from the flowthrough and contained <0.3% of CD4+CD25+ T cells.

Cell Culture, Proliferation, and Cytokine Assays
Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mmol/L 2B-mercaptoethanol, and antibiotics. To assess in vitro proliferation of purified CD4+ T cells, we cultured them in flat-bottomed 96-well microplates (105 cells per well; total volume, 200 µL/well) in the presence of antigen-presenting cells (2×105 cells per well) purified on CD11c-coated magnetic beads (Miltenyi Biotec) and stimulated the cells with purified soluble CD3-specific antibody (1 µg/mL, Pharmingen). Cells were cultured at 37°C for 96 hours and pulsed with 1 µCi of [3H] thymidine (Amersham Biosciences Inc, Piscataway, NJ) for the last 18 hours of culture. Thymidine incorporation was assessed with a TopCount scintillation counter (Perkin Elmer, Waltham, Mass). To assess the in vitro suppressive potential of natural regulatory T cells, we cultured CD4+CD25+ and CD4+CD25− cells alone or in coculture (at a 1:1 ratio) in flat-bottomed 96-well microplates (0.5×104 cells per well; total volume, 200 µL/well). We stimulated cells with purified soluble CD3-specific antibody (1 µg/mL, Pharmingen) in the presence of CD11c+ cells. Cells were cultured at 37°C for 72 hours and pulsed with 1 µCi of [3H] thymidine (Amersham) for the last 18 hours of culture. For cytokine measurements, we cultured purified CD4+ T cells at 1×105 cells per well for 48 hours in anti-CD3–coated microplates (5 µg/mL) in the presence or absence of CD11c+ cells. IL-4, IL-10, and interferon (IFN)-γ productions in the supernatant were measured with specific ELISAs (R&D Systems, Minneapolis, Minn).

Flow Cytometry
We labeled cells with allophycocyanin-conjugated antibody to CD4 (RM4-5, Pharmingen) and phycoerythrin-conjugated CD25-specific antibody (PC61, Pharmingen) and then analyzed the cells by flow cytometry.

Real-Time Polymerase Chain Reaction Analysis
Total RNA was isolated from purified CD4+ cells with the Trizol reagent (Invitrogen, Carlsbad, Calif). Primer sequences for TGF-β are as follows: forward, 5’-GCAAACAGTGGAATCTCAGAGAA-3’; and reverse, 5’-ACGTCACAGACAGCCACTCA-3’. The primers were purchased from Invitrogen. The real-time polymerase chain reaction was performed on an ABI prism 7700 using Taqman Universal polymerase chain reaction master mix (Applied Biosystems, Foster City, Calif) in triplicates. CT for GAPDH was used to normalize the gene expression of samples.

Determination of Serum Antibodies
For determination of total antibodies, F(ab’)2 fragments of anti-mouse IgG (Pierce Biotechnology Inc, Rockford, Ill) or anti-mouse IgM (Pharmingen) were used as capture antibodies. Low-density lipoprotein (LDL; 1.019<d<1.063) was isolated by ultracentrifugation from Apoe−/− mouse EDTA plasma and copper oxidized with 5 µmol/L Cu++ for 24 hours at 37°C. Capture antibodies and oxidized LDL were coated at 10 µg/mL onto ELISA plate wells. Total and antigen-specific IgM was revealed in serial dilution of individual mouse plasma by using anti-mouse IgM (BD Biosciences) and alkaline-phosphatase–conjugated secondary antibodies and p-nitrophenylphosphate disodium salt substrate. Plates were read at 405 nm.

Statistical Analysis
Values are expressed as medians and quartiles or mean±SEM when appropriate. Statistical tests included 2-way ANOVA, Mann-Whitney, or Kruskal-Wallis tests. Values of P<0.05 were considered significant.

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

Results
Measles Virus NP Reduces Atherosclerotic Lesion Development
We first examined the effects of NP administration on the development of early atherosclerotic lesions. Male Apoe−/− mice have very small lipid lesions in the aortic sinus at the age of 12 weeks. We treated male Apoe−/− mice with intraperitoneal injections of 20 µg purified recombinant NP every other week. Mice were killed 24 to 48 hours after the
immunosuppressive effects of NP reached a threshold at 12 µg and were maximal with 25 µg NP. Control male ApoE−/− mice received repeated injections of PBS. Weights (29.5 ± 1.5 versus 29.5 ± 0.5 g in control and NP groups, respectively; P=0.95) and serum total cholesterol levels (4.8 ± 0.3 versus 4.3 ± 0.7 g/L in control and NP groups, respectively; P=0.61) were similar between control and NP-treated mice. We observed an important and significant 50.3 ± 8.3% reduction (P < 0.05) in the size of atherosclerotic lesions in mice treated with NP compared with controls (Figure 1A). Lesions of mice treated with NP also showed marked 57.9 ± 7.4% reduction in the accumulation of macrophages (P < 0.05), the main component of inflammatory lipid lesions, indicating reduced plaque inflammation (Figure 1B).

Measles Virus NP Inhibits the Progression of Established Atherosclerotic Lesions
In the vast majority of cases, individuals in need of an antiatherosclerosis therapy already have established atherosclerotic plaques. An effective therapy in this population should induce plaque stabilization (reduction in macrophages and enhancement of smooth muscle cell content) and/or limit plaque progression. Thus, we examined the effects of NP treatment on the progression and composition of established atherosclerotic plaques in mice. Male ApoE−/− mice have established lipid lesions in the aortic sinus at the age of 30 weeks (Figure 2). We treated 30-week-old male ApoE−/− mice with 20 µg purified recombinant NP intraperitoneally every other week, and the mice were killed 24 to 48 hours after the seventh NP injection. Control male ApoE−/− mice received repeated injections of PBS. Weights (35.3 ± 1.0 versus 36.9 ± 1.2 g in control and NP groups, respectively) and serum total cholesterol levels (6.1 ± 0.6 versus 6.7 ± 0.7 g/L in control and NP groups, respectively) were similar between control and NP-treated mice. We observed an important and significant 41.1 ± 3.2% reduction (P < 0.001) in the size of atherosclerotic lesions in mice treated with NP compared with controls (Figure 2). Interestingly, NP treatment of ApoE−/− mice at the age of 30 weeks almost blocked further age-related plaque progression. During the 12-week period of treatment, mice treated with NP showed a 6-fold reduction in lesion progression compared with control mice (mean increase in lesion size, 12.2 ± 3.9% in NP-treated mice versus 84.2 ± 11.5% in control mice, P < 0.001) (Figure 2). NP treatment also blocked further macrophage accumulation and necrotic core size enlargement and significantly enhanced smooth muscle cell and collagen contents, suggesting a switch toward a stable plaque phenotype (Figure 2). We also received repeated injections of PBS. Weights (35.3 ± 1.0 versus 36.9 ± 1.2 g in control and NP groups, respectively) and serum total cholesterol levels (6.1 ± 0.6 versus 6.7 ± 0.7 g/L in control and NP groups, respectively) were similar between control and NP-treated mice. We observed an important and significant 41.1 ± 3.2% reduction (P < 0.001) in the size of atherosclerotic lesions in mice treated with NP compared with controls (Figure 2). Interestingly, NP treatment of ApoE−/− mice at the age of 30 weeks almost blocked further age-related plaque progression. During the 12-week period of treatment, mice treated with NP showed a 6-fold reduction in lesion progression compared with control mice (mean increase in lesion size, 12.2 ± 3.9% in NP-treated mice versus 84.2 ± 11.5% in control mice, P < 0.001) (Figure 2). NP treatment also blocked further macrophage accumulation and necrotic core size enlargement and significantly enhanced smooth muscle cell and collagen contents, suggesting a switch toward a stable plaque phenotype (Figure 2). We also
examined lesion progression in the thoracic aorta, another atherosclerosis-prone site. Before treatment, lesions occupied 14.9±2.4% of the total aortic area in 30-week-old male ApoE<sup>-/-</sup> mice. Twelve weeks later, we found a marked inhibition of plaque progression in NP-treated mice (20.7±1.8%; \( P=0.13 \) versus before treatment) compared with control-treated mice (29.8±2.1%; \( P=0.014 \) versus before treatment, \( P=0.02 \) versus NP group) (Figure 3). Thus, NP treatment reduces the development of early lipid lesions and significantly inhibits the progression of established plaques.

**Measles Virus NP Promotes an Antiatherogenic Tr1-Like Phenotype**

We next examined potential mechanisms responsible for NP protective effects in atherosclerosis. The induction of antibodies against oxidized lipoproteins may play a role in limiting lesion development.\(^1\)\(^2\)\(^3\) Levels of IgM antibodies against oxidized LDL did not differ between control and NP-treated groups (median, 0.113 [quartiles, 0.105 to 0.117] in control versus 0.121 [quartiles, 0.117 to 0.150] in NPc) and were slightly elevated in the NP-treated group (0.128 [quartiles, 0.113 to 0.154]; \( P=0.02 \) versus control). However, no correlation was present between IgM anti–oxidized LDL levels and lesion size \( (r^2 = 0.006, P=0.75) \), suggesting no or a marginal role for this immune response in the atheroprotective effect of NP.

An important feature of the lipid lesions of ApoE<sup>-/-</sup> mice treated with measles virus NP was a very important reduction in the accumulation of CD3-positive T lymphocytes (Figure 1C). In addition, T-cell infiltration closely correlated with lesion size \( (r^2 = 0.43, P=0.03) \), suggesting a T cell–mediated protective effect of NP on atherosclerosis. Thus, we examined the effects of NP treatment (6 weeks) on the proliferation and cytokine production of purified CD4<sup>+</sup> T cells in the presence of dendritic cells. We observed a significant reduction in CD4<sup>+</sup> T-cell proliferation in vitro in response to CD3 stimulation when cells were recovered from NP-treated mice compared with control-treated mice (Figure 4A). Incubation of dendritic cells from control mice with CD4<sup>+</sup> T cells of NP-treated mice rescued T-cell proliferation, whereas incubation of dendritic cells from NP-treated mice with CD4<sup>+</sup> T cells of control mice inhibited T-cell proliferation (Figure 4A), suggesting a dendritic cell–dependent effect of NP on T-cell proliferation. The reduction in T-cell proliferation was not associated with a better natural regulatory T-cell function because we found similar levels of CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> cells in NP-treated and control mice (Figure II of the online-only Data Supplement and data not shown). In addition, TGF-\( \beta \) mRNA levels in purified CD4<sup>+</sup> T cells did not differ between control and NP-treated mice (TGF-\( \beta \) ratio, 0.76±0.16 versus 0.75±0.06, respectively).

Besides CD4<sup>+</sup>CD25<sup>+</sup> naturally occurring regulatory T cells, other inducible CD4<sup>+</sup> cells with potent immunoregulatory properties have been described. In particular, T regulatory cells type 1 (Tr1) are CD4<sup>+</sup> T lymphocytes that are...
defined by their ability to produce IL-10 but not IL-4 and to suppress Th1 and Th2 cells.16,17 Therefore, we assessed cytokine production in T-cell supernatants after CD3 stimulation in the presence of dendritic cells. We found an increase in T-cell production of IL-10 (Figure 4B) and a significant reduction in both IL-4 (Figure 4C) and Ifn-γ (Figure 4D) compared with controls (96 ± 3 versus 22 ± 1 pg/mL, respectively, n = 3 per group; P < 0.05). We found no difference in IL-12 production (data not shown).

We also assessed cytokine production by purified CD4 cells stimulated with a combination of CD3/CD28 antibodies in the absence of DC. Under these conditions, we also found increased IL-10 production in CD4 cells of NP-treated animals compared with controls (608 ± 99 versus 261 ± 40 pg/mL, respectively; P < 0.05) with no difference in Ifn-γ production (7795 ± 370 versus 6986 ± 402 pg/mL, respectively). These results suggest that CD4 T cells recovered from NP-treated animals have acquired a relatively stable capacity to produce high levels of IL-10, consistent with a regulatory phenotype.

To examine whether the induction of a Tr1-like immune response by NP was required for NP protective effects in atherosclerosis, we repeated NP injections in ApoE-/-Rag2-/- mice, which are deficient in lymphocytes. Interestingly, we found no effect of NP treatment on lesion size in ApoE-/-Rag2-/- mice (Figure 5), suggesting that the induction of a regulatory T-cell immune response after NP treatment is required for its antiatherosclerotic effect.

Discussion

Recent studies have clearly suggested a central role for regulatory T cells in the control of the immunoinflammatory response in atherosclerosis, leading to limitation of lesion development.3,18 Deficiency in natural CD4+CD25+Foxp3+ T regulatory (Treg) cells accelerates atherosclerosis in mice,10,19 and defective natural Treg cell function has been associated with the presence of stable or unstable coronary artery disease.20 In addition, strategies leading to improvement in natural Treg cell number or Treg-related immune response lead to inhibition of lesion development and block the progression of established lesions in mouse models of atherosclerosis.21,22

Besides naturally occurring Treg cells, another subset of regulatory T cells, called adaptive Treg cells, develops in the periphery in response to antigen stimulation and may play a significant role in the control of atherosclerosis.9 The adaptive Treg cells do not necessarily express CD25 and show Foxp3-independent but cytokine-dependent suppressive function. The IL-10–producing Treg cells are known as Tr1 cells. These cells can be generated in vivo by long-term antigenic stimulation or mucosal administration of antigen23,24; in vitro, particularly in the presence of dendritic cells secreting high levels of IL-10,16,25,26; or after coengagement of CD3 and CD46.17 Interestingly, measles virus NP has been shown to inhibit the production of IL-12 by dendritic cells but preserves IL-10 production,13 leading to significant inhibition of antigen-specific immune responses in various models of hypersensitivity. This alteration of dendritic cell function in response to measles virus NP suggests a potential role for the latter in the induction of a Tr1-like immune response. In this study, we show that long-term administration of measles virus NP leads to an increase in IL-10 production by CD4 T cells in vitro associated with a reduction in T-cell proliferation and expansion of Th1-like cells in the presence of dendritic cells. This cytokine profile also could be induced in vitro by incubation of CD4 T cells recovered from PBS-treated mice with dendritic cells recovered from NP-treated mice, suggesting that NP treatment has modulated dendritic cell function toward a potentially tolerogenic phenotype. However, it is important to examine in more detail the characteristics of these cells, including the expression of CD45RB, myosin heavy chain-II, and costimulatory molecules, as well as the mechanisms responsible for the induction of Tr1-like cells. In addition, whether such a Tr1 switch could account for the suppression of immune-mediated diseases observed during measles virus infection12 is presently unknown.
Currently, no valid methodology exists to directly isolate or deplete Tr1 cells to examine their direct role in immune-mediated diseases. However, we believe that our data argue for a T cell–mediated protective effect of NP in atherosclerosis. First, the involvement of the B cell is probably marginal for a T cell–mediated protective effect of NP in atherosclerosis. However, we believe that our data argue for a T cell–mediated protective effect of NP in atherosclerosis. We show that repetitive administration of measles virus NP to mice susceptible to atherosclerosis induces a regulatory immune response and limits both lesion development and progression. Our findings identify a novel mechanism of immune modulation by measles virus NP through the promotion of a Tr1-like response and suggest that this property of NP or its fragments may be harnessed for treating atherosclerosis, the first cause of heart disease and stroke.

Conclusions

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Acknowledgments

The antiatherogenic effect of measles virus NP is lost in mice with lymphocyte deficiency. Quantitative analysis of serum total cholesterol (A) and atherosclerotic lesion size (B) in the aortic sinus of Apoe/Rag2–/– mice fed a cholate-free high-fat diet and injected intraperitoneally at the age of 8 weeks with either PBS (Cont; n=6) or 20 µg measles virus NP (n=8) every other week for 6 weeks. NP treatment did not alter lesion size in mice with lymphocyte deficiency.
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Disclosures
None.

References

CLINICAL PERSPECTIVE
Atherosclerosis develops in response to arterial wall injury caused by several agents, namely modified lipids, and ultimately leads to tissue ischemia, infarction, and death. Immune pathogenic and regulatory responses control the development and progression of atherosclerosis. Recent studies clearly suggest that regulatory T cells, a subset of T cells that protect against autoimmune diseases, play a critical role in the control of the immunoinflammatory response in atherosclerosis and substantially limit lesion development. Thus, we hypothesized that strategies promoting an endogenous regulatory immune response would be of major interest in developing novel therapies to treat atherosclerosis. Such strategies are in fact part of the armory of some pathogens, particularly measles virus, the constituents of which are able to inhibit immune responses. Here, we show for the first time that repetitive administration of measles virus nucleoprotein to apolipoprotein E−deficient mice, a validated experimental model of atherosclerosis, promotes an antiinflammatory T-regulatory-cell type 1–like response (characterized by enhanced interleukin-10 production) and inhibits macrophage and T-cell accumulation within the lesions. Treatment with measles virus nucleoprotein significantly reduces the development of new atherosclerotic plaques and markedly inhibits the progression of established lesions. Our findings identify a novel mechanism of immune modulation by measles virus nucleoprotein through the promotion of a regulatory T-cell response and suggest that this property may be harnessed for treating atherosclerosis, the first cause of heart disease and stroke. Additional studies are required to establish the safety of such therapeutic approach.
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Data Supplement Figure I. Representative data showing induction of anti-NP/NPc antibodies following repetitive treatment of Apoe-deficient mice with phosphate buffered saline (control), NP or NPc. NPc treatment is not immunogenic.
Supplementary Figure II. Assessment of natural regulatory T cell function. Panel (a), shows similar levels of CD4+CD25+ in the spleens of control and NP-treated Apoe–/– mice using flow cytometry. Values are mean ± s.e.m. of n=3 mice per group. Panel (b), shows suppression of proliferation of CD4+CD25– effector cells by CD4+CD25+ cells from control or NP-treated mice. Proliferation was assessed by ³[H] thymidine incorporation after stimulation with soluble anti-CD3 in the presence of purified CD11c+ dendritic cells. Results are expressed as percentage inhibition of CD4+CD25– effector cells. Cells were purified and pooled from 3 mice in each group. Values are mean ± s.e.m. of triplicate measurements.