Immunotherapy with tolerogenic apolipoprotein B-100–loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice

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Immunotherapy With Tolerogenic Apolipoprotein B-100–Loaded Dendritic Cells Attenuates Atherosclerosis in Hypercholesterolemic Mice

Andreas Hermansson, PhD; Daniel K. Johansson, MSc; Daniel F.J. Ketelhuth, PhD; John Andersson, PhD; Xinghua Zhou, MD, PhD*; Göran K. Hansson, MD, PhD*

Background—Atherosclerosis is a chronic inflammatory disease characterized by a massive intimal accumulation of low-density lipoprotein that triggers chronic vascular inflammation with an autoimmune response to low-density lipoprotein components.

Methods and Results—To dampen the inflammatory component of atherosclerosis, we injected hypercholesterolemic huB100tg\times Ldlr\textsuperscript{-/-} mice (mice transgenic for human apolipoprotein B100 [ApoB100] and deficient for the low-density lipoprotein receptor) intravenously with dendritic cells (DCs) that had been pulsed with the low-density lipoprotein protein ApoB100 in combination with the immunosuppressive cytokine interleukin-10. DCs treated with ApoB100 and interleukin-10 reduced proliferation of effector T cells, inhibited production of interferon-γ, and increased de novo generation of regulatory T cells in vitro. Spleen cells from mice treated with DCs plus ApoB100 plus interleukin-10 showed diminished proliferative responses to ApoB100 and significantly dampened T-helper 1 and 2 immunity to ApoB100. Spleen CD4\textsuperscript{+} T cells from these mice suppressed activation of ApoB100-reactive T cells in a manner characteristic of regulatory T cells, and mRNA analysis of lymphoid organs showed induction of transcripts characteristic of these cells. Treatment of huB100tg\times Ldlr\textsuperscript{-/-} mice with ApoB100-pulsed tolerogenic DCs led to a significant (70%) reduction of atherosclerotic lesions in the aorta, with decreased CD4\textsuperscript{+} T-cell infiltration and signs of reduced systemic inflammation.

Conclusions—Tolerogenic DCs pulsed with ApoB100 reduced the autoimmune response against low-density lipoprotein and may represent a novel possibility for treatment or prevention of atherosclerosis. (Circulation. 2011;123:1083-1091.)

Key Words: immunology ■ atherosclerosis ■ inflammation ■ cytokines ■ apolipoproteins ■ lipoproteins

In atherosclerosis, low-density lipoprotein (LDL) accumulates within the arterial wall, attracting immune cells and leading to chronic inflammation.1 This process involves an autoimmune response to the protein moiety of LDL, apolipoprotein B100 (ApoB100).2–5 Antigen-presenting dendritic cells (DCs) traverse the arterial wall and the atherosclerotic plaque, where they may take up LDL components before migrating to lymphoid organs. This could initiate and boost cellular immune responses by presenting ApoB100 to T cells in the draining lymph nodes.6–10 Such responses contribute significantly to the progression of atherosclerosis.11–13

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The evolving plaque is dominated by a T-helper 1 (Th1)–type response, with production of proinflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor (TNF-α), and interleukin (IL)-12,14 but also chemokines such as CCL2, CCL5, and CXCL4,15 which stimulate the influx of various types of leukocytes.16,17 Several therapeutic strategies have been devised to dampen the inflammatory process in the plaque, but the limited mechanistic understanding of molecular mechanisms has hampered their translation into clinical utility.2 However, advances in understanding the plasticity of the DC system may open up novel possibilities for the treatment of atherosclerosis.

DCs orchestrate the defense against infectious agents, as well as the maintenance and regulation of peripheral tolerance.18,19 Although immunogenic myeloid DCs play a major role in the initiation of inflammation and regulation of adaptive immunity, the tolerogenic properties of such DCs can be manipulated by immunomodulatory mediators such as cytokines, which provides opportunities for prevention and
therapy of autoimmune diseases.20,21 Numerous approaches have been adopted during recent years to generate tolerogenic DCs for treatment of autoimmune disorders and improvement of allograft survival.21 One of the most promising strategies includes induction of antigen-specific tolerogenic DCs by incubation with the immunosuppressive cytokine IL-10. Treatment of DCs with IL-10 downregulates secretion of inflammatory cytokines such as TNF-α, IL-6, and IL-12; induces anergy of antigen-specific T cells; and promotes generation of regulatory T cells (Treg).21–25 The utility of these immunoregulatory DCs as treatment of atherosclerotic disease has not been examined previously.

Here, we show that IL-10 imposes a regulatory phenotype on DCs, and that such tolerogenic DCs induce T-cell tolerance to ApoB100, blunt Th1 responses, and suppress autoimmune inflammation in hypercholesteremic mice in an ApoB100-specific manner. Treatment of disease-prone hypercholesteremic mice with a single injection of tolerogenic DCs led to significantly reduced atherosclerosis.

Methods

For an expanded Methods section, please refer to the online-only Data Supplement.

Preparation of ApoB100

LDL was isolated by ultracentrifugation from pooled plasma of healthy donors, as described previously,28 and kept in a native state. ApoB100 was isolated by previously described methods.29,30 ApoB100 preparations were more than 90% pure, as evaluated by gel-filtration high-performance liquid chromatography (GE Healthcare, Uppsala, Sweden).

Isolation and Loading of DCs

Myeloid DCs were generated as described previously.31,32 Briefly, bone marrow was isolated from huB100tg/Ldlr−/− mice (mice transgenic for human ApoB100 crossed with mice that were deficient for the LDL receptor). Bone marrow cells were depleted of red blood cells and cultured for 8 days in tissue culture dishes at 37°C and 7.5% CO2. DMEM culture medium was supplemented with 10% FCS, penicillin 50 U/mL, streptomycin 50 μg/mL, L-glutamine 2 mmol/L, sodium pyruvate 1 mmol/L, granulocyte-macrophage colony-stimulating factor 10 ng/mL (Peprotech, Rocky Hill, NJ), and IL-4 10 ng/mL (Peprotech). Purification of DCs from the differentiated bone marrow cells was performed with a CD11c magnetic cell-sorting kit (Miltenyi Biotech, Göteborg, Sweden) according to the manufacturer’s instructions.

For cell-transfer experiments, the purified DCs were incubated in tissue culture dishes with IL-10 (30 ng/mL), with or without ApoB100 antigen in 200 μL of DMEM supplemented with 2.5% mouse serum, penicillin 50 U/mL, streptomycin 50 μg/mL, L-glutamine 2 mmol/L, and sodium pyruvate 1 mmol/L for 72 hours at 37°C in a humid 5% CO2 atmosphere. One μCi of 3H-thymidine (Sigma-Aldrich) was added after 60 hours, and DNA replication was measured with a scintillation counter (Wallac, Turku, Finland). Cytokines secreted in the cell culture supernatants after 72 hours were measured with an enzyme-linked immunosorbent assay kit (Mabtech, Nacka Strand, Sweden) or with a cytokometric bead array flex set kit (BD Biosciences).

Flow Cytometry and Intracellular Cytokine Staining

To measure surface-marker expression, single-cell suspensions were prepared and stained with primary labeled antibodies (BD Biosciences) according to the manufacturer’s instructions. For further characterization of cytokine profiles, 1×104 CD4+ T cells were incubated for 96 hours with 4×105 DCs treated with or without IL-10 in the presence of anti-CD3 (2 μg/mL) and restimulated for the last 4 hours of culture at 37°C in 7.5% CO2 with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (1 μg/mL), and GolgiPlugs (1 μL per 1 mL; BD Biosciences). All cells were incubated with FcγR block (BD Biosciences) followed by surface staining for CD4 and intracellular staining for IFN-γ, IL-10 (BD Biosciences), or FoxP3 (for cytokine P3; eBioscience, San Diego, CA) according to the manufacturer’s instructions. In some experiments, cell proliferation was measured by staining with carboxyfluorescein succinimidyl ester (Invitrogen).

Plasma Analyses

The titers of specific antibodies to ApoB100 were measured by enzyme-linked immunosorbent assay as described previously.2 Plasma cholesterol and triglycerides were measured with enzymatic colorimetric kits (Randox Laboratory Ltd, Crumlin, United Kingdom) according to the manufacturer’s protocol.

Statistical Analysis

Values are expressed as mean±SEM unless otherwise indicated. The nonparametric Mann-Whitney U test was used for pairwise comparisons and the Kruskal-Wallis test for multiple comparisons. Differences between groups were considered significant at P<0.05.

Results

Tolerogenic DCs Promote Regulatory T Cells and Have an Impaired Ability to Induce Effector T-Cell Responses

DCs were prepared from bone marrow of huB100tg/Ldlr−/− mice and matured by lipopolysaccharide treatment in the...
presence or absence of IL-10. These DCs displayed a characteristic CD11c^+ Flt3^- I-A^b-positive phenotype with >90% CD11c^+ cells (online-only Data Supplement Figure 1). IL-10 treatment of DCs did not affect the mean cell-surface expression of the costimulatory factors CD80 and CD86 or the coinhibitory molecules programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2; online-only Data Supplement Figure 1). Secretion of TNF-α, monocyte chemotactic protein-1, and IL-12 from DCs was increased markedly after uptake of ApoB100 but was repressed significantly on concomitant treatment with IL-10 (Figure 1).

To assess the functional activity of IL-10–treated DCs, we added such cells to polyclonally activated spleen CD4^+ T cells. This led to significantly reduced IFN-γ production and to modestly increased IL-10 production by these T cells (Figure 2A). The T cells constitutively expressed the costimulatory factor CD28 and low levels of CTLA-4 (cytotoxic T-lymphocyte antigen 4) and ICOS (inducible T-cell co-
stimulator). The coinhibitory receptor PD-1, which is involved in T-cell inhibition, autoimmunity, and peripheral tolerance, was slightly upregulated on T cells stimulated by IL-10–treated DCs (online-only Data Supplement Figure II).

Compared with nontreated DCs, exposure to IL-10–treated DCs more than doubled the number of FoxP3+CD4+ Treg cells among purified spleen CD4+ T cells that had been depleted of preexisting CD25+ Treg, which suggests de novo generation (Figure 2B; online-only Data Supplement Table I). IL-10–treated DCs promoted significantly less effector T-cell proliferation on polyclonal stimulation than did regular myeloid DCs (Figures 2B and 2C; online-only Data Supplement Table II).

We have found recently that atherogenic T cells recognize ApoB100-derived peptides of native LDL in an I-Aβ-restricted manner. When DCs pulsed with ApoB100 and IL-10 were incubated with the ApoB100-specific T-cell hybridoma 48.5, they suppressed its activation compared with its response to DCs without antigen. **P<0.01 vs all other groups (n=7 to 9 mice per group).

**Figure 3.** Tolerogenic dendritic cells (DCs) reduce atherosclerotic lesion formation in the thoracic aorta of huB100tg/Ldlr−/− mice. A, Mice received 1 intravenous injection with DCs loaded with or without apolipoprotein B100 (ApoB100) and/or interleukin-10 (IL-10) and were fed a Western diet for 10 weeks. Dissected aortas were stained with Sudan IV, and percent lesion area of total vessel area was calculated with ImageJ image analysis software. B through F, En face photographs of aortas from treatment groups: B, DCs without antigen; C, DC+ApoB100; D, DC+ApoB100+IL-10; E, DC+IL-10; and F, no DCs. **P<0.01 vs all other groups (n=7 to 9 mice per group).

**Figure 4.** Tolerogenic dendritic cells (DCs) decrease infiltration of CD4+ T cells in aortic root lesions of huB100tg/Ldlr−/− mice and stabilize lesions. Mice received 1 intravenous injection with DCs loaded with or without apolipoprotein B100 (ApoB100) and/or interleukin-10 (IL-10) and were fed a Western diet for 10 weeks. A, Number of CD4+ cells per square millimeter of lesion area. B, Percent of Sirius red–stained area in the aortic root of treated groups. C through F, CD4 immunostaining of representative cryosections from the aortic root. Immunoperoxidase labeling; original magnification ×100. G through J, Sirius red staining of representative cryosections from the aortic root; original magnification ×100. C and G, No antigen; D and H, DC+ApoB100; E and I, DC+IL-10; and F and J, DC+IL-10. **P<0.05 (n=5 to 7 mice per group).
response to DCs pulsed with ApoB100 in the absence of IL-10 (Figure 2D). When DCs from I-A\(^d\) donors were used, or when antibodies against I-A\(^d\) were added to the culture, the response was abrogated, which demonstrates that I-A\(^d\) restricted antigen presentation of ApoB100 by DCs to T cells

Table. Body Weight, Plasma Cholesterol, and Triglyceride Levels

<table>
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<tr>
<th>Group</th>
<th>Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
<th>Weight, g</th>
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<td>659±210</td>
<td>27.3±2.9</td>
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<tr>
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<tr>
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<td>712±165</td>
<td>28.1±1.6</td>
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<tr>
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<td>718±233</td>
<td>28.2±2.5</td>
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<tr>
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<td>683±233</td>
<td>31.3±2.3</td>
</tr>
<tr>
<td>(P)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

DC indicates dendritic cell; ApoB100, apolipoprotein B100; IL-10, interleukin-10; and NS, not significant.

Values are expressed as mean±SD. \(n=7–9\) mice per group.

Figure 5. Tolerogenic dendritic cells (DCs) generate antigen-specific suppressor regulatory T cells (Treg) and downregulate antigen-specific T helper responses and inflammation in vivo. A, DCs pulsed with or without apolipoprotein B100 (ApoB100) and interleukin-10 (IL-10) were transferred intravenously to recipient mice. One week later, \(2\times10^4\) cells of the ApoB100-specific T hybridoma 48.5 were seeded together with \(1\times10^6\) DCs and 20 \(\mu\)g/ml ApoB100 in 96-well plates. CD4\(^+\) T cells purified from spleens of DC-treated mice were coincubated at different ratios with the T hybridoma effectors cells. Antigen-specific T-cell activation was measured 24 hours later by interleukin-2 (IL-2) secretion in the supernatant. Black bars indicate T effector cells and DCs only. Ratios are shown as suppressor-to-effector ratio. \(*P<0.05\) vs effectors only (n=4). B, DCs pulsed with or without ApoB100 and IL-10 were transferred intravenously to recipient mice that were immunized 1 week later with a subcutaneous injection of 100 \(\mu\)g of ApoB100 to boost the cellular immune response to this protein. After another week, the in vitro recall cytokine response against ApoB100 (40 \(\mu\)g/mL) was measured in cultures of \(5\times10^5\) splenocytes from the treated mice. Cytokine secretion in cell supernatants was analyzed after 48 hours with enzyme-linked immunosorbent assay. IFN\(_\gamma\) indicates interferon-\(\gamma\); IL-5, interleukin-5; and TNF\(_{\alpha}\), tumor necrosis factor-\(\alpha\). \(*P<0.05\) (n=5 mice per group).

Tolerogenic DCs Presenting ApoB100 Reduce Atherosclerosis in Hypercholesterolemic Mice

HuB100\(^{gb}\)×\(Ldlr^{-/-}\) mice were used to test the effect of tolerogenic DCs on atherosclerosis. These mice express full-length human ApoB100 in the liver and display humanized hyperlipidemic lipoprotein profiles. The huB100\(^{gb}\)×\(Ldlr^{-/-}\) model permits the use of human ApoB100 as antigen.\(^5\) Mice received a single intravenous injection of DCs that had been pulsed with either (1) medium, (2) ApoB100 alone (ie, antigen-loaded myeloid DCs), (3) ApoB100 plus IL-10 (antigen-loaded tolerogenic DCs), or (4) IL-10 alone (unloaded tolerogenic DCs). Finally, a fifth group of mice remained untreated. A significant
ApoB100-Loaded Tolerogenic DCs Induce Antigen-Specific CD4+ T-reg Cells That Suppress T-Cell Effector Responses to ApoB100

To investigate the functional activity of T-reg cells in the huB1008 X Ldlr−/− mice treated with tolerogenic DCs, we purified CD4+ T cells from spleens of treated mice and tested whether they could suppress activation of a T-cell hybridoma that recognizes ApoB100 in an I-Aβ-restricted manner. CD4+ T cells from mice injected with tolerogenic ApoB100-pulsed DCs significantly suppressed activation of the ApoB100-specific T cells (Figure 5A). None of the CD4+ T-cell populations from mice injected with DCs and treated with antigen or cytokine alone could suppress these ApoB100-specific T cells (Figure 5A).

ApoB100-Loaded Tolerogenic DCs Downregulate Proinflammatory T-Cell Responses in an Antigen-Dependent Manner and Mobilize Antiinflammatory Activity In Vivo

Splenocytes from huB1008 X Ldlr−/− mice proliferate in response to human ApoB100 and display a Th1 cytokine profile. We transferred tolerogenic DCs to test whether this response can be suppressed in vivo. When recipients were injected subcutaneously with 100 μg of ApoB100 1 week after injection of ApoB100-pulsed DCs, a vivid recall response was registered to the protein, with splenocytes secreting increased amounts of IFN-γ, TNF-α, and IL-6 in response to ApoB100 (Figure 5B). However, spleens of mice injected with ApoB100-loaded tolerogenic DCs displayed significantly decreased production of IFN-γ, TNF-α, and IL-6, as well as IL-5 (Figure 5B). This indicates that the tolerogenic treatment reduced antigen-specific responses in immunized mice.

mRNA levels for FoxP3, IL-10, and transforming growth factor-β1 were increased and IL-12 was decreased in spleens of mice that received ApoB100-loaded tolerogenic DCs (Figure 6). Transcripts for ICOS ligand, PD-1, and PD-L1 were increased after treatment with IL-10-pulsed DCs irrespective of whether they were loaded with antigen (online-only Data Supplement Figure VI). Lymph nodes of mice treated with tolerogenic DCs showed significantly lower levels of IL-12 and IL-5 and increased levels of ICOS ligand compared with mice that received DCs pulsed with ApoB100 alone (online-only Data Supplement Figure VI). These data show that tolerogenic DCs induce a regulatory and antiinflammatory machinery in the lymphoid organs of treated mice.

The Atheroprotective Effect of Tolerogenic DCs Is Associated With Decreased Cellular But Not Humoral Immunity to ApoB100

Hypercholesterolemic mice injected with ApoB100-loaded tolerogenic DCs displayed significantly suppressed proliferative spleen T-cell responses to ApoB100 at 40 μg/mL (Figure 7A). Plasma levels of IFN-γ were also decreased significantly in these mice compared with mice that received ApoB100-loaded DCs (Figure 7B). IgG antibodies to ApoB100 were increased significantly in the 2 groups of mice that received DCs pulsed with ApoB100, irrespective of whether they were treated with IL-10 to become tolerogenic (Figure 7C). A similar trend was seen for IgG antibodies to oxidized LDL (data not shown). No significant differences were seen for IgM titers against ApoB100 in plasma of
injected mice (Figure 7D). Therefore, the atheroprotective effect of ApoB100-loaded tolerogenic DCs is associated with inhibition of effector T-cell responses but not antibody production.

**Discussion**

The significant role of adaptive immunity in atherosclerosis is well established and makes it likely that antigen-presenting DCs are of importance for disease development. This was recently supported by studies that showed that transfer of LDL-loaded DCs into hypercholesterolemic mice accelerated atherosclerosis,\(^31\) and that an extended lifespan of DCs affected plaque inflammation and cholesterol metabolism.\(^37\) One may surmise that manipulation of DC activity could be used to reduce the autoimmune response to LDL components and ameliorate atherosclerosis.

We now show that DCs can be made tolerogenic to LDL autoimmunity by treating them with IL-10 while loading them with ApoB100. Such ApoB100-loaded tolerogenic DCs inhibited the proliferative and proinflammatory T-cell response to ApoB100 and promoted the development of regulatory T cells. When injected into atherosclerosis-prone huB100/\(^8\)×Ldlr\(^/-\) mice, they induced antiinflammatory activity, reduced immune cell infiltration into lesions, and mediated a significant reduction of atherosclerotic lesion size compared with immunogenic ApoB100-loaded DCs or vehicle controls.

The atheroprotective effect of ApoB100-loaded tolerogenic DCs was paralleled by reduced plasma levels of IFN-\(\gamma\) and reduced CD4\(^+\) T-cell infiltration into lesions but affected neither entry of CD11c\(^+\) DCs, expression of major histocompatibility complex class II (I-A) or vascular cell adhesion molecule-1 in lesions, nor plasma lipid levels. This suggests that activation and recruitment to lesions of effector T cells is of major importance for the atheroprotective effect of these tolerogenic DCs. Because FoxP3\(^+\) Treg and the coinhibitory molecule PD-1 were increased in T-cell populations exposed to tolerogenic DCs and a significant inhibition of helper T-cell immunity was seen in mice injected with such DCs, the present data point to suppression of cell-mediated immune responses as a likely pathway. This is in line with previous studies showing inhibitory effects on effector T-cell immunity when Treg function was stimulated by IL-10-treated tolerogenic DCs.\(^{21,26,39}\) It has been suggested that the effects of such tolerogenic DCs are mediated by downregulation of costimulatory molecules, major histocompatibility complex class II, and IL-12.\(^{21}\) We did not see any such difference in surface-marker expression; however, IL-12 expression was repressed in tolerogenic DCs and in lymphoid organs of mice injected with these DCs. IL-12 is one of the major determinants for differentiation of naïve T cells into effector Th1 cells, with ensuing production of IFN-\(\gamma\).\(^{39}\) This further supports the present data that the effect of tolerogenic DCs on atherosclerosis is mediated by suppression of effector T-cell immunity.

The notion that antigen-specific tolerogenic DCs operate through cell-mediated immunity when reducing atherosclerosis is also supported by the reduced T-cell proliferation to apoB100 in treated mice in the present study. In contrast, B-cell responses to ApoB100 were not affected significantly. This suggests that humoral immunity is less important than cellular immunity for the atheroprotective effect of tolerogenic DCs.

Several lines of evidence support a role for antigen specificity in atheroprotective immunity. Only tolerogenic
DCs loaded with apoB100 antigen had a protective effect; the fact that just a single injection of such DCs was sufficient to achieve protection 10 weeks later points to a role for immunologic memory. Of note, DCs prepared in this way present not only the loaded antigen (in this case, apoB100) but also BSA, which is present in the medium needed for DC differentiation. Presentation of BSA did not affect disease, because lesions of mice that received tolerogenic DCs without apoB100 did not differ from those of control mice that did not receive any DCs. Instead, atheroprotection was dependent on presentation of ApoB100 by tolerogenic DCs.

We and others have previously shown that repeated immunization with antigen-loaded immunogenic DCs increases atherosclerosis. This effect was observed both when DCs were loaded with oxidized LDL and when they were loaded with an artificial antigen expressed transgenically in the artery wall. In contrast, it was reported recently that immunogenic DCs loaded with oxidized LDL reduced lesions induced when a collar was surgically attached to the carotid artery of hypercholesterolemic mice, whereas aortic lesions were unchanged.

The present study may shed light on these seemingly contradictory studies. When ApoB100-pulsed immunogenic DCs were administered, a modest, nonsignificant increase in lesion size was observed. Tolerogenic modulation of DCs by IL-10 treatment led to a significant reduction of lesions under otherwise similar conditions. Therefore, the local concentration of IL-10 and other mediators that influence DC phenotype could conceivably determine the outcome of DC transfer experiments. Because IL-10 expression increases with increasing plasma cholesterol levels, the choice of hypercholesterolemic atherosclerosis model may also affect the balance between conventional and tolerogenic DCs. When these findings are translated into clinical use for humans, the immunologic properties of donor and recipient will also be of major importance, because only human leukocyte antigen–matched tolerogenic DCs are likely to confer atheroprotection.

Conclusions

We present here a novel treatment regimen for atherosclerotic disease. Using a tolerogenic DC therapy that targets immune reactions to the ApoB100 protein present in LDL, it was possible to attenuate systemic inflammation and reduce the atherosclerotic plaque burden by about 70%. Because both LDL and markers of inflammation are major risk factors for coronary and cerebrovascular disease, and because current treatment benefits only a limited proportion of all those considered at risk, the present findings should encourage development of DC therapy as an additional approach to cardiovascular prevention and therapy.

Acknowledgments

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Disclosures

Drs Hermansson and Hansson are the inventors of patents on the concept of tolerogenic dendritic cells in atherosclerosis. The remaining authors report no conflicts.

References

In recent years, the perception of atherosclerotic cardiovascular disease has changed from that of a vascular lipid disorder to a chronic inflammatory condition elicited by lipoprotein retention in the vessel wall. Components of the accumulating low-density lipoprotein particles are immunogenic and can activate T cells and macrophages that promote inflammation, lesion growth, and plaque vulnerability. We devised a cell-therapy strategy to dampen inflammation and reduce atherosclerosis by injecting tolerogenic dendritic cells into hypercholesterolemic mice. Before transfer, dendritic cells were loaded with apolipoprotein B100, the protein part of low-density lipoprotein, and subsequently exposed to the anti-inflammatory cytokine interleukin-10. Such dendritic cells suppressed the activity of apolipoprotein B100–reactive T cells. A single injection of tolerogenic dendritic cells loaded with apolipoprotein B100 significantly reduced atherosclerosis and increased plaque-stabilizing collagen in hypercholesterolemic mice. Because similar immune responses occur in human atherosclerosis, tolerogenic dendritic cell therapy may represent a new strategy for reduction and stabilization of atherosclerotic lesions in humans.
Preparation of ApoB100

LDL (d = 1.019 – 1.063 g/mL) was isolated by ultracentrifugation from pooled plasma of healthy donors, as described\(^1\). After isolation, LDL was dialyzed extensively against PBS. One millimolar EDTA was added to an aliquot of LDL to generate unmodified LDL. ApoB100 was isolated by using a modification of previously described methods\(^2-3\). Briefly, 0.4 ml methanol, 0.1 ml chloroform, and 0.3 ml water were added to 0.1 ml of LDL (1mg/mL); the suspension was then vortexed and centrifuged at 9000 x g for 1 min. The upper phase was removed and 0.3 ml of methanol added to the lower phase and interphase with precipitated protein, which was mixed again and centrifuged at 9000 x g for 2 min to pellet the protein.

To obtain soluble, purified ApoB100, the protein pellet was resuspended in a minimum volume of 10% SDS (Bio-Rad Laboratories, Hercules, CA, USA) until it solubilized. These preparations first were filtered on a PD-10 column (GE Healthcare, previously Amersham Biosciences, Uppsala, Sweden) to remove excess SDS. They were then purified on a Superdex-200 size-exclusion column (0.5 mL/min, in Tris-HCl, pH 7.4). The first peak that contained ApoB100 was collected. ApoB100 preparations were greater than 90% pure, as evaluated by gel-filtration HPLC (GE Healthcare, Uppsala,
Sweden). Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

**In vitro cell culture assay**

Splenocytes from treated mice were isolated and resuspended in 96-well plates. 5 x 10^5 splenocytes were incubated with or without ApoB100 antigen in 200 μL of DMEM supplemented with 2.5% mouse serum, 50U/ml penicillin, 50μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate for 72 hours at 37°C in a humid 5% CO₂ atmosphere. One μCi ³H-thymidine (Sigma-Aldrich, St.Louis, MO, USA) was added after 60 h, and DNA replication measured with a scintillation counter (Wallac, Turku, Finland). Results are expressed as stimulation index = ((s – c)/c), where s is the cpm of the sample with antigen and c is the cpm of the sample without antigen. Cytokines secreted in the cell culture supernatants after 72 hours were measured with a mouse IFNγ ELISA kit (Mabtech, Sweden) and by using the cytometric bead array flex set kit (BD Biosciences, CA, USA) for IL-21, MCP-1, IL-10, IL-6, TNFα, IL-12 and IL-5. In some experiments, 4x10^5 antigen-pulsed and cytokine-treated DC were incubated with 1x10^5 cells of the T cell hybridoma 48.5, which can recognize human ApoB100 in an I-A^b restricted manner and release IL-2 upon activation. This cytokine was measured with mouse IL-2 ELISA kit (R&D systems, USA).

**Tissue processing, immunohistochemistry and lesion analysis**

Blood from sacrificed mice was collected by cardiac puncture and vascular perfusion performed with sterile RNase-free PBS. Abdominal aorta, one third of the spleen, and
draining lymph nodes were dissected and snap-frozen for later RNA isolation. Hearts and aortic root were dissected and preserved for immunohistochemistry. Lesion analysis was performed as described. Briefly, hearts were serially sectioned from the proximal 1 mm of the aortic root on a cryostat. Primary antibodies to CD4, CD8, CD11c, I-Ab and VCAM-1 (all rat anti-mouse from BD Biosciences, Franklin Lakes, NJ, USA) were applied to acetone-fixed cryosections followed by detection with the ABC alkaline phosphatase kit (Vector Laboratories, Burlingame, CA, USA). En face lipid accumulation was determined in the aortic arch from immunized mice using Sudan IV staining. Briefly, dissected arches were fixed in 4% neutral buffered formalin. Samples were then cut longitudinally, splayed, pinned and subjected to Sudan IV staining (red color). Images were captured using a Leica DC480 camera connected to a Leica MZ6 stereo microscope (Leica, IL, USA). The additive area of all the plaques in a given aortic arch was calculated as a percent of the total surface area of the arch (not including branching vessels). Quantitation of plaques was performed using Image J software (NIH, Bethesda, USA). Immunohistochemical data was obtained using Qwin computerized analysis (Leica, IL, USA) of stained sections.

Flow cytometry and Intracellular Cytokine Staining

Flow cytometry was performed on a CyAn™ (Dako, Glostrup, Denmark); data were analyzed using Summit v4.3 software (Dako). Primary labeled antibodies were from BD Biosciences (anti-CD4, -CD11c, -Flt3, -CD80, -CD86, -I-A b, -PD-L1, -PD-L2, -PD-1, -CTLA-4, -CD28, -ICOS, -IFNγ, and anti-IL-10) or from eBioscience (anti-FoxP3). To characterize the cytokine expression profiles of CD4+ T cells from mouse spleen
incubated with treated DC, cell suspensions were prepared as described before and CD4+ T cells (> 95% purity) were isolated by negative selection over a magnetic column using MACS microbeads (CD4+ negative selection kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. 1x10^4 CD4+ T cells were incubated for 96h with 4x10^4 DC treated with or without IL-10 in the presence of anti-CD3 (2µg/ml) and were restimulated for the last 4h of culture at 37 °C in 7.5% CO₂ with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml), ionomycin (1 µg/ml; Sigma) and GolgiPlug (1ul per 1 ml; BD Bioscience). All cells were incubated with FcγR block (BD Bioscience) followed by surface staining for CD4) and intracellular staining for IFNγ, IL-10 (BD Bioscience) or FoxP3 (eBioscience) according to the manufacturer’s instructions. In some experiments cell proliferation was measured by staining with carboxyfluorescein succinylester (CFSE; Invitrogen, Carlsbad, CA, USA).

**RNA isolation, cDNA synthesis, and real-time PCR**

RNA was isolated from the indicated tissues or cells using the RNeasy kit (Qiagen, Hilden, Germany). Total RNA was analysed on a BioAnalyzer (Agilent Technologies, Waldbronn, Germany). Reverse transcription was performed with Superscript-II and random hexamers (both from Invitrogen, Carlsbad, USA) and amplified by real time-PCR using Assay-on-demand primers and probes for FoxP3, IL-5, IL-10, IL-12, TGFβ1, ICOS-L, PD-1, PD-L1 and TATAbox (Applied Biosystems, Foster City, CA, USA) in an ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Data was analyzed on the basis of the relative expression method with the formula 2^-ΔΔCT, where ΔΔCT = ΔCT (sample) – ΔCT (calibrator = average CT values of all samples within each
group), and ΔCT is the CT of the housekeeping gene (TATAbox) subtracted from the CT of the target gene.

**Plasma analyses**

The titers of specific antibodies to ApoB100 were measured by ELISA. Briefly, 50 µL of ApoB100 (10µg/mL in PBS pH 7.4) was added to 96-well ELISA plates and incubated overnight at 4°C. Coated plates were washed with PBS and blocked with 1% gelatin (Gibco Invitrogen, Carlsbad, CA, USA) in PBS for 1 hr at room temperature. Next, plates were washed and incubated for an additional two hours with mouse plasma, diluted in Tris-buffered saline (TBS)/gelatin 0.1%. After washing, total IgG or IgM levels were measured using enzyme-conjugated anti-mouse antibodies (BD Biosciences, Franklin Lakes, NJ, USA). Colorimetric reactions were developed using TMB (BD Biosciences, Franklin Lakes, NJ, USA) and absorbance measured using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Plasma cholesterol and triglycerides were measured using enzymatic colorimetric kits (Randox Lab. Ltd. Crumin, UK) according to the manufacturer’s protocol.
**Supplemental Tables**

**Supplemental Table I. Mean fluorescent intensity (MFI) of FoxP3 expressed in CD4+ T cells after DC co-culture. Flow cytometry analysis of immunostained cells.**

<table>
<thead>
<tr>
<th>Cell surface marker</th>
<th>DC (MFI ± CV)</th>
<th>DC + IL-10 (MFI ± CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoxP3</td>
<td>460 ± 147</td>
<td>707 ± 93</td>
</tr>
</tbody>
</table>

**Supplemental Table II. Number of dividing CD4+ T cells after DC co-culture. Flow cytometry analysis of CFSE stained cells (mean ± SEM).**

<table>
<thead>
<tr>
<th>Cell divisions</th>
<th>DC (number of cells)</th>
<th>DC + IL-10 (number of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 1</td>
<td>2,114 ± 904</td>
<td>11,026 ± 7,231</td>
</tr>
<tr>
<td>2 – 3</td>
<td>31,559 ± 21,540</td>
<td>93,389 ± 19,618</td>
</tr>
<tr>
<td>&gt;3</td>
<td>73,618 ± 26,332</td>
<td>21,100 ± 19,618</td>
</tr>
</tbody>
</table>
Supplemental Figures

Supplemental Figure I
Surface marker analysis of tolerogenic dendritic cells (DC). DC were differentiated from mouse bone marrow in the presence of GM-CSF and IL-4 according to a method adapted from Son et al. CD11c+ dendritic cells were purified using magnetic cell sorting and matured with LPS. 2x10^5 cells incubated in the presence or absence of IL-10 for 24h were analysed for cell specific surface markers CD11c, Flt3 and I-A^b as well as co-stimulatory markers CD80/CD86 and co-inhibitory PD-L1/PD-L2. Dotted line shows negative control, grey profile indicates cells without IL-10 and black line shows cells with IL-10. Histograms are representative of several experiments.

Supplemental Figure II
Effect of tolerogenic DC on surface marker expression of activated CD4+ T cells. 1x10^4 CD11c+ dendritic cells treated with or without IL-10 were co-incubated for 96h with 4x10^4 CD4+ T cells purified from mouse spleen and activated with anti-CD3. Staining of co-stimulatory surface markers CD28 and ICOS, and co-inhibitory markers CTLA-4 and PD-1, on CD4+ T cells 96h after co-incubation with dendritic cells. Grey profile indicates co-cultured dendritic cells matured without IL-10 and black line shows IL-10-treated dendritic cells.
**Supplemental Figure III**

5x10⁴ cells of the T cell hybridoma 48.5, which specifically recognizes ApoB100, were co-incubated with 2x10⁵ DC in the presence of ApoB100 and different concentrations of an antibody against I-Ab. The DC originated from donor mice expressing I-Ab. T cell effector function was measured 24h later by IL-2 secretion in the supernatant.

**Supplemental Figure IV**

No effect of tolerogenic DC on infiltration of CD8+ T cells and DC, or on expression of VCAM-1 and I-A\(^b\) in aortic root lesions of huB100⁹⁸xLdlr/-/- mice. Mice received one injection I.V. with DC loaded with or without ApoB100 and/or IL-10 and fed Western diet for 10 weeks. Graphs show the number of cells per mm\(^2\) lesion area or the percent stained lesion area. (n=5-7 mice per group).

**Supplemental Figure V**

No effect of DC transfer on FoxP3 mRNA in aortas of huB100⁹⁸xLdlr/-/- mice. Mice received one injection I.V. with DC loaded with or without ApoB100 and/or IL-10 and fed Western diet for 10 weeks. FoxP3 mRNA was analyzed by real-time reverse-transcription PCR. Bar graphs represent mean ± SEM of target mRNA relative to TATA box protein mRNA.

**Supplemental Figure VI**

In vivo transfer of IL-10-treated ApoB100-pulsed DC mobilizes anti-inflammatory activity in lymphoid organs. mRNA transcript levels was evaluated with real-time PCR
from (A) spleen and (B) inguinal lymph nodes of recipient mice that had been injected
I.V with DC pulsed and treated with or without ApoB100 and IL-10, and primed one
week later with ApoB100. Bar graphs represent the mean ± SEM expression of target
gene relative to TATAbox expression. * = p<0.05, as compared to DC or DC + ApoB100
(n=5 mice per group).

Supplemental References

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143
1998;102:910-918
Supplemental Figure I

CD11c
Flt3
CD80
CD86

I-A\text{b}
PD-L1
PD-L2

Dashed line - isotype control
Solid line - IL-10 treated DC
Grey profile - DC control
Supplemental Figure II
Supplemental Figure III
Supplemental Figure V

The figure shows a bar graph comparing FoxP3 mRNA levels in different conditions. The x-axis represents different groups: DC, DC+ApoB100, DC+ApoB100+IL-10, and DC+IL-10. The y-axis represents the FoxP3 mRNA levels. The graph indicates that there is no significant difference (n.s.) in FoxP3 mRNA levels among the groups.
Supplemental Figure VI

A

B
총상경화 예방 치료의 새로운 접근방식:
ApoB100 면역치료로 총상경화를 예방한다

김상현 교수 서울대학교 보라매병원 순환기내과

Summary

배경
총상경화는 저밀도지단백 콜레스테롤이 혈관 내막하부
위에 축적되면서 염증작용을 통해 저밀도지단백 콜레스
테롤에 대한 자가 면역반응이 발생되고 이에 따라 만성
혈관 염증으로 진행하는 일련의 과정이다.

방법 및 결과
총상경화 염증반응을 억제하기 위한 면역 향용 치료 연
구로서, 저밀도지단백 수용체가 없는 취에 인간 아포지
단백 B100 유전자 전이를 조작하여(huB100”Xldir”),
연구를 진행하였다. 이 취의 골수에서 분리하여 얻은 가
지세포(dendritic cell)에 저밀도지단백 콜레스테롤-
아포지단백 B100과 함께 면역 억제효과가 있는 IL-
10(interleukin 10)을 가하여 감내 장치치하였다. 이후, 이
렇게 전처리한 가지세포를 취에게 정맥주사한 후에, 10
주 동안 고지방식이로 사육하였다. 이러한 면역 향용 치
료에 따라 가지세포는 아포지단백 B100 투여에 따른 작
용 T세포의 증식과 인터페론 감마의 생산을 억제하였
고, 조절 T세포의 증식을 증가시켰다. 또한, TNF-alpha,
MCP-1, IL-12의 분비가 감소되었다. 취의 비정상세포들은
아포지단백 B100 투여에 대한 중증반응이 억제되었으
며, 아포지단백 B100에 대한 Th1, Th2 면역반응도 유의
하게 억제되었다. 비장 CD4+ T세포는 아포지단백 B100
에 대한 T세포 반응의 저하를 보였다. 면역치료를 받은
취의 혈청 흩부대동맥 총상반응 대조군 취에 비해 크기
가 70% 정도 유의하게 감소하였으며, CD4+ T세포의 침
투가 감소하고 전신 염증 반응이 감소된 소견을 보였다.

결론
아포지단백 B100 면역 향용 치료를 받은 가지세포는 저
밀도지단백 콜레스테롤에 대한 자가면역 반응을 유의하
게 감소시켰다. 외부, 이러한 면역 향용 치료가 총상경화
의 치료와 예방을 위한 새로운 방법으로 개발될 수 있다.
Commentary

즉시거는 임질도지단백 콜레스테롤의 혈관 내 점착
교환학과 연관, 그리고 변형된 지질도지단백 콜레스테롤에
한 연역반응과 만성 염증반응의 결과이다. 침착된 임질
도지단백 콜레스테롤은 산화 등 변형과정을 겪게 되고,
특히 아포지단백 B100 성분은 연역반응에서 중요한
항원으로 작용한다. 가시세포는 항원을 면역세포 특히
T세포에 제공하고 여러 면역관련 세포가 방어
T세포의 중심과 면역반응을 유발하고 유지한다. Th1
면역반응을 통해서 인터페론-감마와 TNF-alpha, IL-12
CCL2, CCL5, CXCL4 등이 생산되어 염증반응이 활성화
된다.

특성화된 자세한 과정에 IL-10을 참가함으로써, 면
역반응을 약화시키는 면역 허용 가지세포가 유도된다는
의를 통해, 면역 허용 지표의 성장성에 대한 효과
을 연구하였다. 즉, 특성화된 자세한 과정에 간염이 필요
면역 허용 기능은 유도
가능한 가지세포의 특성을 이용하여 억제지를 사용한 것이
다. IL-10 투여에 의하여 가지세포의 발현이 변하지는 않
었고, CD80, CD86, PD-L1, PD-L2, 면역 허용 변화를 보이
지 않았다. 하지만, 전처리된 가지세포를 T세포에 노출시
켜서 인터페론 감마의 생산 감소와 PD-1의 발현 증가가
나타났고, 전처리 T세포의 중심이 감소하면서 조직 T세포
의 중심은 증가하였다. 하지만, 활성 콜레스테롤 수치는
변화가 없었고 아포지단백 B100 혹은 산화 지질도지단
백 콜레스테롤에 대한 IgG 항체들은 유의하게 증가하였
다. 따라서 면역 허용 가지세포를 이용한 면역지표가 주
로 세포성 면역작용 감소에 의한 효과임을 알 수 있다.
그렇다면, IL-10을 주가하지 않고 항원으로 전처리된 가
지세포를 투여하면 췌장성화의 여파가 될까? 이에 대해서
는 기존의 연구들이 일부 상반된 결과를 보고하였다.
변정 지질도지단백 콜레스테롤을 감쳐서는 타
동맥 등의 췌장성화가 증가하였다고 보고되었고, 경동맥
막질체를 설치한 고지혈성의 실험 연구에서는 경동맥
의 췌장성화가 오히려 감소하였다. 보고가 있었다. 이

이에 대해서는 항후 추가적인 연구를 통해 결과와 기전에
대한 개념 정립이 필요할 것이다.

이 연구에서는 IL-10을 항원 아포지단백 B100과 함께
전처리한 가지세포를 19형 항체주사하고 10주 동안 고지
혈관 이식을 사용한 채 모델을 분석하였고, 세포성 면역 반
응의 역제와 함께 대동맥 췌장성화의 크기 감소를 보
였다. 1회 주사에 의해 10주 동안 작용한 장기적인 효과
의 특성, 그리고 면역 허용 지표는 새로운 방법 특성
을 통해 이러한 면역 지표가 항후 췌장성화 예방에
있어서 새로운 접근 방식으로 대두될 가능성을 보이고
있음을 알 수 있고, 항후 추가로 해결해야 할 문제들, 특
히 안전성 관련 문제들이 중요할 것으로 생각된다.

References
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