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Molecular Cardiology

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/Ldlr−/− 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+ 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/Ldlr−/− mice with ApoB100-pulsed tolerogenic DCs led to a significant (70%) reduction of atherosclerotic lesions in the aorta, with decreased CD4+ 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).21–27 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 hypercholesterolemic mice in an ApoB100-specific manner. Treatment of disease-prone hypercholesterolemic mice with a single injection of tolerogenic DCs led to significantly reduced atherosclerosis.

Methods

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.2,9,29,30 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, and gentamicin sulfate 50 μg/mL (Sigma-Aldrich) at 37°C in a humid 5% CO2 atmosphere. One μCi of 3H-hybridine (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 cytometric 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 4×104 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; Sigma), and GolgiPlug (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 (forkhead box 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. 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

Animal Experiments and Evaluation of Disease
Eleven-week-old male huB100tg/Ldlr−/− mice (C57BL/J6129-ApoB100tg/Ldlr−/− mice, kindly provided by Dr Jan Boren, Göteborg University), were injected intravenously with 2.5×104 DCs pulsed with antigen and/or cytokine. The mice were fed a high-fat diet (0.15% cholesterol R638, Lantmänn, Malmö, Sweden) for 10 weeks starting 5 days after cell therapy. In some experiments, the mice were also immunized subcutaneously with 100 μg of ApoB100 emulsified with complete Freund’s adjuvant 1 week after DC injection. All experiments were approved by the local ethics committee.

Mice were euthanized under CO2 anesthesia, blood was collected by cardiac puncture, and the vasculature was perfused with sterile RNase-free PBS. The descending thoracic aorta was dissected and fixed in 4% formaldehyde, opened longitudinally, pinned onto black wax plates, and stained with Sudan IV (Merck AG, Darmstadt, Germany). Lesion area and total aortic area were calculated with ImageJ software (National Institutes of Health, Bethesda, MD). Part of the spleen was used for cell experiments and snap-frozen for RNA isolation. Inguinal lymph nodes and abdominal aortas were snap-frozen and used for RNA isolation. Immunohistochemistry was performed on acetone-fixed cryosections from the aortic root of dissected mouse hearts as described previously.35
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-

Figure 1. Tolerogenic dendritic cells (DCs) have an impaired capability to induce T-cell effector responses in vitro. DCs were differentiated from mouse bone marrow in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4. CD11c^+ DCs were purified by magnetic cell sorting and matured with lipopolysaccharide. To test the DCs for their capability to induce proinflammatory cytokines in the presence of antigen or antiinflammatory cytokine, 2 × 10^5 cells were seeded in 96-well plates and incubated together with or without apolipoprotein B100 (ApoB100) in the presence or absence of interleukin-10 (IL-10) for 24 hours, as indicated by (+) or (−) in the graphs. Bar graphs show secretion to culture media of (A) TNF-α (tumor necrosis factor-α) B) IL-12 (interleukin-12) and (C) MCP-1 (monocyte chemotactic protein-1). ***P<0.001 vs DC+ApoB100 (n=6 for each experimental group).

Figure 2. Interleukin-10 (IL-10)–treated dendritic cells (DCs) inhibit T-cell proliferation and effector function but stimulate de novo generation of regulatory T cells (Treg). A total of 1 × 10^6 CD11c^+ DCs treated with or without IL-10 were coincubated for 96 hours with 4 × 10^5 CD4^+ T cells purified from mouse spleen and activated with anti-CD3. A–C, Fluorescent-activated cell sorter data from a representative experiment that was performed twice with similar results. A, Intracellular staining of CD4^+ T cells after 96 hours of coincubation. Cells were restimulated with phorbol 12-myristate 13-acetate and ionomycin and added together with brefeldin A for the last 5 hours of culture. CD4^+ T cells were then stained with anti-interferon (IFN)-γ and anti-IL-10 according to the manufacturer’s protocol (BD Biosciences). B, Spleen CD4^+ T cells were depleted of CD25^+ natural Treg, stained with carboxyfluorescin succinimidyl ester (CFSE) to visualize cell proliferation, cocultured for 96 hours, and stained for FoxP3. C, Overlay histogram showing CFSE staining in FoxP3^+ effector T cells cultured with DCs (shaded area) and DCs pretreated with IL-10 (black line), with peaks reflecting cell division (due to dilution of CFSE dye in each cell division, highly proliferating cells are toward the left and nonproliferating cells are toward the right). Max indicates maximum. D, 5 × 10^6 cells of the T-cell hybridoma 48.5, which specifically recognizes apolipoprotein B100 (ApoB100), were coincubated with 2 × 10^5 washed DCs that had been pretreated for 24 hours in the presence or absence of IL-10 and ApoB100, as indicated by (+) or (−) in the graph. The DCs originated from donor mice expressing either I-A^b or I-A^d. T-cell activation was measured 24 hours later by interleukin-2 (IL-2) secretion. **P<0.01 vs DC+ApoB100 (n=4); ns indicates not significant.
stimulator). The coinhibitory receptor PD-1, which is involved in T-cell inhibition, autoimmunity, and peripheral tolerance,⁴⁶ 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.⁵ 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 re-

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^b were added to the culture, the response was abrogated, which demonstrates that I-A^b restricted antigen presentation of ApoB100 by DCs to T cells

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<td>683±233</td>
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*P < 0.05 vs effector cells

Tolerogenic DCs Presenting ApoB100 Reduce Atherosclerosis in Hypercholesterolemic Mice

HuB100^b^×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^b^×Ldlr^-/-^ model permits the use of human ApoB100 as antigen. 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

![Figure 5](http://circ.ahajournals.org/)
ApoB100-Loaded Tolerogenic DCs Induce Antigen-Specific CD4+ Treg Cells That Suppress T-Cell Effector Responses to ApoB100

To investigate the functional activity of Treg cells in the huB100/5×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-Aα-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 Antinflammatory Activity In Vivo

Splenocytes from huB100/5×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/HLdcr−/− 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-γ and reduced CD4+ T-cell infiltration into lesions but affected neither entry of CD11c+ DCs, expression of major histocompatibility complex 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.21,26,38 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-γ.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. 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 ≈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.

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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 antiinflammatory 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.
Supplemental Methods

**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,
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. $5 \times 10^5$ 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% CO$_2$ atmosphere. One µCi $^3$H-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, $4 \times 10^5$ antigen-pulsed and cytokine-treated DC were incubated with $1 \times 10^5$ 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 described⁴. 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, -Ft3, -CD80, -CD86, -I-A<sup>b</sup>, -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 ug/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 succinylesther (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, TGFB1, 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</th>
<th>DC + IL-10</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</th>
<th>DC + IL-10</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

5x10^4 cells of the T cell hybridoma 48.5, which specifically recognizes ApoB100, were co-incubated with 2x10^5 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 huB100^tg xLdlr/- 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 huB100^tg xLdlr/- 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

Supplemental Figure I

CD11c

Flt3

CD80

CD86

I-Aβ

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

![Graph showing FoxP3 mRNA levels with different conditions: DC, DC+ApoB100, DC+ApoB100+IL-10, DC+IL-10. The graph indicates that DC+ApoB100+IL-10 has a n.s. (not significant) result compared to other conditions.]
져항에서 예방 치료의 새로운 접근방식: ApoB100 면역치료로 측정경화를 예방한다

김상현 교수 서울대학교 보라매병원 순환기내과

Summary

배경

저항성혈관질환의 치료에서 저항성혈관의 내피학적변화가 혈관 내막하부위에 촉각되면서 염증작용이 통합되지 않는 저항성혈관의 폐쇄시작에 대해 차단적혈관의 측정경화에 대한 치료법을 바탕으로 연구하는 것이 주요한 과제이다.

방법 및 결과

저항성혈관의 측정성비율을 측정하기 위한 면역 혈청 치료 연구로서, 저항성혈관의 수용체가 없는 저항성혈관 B100 유전자 전이를 조작하여(huB100“Xldlr”), 연구를 진행하였다. 이 특수한 유전자에 의하여 미분화된 가정세포(dendritic cell)에 저항성혈관 경포를 하여 저항성혈관의 측정성비율을 측정할 수 있었다. 이연구에 참여한 저항성혈관의 측정성비율을 측정한 결과, 10주 동안 저항성혈관이 보존되어 있었다. 이 연구의 효과는 저항성혈관의 측정성비율을 측정할 수 있었다. 또한, TNF-alpha, MCP-1, IL-12의 분비가 감소되었다. 저항성혈관의 B100 투여에 대한 측정성비율이 감소하였으며, 저항성혈관 B100에 대한 면역 지하와 면역 지하에 의한 면역 지하가 높았다. 면역 지하를 받은 저항성혈관의 측정성비율이 대조군에 비해 크기 70% 정도 유의하게 감소하였으며, CD4+ T세포의 체내 감소가 효과적이다.

결론

저항성혈관의 B100 면역 혈청 치료를 받은 저항성혈관 경포를 저항성혈관의 측정성비율에 대한 저항성혈관의 측정성비율을 유의하게 감소시켰다. 또한, 면역 혈청 치료가 저항성혈관의 측정성을 감소시켰다. 또한, TNF-alpha, MCP-1, IL-12의 분비가 감소되었다. 저항성혈관의 B100 투여에 대한 측정성비율이 감소하였으며, 저항성혈관 B100에 대한 면역 지하와 면역 지하에 의한 면역 지하가 높았다. 면역 지하를 받은 저항성혈관의 측정성비율이 대조군에 비해 크기 70% 정도 유의하게 감소하였으며, CD4+ T세포의 체내 감소가 효과적이다.
Commentary

즉시점착은 저밀도지단백 콜레스테롤의 혈관 벽내 침착과 변형을 억제하고 변형된 저밀도지단백 콜레스테롤과 관련된 면역반응을 억제해 결과로 침착된 저밀도지단백 콜레스테롤의 산화 등이 변형을 억제하는 것으로 보고, 특히 아포 지단백 B100 성분은 면역반응에서 중요한 역할을 한다. 가시세포는 단백질 면역세포 특히 T세포와 해치고 여러 면역관련 세포의 분화를 전달하는 유전자(PI3)를 유지한다. 면역반응을 통해 인터레티론 감마와 TNF-alpha, IL-2, CCL2, CCL5, CXCL4 등이 생산되며 면역반응이 활성화된다.

특정항원에 대한 감각 과정에 IL-10을 참가함으로써 면역반응을 억제시키는 면역 억제가 가시세포에서 유도된다는 정의를 통해, 면역 억제 지표의 즉시점착에 대한 효과를 연구하였다. 즉, 특정 항원에 대해 감각되어 해당 면역반응을 축소하는 기능이 있다. 면역 억제 기능은 유도된 가시세포의 특성을 이용하여 면역억제를 도시하기도 한다. IL-10 투여에 의해 가시세포의 발현이 변하지 않았고, CDB0, CDB8, PD-L1, PD-L2로 보다 큰 변화를 보이지 않았다. 하지만, 전처리된 가시세포를 T세포로 노출시키면 인터리포레 감마의 생산 감소와 PD-L1의 발현 증가가 나타났고, T세포의 증식이 감소하면서 조절 T세포의 증식이 증가하였다. 하지만, 혈중 콜레스테롤 수치는 변화가 없었고 아포지단백 B100 혹은 산화 저밀도지단백 콜레스테롤에 대한 IgG 항체들의 유의하게 증가하였다. 따라서 면역 억제 가시세포에 사용된 면역지표가 주로 세포성 면역 억제 감소에 의한 효과임을 알 수 있다. 그렇다면, IL-10을 추가하지 않고 항원으로 전처리된 가시세포를 투여하면 즉시점착을 어떻게 될까? 이에 대해서는 기존의 연구들이 일부 상반된 결과를 보고하였다. 변형 저밀도지단백 콜레스테롤을 감지시킨 취해에서는 탄동맥 등이 작용하였고 증가하였다. 검증적 억제제를 사용한 고지방성식은 실험연구에서는 경동맥의 작용을 오래 지속될 것으로 보고되었다. 이에 대해서는 원추 추가적인 연구를 통해 결과와 기전에 대한 개념 정립이 필요할 것이다.

이 연구에서는 IL-10을 흡수한 아포지단백 B100을 함께 전처리한 가시세포를 T세포의 작용부위와 10주 동안 고지방성식을 사용한 실험을 분석하였고, 세포성 면역 반응의 억제와 함께 대동맥 즉시점착의 크기 감소를 보였다. 1회 투여에 의해 10주 동안 적극적인 효과의 특성, 그리고 면역 억제 지표는 새로운 방법 특성을 통해 이러한 면역 지표가 향후 즉시점착 예방에 있어서 새로운 접근 방식으로 대두될 가능성을 보이고 있음을 알 수 있고, 향후 추가적 해석해야 할 문제들, 특히 안전성 관련 문제들이 중요할 것으로 생각된다.

References