Basic Science Reports

Induction of a Regulatory T Cell Type 1 Response Reduces the Development of Atherosclerosis in Apolipoprotein E–Knockout Mice

Ziad Mallat, MD, PhD; Andrea Gojova, PhD; Valérie Brun, PhD; Bruno Esposito; Nathalie Fournier, PhD; Françoise Cottrez, PhD; Alain Tedgui, PhD; Hervé Groux, PhD

Background—T helper type 1 (Th1) response plays a permissive role in atherosclerosis. We hypothesized that adoptive transfer of a novel subtype of T lymphocytes called regulatory T cells type 1 (Tr1) would inhibit Th1 responses by inducing a bystander immune suppression and therefore limit the development of atherosclerosis.

Methods and Results—Clones of ovalbumin (OVA)–specific Tr1 cells expanded in vitro were administered intraperitoneally (10^6 cells per mouse) with their cognate antigen (50 μg of OVA subcutaneously in complete Freund’s adjuvant [CFA]) to female apolipoprotein E–knockout mice. A group of mice received only (OVA/CFA) immunization without Tr1 cells. Two other control groups received no immunization and were injected with either Tr1 cells or saline. After 9 weeks of treatment, mice injected with (OVA/CFA)+OVA-specific Tr1 cells showed a significant decrease in Th1 responses, as revealed by a decrease in OVA-specific IgG2a serum levels (P<0.0001), a decrease in the production of interferon-γ (P<0.001), and an increase in interleukin-10 production (P<0.001) by cultured spleen and lymph T cells compared with controls. In addition, cytokine production by concanavalin A–stimulated spleen cells showed a clear switch to a regulatory immune response in mice treated with (OVA/CFA)+Tr1. This was associated with a significant reduction in atherosclerotic lesion size in both the thoracic aorta and aortic sinus of mice treated with (OVA/CFA)+Tr1 compared with controls (P=0.002 to P<0.0001). Plaques of mice injected with (OVA/CFA)+Tr1 showed significantly lower accumulation of macrophages and T cells than plaques of control mice.

Conclusions—Tr1-type regulatory immune response reduces the development of experimental atherosclerosis.

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Key Words: atherosclerosis □ inflammation □ immunity □ lymphocytes □ immunization

Atherosclerosis is a chronic disease of the arterial wall in which both innate and adaptive immunoinflammatory mechanisms are involved.1–3 The profile of the T-cell response in atherosclerosis is of the Th1 helper type 1 (Th1),3 whose proatherogenic potential has been demonstrated in experimental models of atherosclerosis.4–10 As in other chronic immunoinflammatory diseases, it has been suggested that the development and progression of atherosclerosis may result from an imbalance between Th1 and Th2 responses.5,11 However, evidence is now accumulating for a novel functionally distinct subpopulation of T cells, called regulatory T cells, that exert important regulatory functions in various immunoinflammatory diseases (see Groux et al,12 Roncarolo et al,13 and McGuirk et al14 for review). Several subsets of regulatory T (Tr) cells with distinct phenotypes and distinct mechanisms of action have now been identified. These include Tr1 cells,15–20 which secrete high levels of interleukin (IL)-10 and low to moderate levels of transforming growth factor (TGF)-β; Th3 cells,21,22 which secrete primarily TGF-β; and CD4+CD25+ T cells, which inhibit immune responses through cell-to-cell contact.23 We have shown that repeated stimulation of naïve T cells from ovalbumin (OVA) T-cell receptor–transgenic mice with OVA and IL-10 results in the generation of T-cell clones with a unique cytokine profile distinct from that of Th0, Th1, or Th2 cells.15 These Tr1 cells produce IL-10 and some IL-5 and interferon-γ (IFN-γ) with or without TGF-β but with little or no IL-2 or IL-4 and proliferate poorly after polyclonal T-cell receptor–mediated activation. Functional studies on Tr1 cells have indicated that Tr1 cells have immunosuppressive properties and have been shown to prevent the development of Th1-mediated autoimmune diseases.15 Coculture of naïve CD4+ T cells and human Tr1 clones in the presence of allogenic antigen-presenting cells results in the suppression of prolif-
Tr1 Cell Transfer Reduces Atherosclerosis

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Table 1. Proliferation and Cytokine Production by OVA-Stimulated Lymph Node Cells

<table>
<thead>
<tr>
<th></th>
<th>Saline (n=5)</th>
<th>OVA/CFA (n=9)</th>
<th>Tr1 (n=6)</th>
<th>Tr1+OVA/CFA (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ, ng/mL</td>
<td>0.01±0.001</td>
<td>3.34±1.2*</td>
<td>0.02±0.001</td>
<td>1.4±0.2†</td>
</tr>
<tr>
<td>IL-10, ng/mL</td>
<td>0.1±0.02</td>
<td>0.34±0.009*</td>
<td>0.09±0.01</td>
<td>1.3±0.3†</td>
</tr>
<tr>
<td>IL-4, pg/mL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-5, ng/mL</td>
<td>0.01±0.001</td>
<td>0.16±0.02*</td>
<td>0</td>
<td>0.1±0.01†</td>
</tr>
<tr>
<td>Proliferation, cpm</td>
<td>1231±136</td>
<td>25147±1267</td>
<td>1567±637</td>
<td>9547±957†</td>
</tr>
<tr>
<td>IgG2a, ng/mL</td>
<td>0.677±0.09</td>
<td>10555±2254*</td>
<td>0.11±0.01</td>
<td>3567±985†</td>
</tr>
<tr>
<td>IgE, pg/mL</td>
<td>0</td>
<td>57±12*</td>
<td>0</td>
<td>29.1±9†</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD.

*P<0.001 vs saline, †P<0.001 vs OVA/CFA.
TABLE 2. Cytokine Production by ConA-Stimulated Splenic T Cells

<table>
<thead>
<tr>
<th></th>
<th>Saline (n=5)</th>
<th>OVA/CFA (n=9)</th>
<th>Tr1 (n=6)</th>
<th>Tr1 + OVA/CFA (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ, ng/mL</td>
<td>2.63±1.6</td>
<td>3.39±1.5*</td>
<td>2.6±1.3</td>
<td>1.7±0.3†</td>
</tr>
<tr>
<td>IL-10, ng/mL</td>
<td>0.5±0.05</td>
<td>1.05±0.2*</td>
<td>0.58±0.1</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>IL-4, pg/mL</td>
<td>137±27</td>
<td>54±12</td>
<td>148±31</td>
<td>0</td>
</tr>
<tr>
<td>IL-5, ng/mL</td>
<td>0.68±0.03</td>
<td>0.3±0.1*</td>
<td>0.69±0.08</td>
<td>0.12±0.05†</td>
</tr>
</tbody>
</table>

Results represent mean±SD.

*P<0.001 vs saline, †P<0.001 vs OVA/CFA.

Results

Transfer of Tr1 Cells With Their Cognate Antigen Induces Immune Suppression

As expected (Table 1), treatment with OVA in CFA resulted in elevated serum levels of OVA-specific antibodies, predominantly IgG2a, and to a much lesser extent IgE, in comparison with the saline-treated group, in which OVA-specific IgGs were undetectable. Treatment of mice with OVA/CFA induced a Th1-like response, as revealed by the production of high levels of IFN-γ (Th1 cytokine) but low levels of IL-10 and no IL-4 (Th2 cytokines) by OVA-stimulated lymph T cells (Table 1). Injection of regulatory Tr1 cells alone did not alter the immune response (Table 1). However, administration of Tr1 cells to OVA/CFA-immunized animals significantly inhibited the secretion of OVA-specific IgG2a antibodies, as reported previously in a model using alum as an adjuvant. Moreover, the suppressive effect of Tr1 cells was confirmed by the marked decrease in IFN-γ production and proliferative response observed in the (OVA/CFA)+Tr1 group compared with the OVA/CFA after in vitro recall response of T cells (P<0.001). This was associated with enhanced IL-10 production by T cells stimulated with OVA in vitro (P<0.001), suggesting that the transferred OVA-specific Tr1 cells were functionally active (Table 1). TGF-β in the supernatants was below detectable levels.

IL-10 is a cytokine with important immunosuppressive effect in vivo. Therefore, to analyze the consequence of high IL-10 secretion in mice transferred with Tr1 cells and treated with OVA/CFA, we analyzed the cytokine response of T cells after polyclonal activation with conA. As expected, conA stimulation induced a Th0 type response in T cells from saline- or Tr1-treated mice and a Th1-type response in mice treated with OVA/CFA (Table 2). In contrast, in mice treated with regulatory Tr1 cells and OVA/CFA, we observed a marked decrease in IFN-γ production and an increase in IL-10 production by conA-stimulated T cells (Table 2). Interestingly, the ratio of IFN-γ to IL-10 was reduced by 3- to 4-fold in the (OVA/CFA)+Tr1 group compared with the other groups of mice, suggesting that the transfer of regulatory Tr1 cells followed by a systemic delivery of their specific antigen induced a bystander nonspecific regulatory immune response as a result of the chronic stimulation of Tr1 cells with OVA/CFA. However, as shown previously in other inflammatory models, the inhibitory function of Tr1 cells is local, and chronic stimulation of Tr1 cells did not result in systemic secretion of IL-10 in the serum, because <30 pg/mL of IL-10 was measured in the serum of the 4 different groups of mice.

Transfer of Tr1 Cells With Their Cognate Antigen Reduces Atherosclerotic Lesion Size in ApoE-Deficient Mice

Serum total cholesterol and HDL levels did not differ between groups (Table 3). Atherosclerotic lesion size in the thoracic aorta did not differ between the saline and Tr1 groups (Table 3). There was a modest but not statistically significant reduction in lesion size in the thoracic aorta of mice treated with OVA/CFA in comparison with the saline or Tr1 group (P=0.09 and P=0.07, respectively). In contrast, there was a marked and highly significant reduction in lesion size in the (OVA/CFA)+Tr1 group in comparison with either OVA/CFA (42% reduction, P=0.008), Tr1 alone, or saline (55% reduction, P<0.001) (Table 3).

Atherosclerotic lesion size in the aortic sinus did not differ between saline-, Tr1-, and OVA/CFA-treated animals. However, we found a highly significant decrease of plaque size in the (OVA/CFA)+Tr1 group compared with either the saline

TABLE 3. Serum Cholesterol Levels and Atherosclerotic Lesion Size and Composition in the Aortic Sinus of ApoE-Deficient Mice

<table>
<thead>
<tr>
<th></th>
<th>Saline (n=5)</th>
<th>OVA/CFA (n=9)</th>
<th>Tr1 (n=6)</th>
<th>Tr1 + OVA/CFA (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, g/L</td>
<td>4.22±0.2</td>
<td>4.03±0.13</td>
<td>4.05±0.19</td>
<td>3.90±0.15</td>
</tr>
<tr>
<td>HDL cholesterol, g/L</td>
<td>0.17±0.04</td>
<td>0.11±0.02</td>
<td>0.14±0.02</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>Lesion size in thoracic aorta, %</td>
<td>8.4±0.9</td>
<td>6.5±0.7</td>
<td>8.5±1.0</td>
<td>3.8±0.3*</td>
</tr>
<tr>
<td>Lesion size in aortic sinus, μm²</td>
<td>323 564±28 956</td>
<td>317 120±13 464</td>
<td>376 102±22 697</td>
<td>234 511±11 797†</td>
</tr>
<tr>
<td>MOMA2-positive area, %</td>
<td>34.6±1.8</td>
<td>27.3±2.3</td>
<td>31.4±3.6</td>
<td>18.6±1.4†</td>
</tr>
<tr>
<td>CD3-positive, cells/mm²</td>
<td>283.3±31.7</td>
<td>314.5±39.6</td>
<td>257.2±29.4</td>
<td>159.2±25.3§</td>
</tr>
<tr>
<td>α-Actin-positive area, %</td>
<td>5.0±1.1</td>
<td>4.9±0.9</td>
<td>5.8±1.0</td>
<td>4.6±0.9</td>
</tr>
<tr>
<td>Sirius red-positive area, %</td>
<td>37.5±2.4</td>
<td>39.6±2.3</td>
<td>39.6±3.9</td>
<td>35.4±2.2</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P=0.0002 vs Tr1, P=0.008 vs OVA/CFA, and P=0.0003 vs saline.

†P<0.0001 vs Tr1, P=0.001 vs OVA/CFA, and P=0.002 vs saline.

‡P=0.0006 vs Tr1, P=0.006 vs OVA/CFA, and P<0.0001 vs saline.

§P<0.05 vs Tr1, P=0.001 vs OVA/CFA, and P=0.02 vs saline.
Despite the significant decrease in lesion size, plaques of mice treated with (OVA/CFA)/H11001 showed a marked decrease in relative macrophage infiltration (32% to 46% reduction, Table 3 and Figure 1) and T-cell accumulation (37% to 50% reduction, Table 3), with no change in smooth muscle cell or collagen content (Table 3). In addition, we found intense IL-10 staining in the plaques of mice treated with (OVA/CFA)+Tr1, whereas no or barely detectable levels of IL-10 were found in plaques from the other 3 groups (Figure 2). IL-10 staining was also detected in the adventitia of all study groups but was clearly enhanced in the (OVA/CFA)+Tr1 group (Figure 2). Overall, these results suggest that a regulatory T-cell response is associated with a less pronounced inflammatory plaque phenotype.

**Discussion**

A substantial body of evidence suggests an important role for Th1-mediated immunoinflammatory responses in the development and progression of atherosclerotic lesions, at least in experimental models of human-like atherosclerosis. There are also reports from human studies suggesting a strong association between Th1-related responses and plaque instability. Therefore, much of the recent research in the field of atherosclerosis, based on current dogma suggesting that the immunoinflammatory response in atherosclerosis is controlled by distinct Th1 and Th2 subpopulations of T cells, has focused on the potential role of Th2-mediated responses in the modulation of the atherosclerotic process. The finding by several independent groups that IL-10, a Th2-related cytokine, exerts major antiatherogenic effects has particularly reinforced the Th1/Th2 paradigm in immunity to atherosclerosis. However, IL-10 is not specific to Th2 cells. Interestingly, another subtype of T cells, Tr1, with cytokine profiles distinct from either Th1 or Th2 cells, has been identified and shown to play an important role in the regulation of and protection against various Th1- and Th2-mediated immunoinflammatory diseases (see References 12–14 for review). Therefore, we hypothesized that in atherosclerosis, there may be an imbalance between the effector and the regulatory (for instance, Tr1) arms of the immune response and suggested that supplementation with Tr1 cells may lead to the induction of a regulatory immune response.
phenotype and a reduction in Th1- and Th2-mediated responses, ultimately altering plaque development and/or composition.

Here, we showed that these regulatory Tr1 cells, when transferred into mice with their cognate antigen, induced a significant suppression of Th1-mediated responses and led to an increase in IL-10 production by stimulated peripheral T cells. Tr1-mediated immune suppression was observed in response to stimulation with either OVA or conA, suggesting the induction of a systemic bystander immune suppression, which most likely resulted from repeated administration of OVA/CFA. Interestingly, the induction of Tr1 responses was associated with a significant reduction in atherosclerotic plaque size and a marked reduction in the relative accumulation of inflammatory macrophages and T lymphocytes with preservation of smooth muscle cell and collagen contents. It is noteworthy that the transfer and in vivo activation of Tr1 cells led to a profound reduction in the ratio of IFN-γ to IL-10 compared with the other groups (Table 2) and with the appearance of intense IL-10 staining within the plaques, suggesting that this change in the balance between proatherogenic and antiatherogenic mediators may have contributed to the alterations in lesion development and composition. These results show that modulation of the immune response is achievable by transfer and activation of Tr1 cells and leads to limitation of the development of atherosclerosis in apoE-deficient mice.

It could be argued that our data may simply be compatible with a systemic effect of circulating IL-10 and that such an effect may be independent of any regulatory or immunosuppressive function. Although it is very difficult to separate the regulatory functions of Tr1 clones from their capacity to produce IL-10 (several studies have established the critical contribution of the immunomodulatory cytokine IL-10 to the regulatory potential of Tr1; for review, see References 12 and 13), we believe that several data argue against such a hypothesis. First, we have been unable to detect any substantial production or difference in serum IL-10 levels between the various study groups, whereas IL-10 protein accumulation was increased locally within the plaques of the OVA/CFA+Tr1 group. Second, not all IL-10–producing T cells are regulatory T cells. Pinderski et al.10 have recently shown that overexpression of IL-10 by T lymphocytes (IL-10 was placed under the control of the IL-2 promoter) led to a switch toward a Th2 phenotype that is phenotypically and functionally distinct from all Tr1 clones. Unlike the results obtained with the overexpression of IL-10 in the latter study,33 we showed in our work that appropriate activation of Tr1 led to significant reductions in both Th1- (IFN-γ) and Th2-related (IL-4 and IL-5) cytokines and immunoglobulins (IgG2a and IgE, respectively) (Tables 1 and 2). This is consistent with previous studies showing that only regulatory T cells, like Tr1, are able to suppress both Th1- and Th2-related responses.12,13,20,24

Another potential limitation to our study is the confounding effect of CFA on atherosclerotic lesion development. Indeed, previously published studies have reported a reduction in atherosclerosis with CFA/IFA.34,35 These reductions in the aortic arch or the thoracic aorta were moderate (18% and 19%) and were accompanied by changes in total and/or HDL cholesterol levels that may have contributed to limitation of plaque progression. It is noteworthy that in our study, the OVA/CFA group showed a reduction in atherosclerotic lesion size in the thoracic aorta (23%) similar to that reported in studies using CFA/IFA, even in the absence of changes in cholesterol levels. However, in our study, the additional transfer of Tr1 cells resulted in a much more important and marked 55% reduction in atherosclerosis.

In conclusion, our results provide the first proof of the concept that cell transfer of a novel subpopulation of T cells with regulatory functions, here Tr1 cells, is able to modulate the in vivo immune response and leads to substantial limitation of plaque size while inducing changes in plaque composition indicative of a less inflammatory plaque phenotype. This work paves the way for the development and testing of novel therapeutic strategies based on the enhancement and/or transfer of specific regulatory immune cells (ie, Tr1 cells specific for a plaque-derived antigen) to limit the development and prevent the complications of atherosclerosis.

Acknowledgments

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References


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