Xenogenic macrophage immunization reduces atherosclerosis in apolipoprotein E knockout mice

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Yamashita T, Kawashima S, Hirase T, Shinohara M, Takaya T, Sasaki N, Takeda M, Tawa H, Inoue N, Hirata K-I, Yokoyama M. Xenogenic macrophage immunization reduces atherosclerosis in apolipoprotein E knockout mice. Am J Physiol Cell Physiol 293: C865–C873, 2007. First published June 6, 2007; doi:10.1152/ajpcell.00117.2007.—Atherosclerosis is a complex chronic inflammatory disease in which macrophages play a critical role, and the intervention of the inflammatory process in atherogenesis could be a therapeutic strategy. In this study, we investigated the efficacy of xenogenic macrophage immunization on the atherosclerotic lesion formation in a model of murine atherosclerosis. Apolipoprotein E knockout (apoE-KO) mice were repeatedly immunized with formaldehyde-fixed cultured human macrophages (phorbol ester-stimulated THP-1 cells), using human serum albumin as a control protein or HepG2 cells as human control cells, once a week for four consecutive weeks. The vehicle phosphate-buffered saline was injected in the nonimmunized controls. THP-1 immunization induced antibodies that are immunoreactive with mouse macrophages. Although the plasma lipid levels were unchanged by the immunization, the atherosclerotic lesion area in the aortic root was significantly reduced by >50% in 16-wk-old THP-1-immunized apoE-KO mice compared with that in control mice. THP-1 immunization reduced in vivo macrophage infiltration, reduced in vitro macrophage adhesion, and changed cytokine production by macrophages to the antiatherogenic phenotype. Xenogenic macrophage immunization protects against the development of atherosclerosis in apoE-KO mice by modulating macrophage function in which antibodies induced by the immunization are likely to be involved. This method is a novel and potentially useful cell-mediated immune therapeutic technique against atherosclerosis.

atherosclerosis, some of their consequences are presumed to be protective.

Several studies have revealed that monocytes/macrophages play major roles in both innate and adaptive immunity. Macrophages produce free oxygen radicals, proteases, and cytokines, which are associated with the innate immunity, and they also initiate adaptive immune responses by antigen presentation to T lymphocytes, which are the directors and amplifiers of immune responses (5). Thus it is postulated that inhibition of the functions of macrophages and T lymphocytes reduces the accumulation of leukocytes in the subendothelial space and, as a result, prevents atherosclerotic lesion formation.

Recently, the role of B lymphocytes in atherogenesis has received increasing attention (1, 15, 23). It was reported that removal of the spleen, which contains a large number of B lymphocytes, aggravated atherosclerosis in apolipoprotein E-knockout (apoE-KO) mice and that the supplementation of B lymphocytes to the splenectomized mice blocked the disease progression (1). Another study demonstrated that the administration of polyclonal nonspecific immunoglobulins reduced the atherosclerotic lesion formation in apoE-KO mice (15). Several studies indicated that antioxidized low-density lipoprotein (LDL) antibodies (Abs) protect against atherosclerosis (18, 23, 29). On the other hand, immunization with heat shock protein 65, one of the atherosclerotic plaque proteins, induced atherosclerosis, and anti-heat shock protein 65 Abs were thought to exhibit proatherogenic effects (3, 27). The atherosclerotic plaque may contain various antigens that could be targeted by the immune system, and the adequate induction of Abs is potentially beneficial for protection against atherogenesis. Taking these findings together, we hypothesized that since macrophage are the most abundant cells in atherosclerotic plaques, macrophage-related immune modulation via the induction of anti-macrophage protein Abs could inhibit the progression of atherosclerosis.

In the present study, we demonstrated that fixed xenogenic macrophage immunization induced Abs that are immunoreactive with mouse macrophage proteins and reduced the atherosclerotic lesion formation in apoE-KO mice. These data indicate that the induction of the macrophage-associated atheroprotective immunity can provide a new therapeutic strategy to prevent the initiation and progression of atherosclerosis.
MATERIALS AND METHODS

Materials and animals. All drugs and culture media used in this study were purchased from Sigma Chemical (MO). ApoE-KO mice (62 males and 35 females, offspring of homozygous apoE-KO mice, backcrossed onto the C57BL/6 background) were provided normal chow (Oriental Yeast, Tokyo, Japan) and water ad libitum and maintained on a 12:12-h light-dark cycle. All animal experiments were conducted in accordance with the Guidelines for Animal Experiments at Kobe University School of Medicine.

Preparation of cultured human cells for use as immunogens. A human monocyte cell line (THP-1) was cultured in RPMI 1640 containing 10% fetal bovine serum (FBS). THP-1 cells, stimulated with 2 μM phorbol 12-myristate 13-acetate (PMA) for 24 h, were collected and washed three times with phosphate-buffered saline (PBS). A human hepatoma cell line (HepG2) was cultured in DMEM with 10% FBS. The cells were then fixed with 10% formaldehyde (Wako, Osaka, Japan) for 24 h at 4°C. After the cells were washed three times with PBS and incubated for 2 h at 37°C to remove the formaldehyde, they were washed again and resuspended in PBS for use as immunogens.

Experimental protocol. The apoE-KO mice were weaned at 4 wk and divided into the following four groups for which injections were initiated at 5 wk of age. The THP-1-immunized group received THP-1 cells as immunogens (5 x 10⁶ cells/200 μl PBS for each mouse); the nonimmunized group received vehicle (PBS; 200 μl); the human protein-immunized control group received human serum albumin (HSA; 0.8 mg; A8763, Sigma); and the human non-macrophage cell-immunized control group received HepG2 cells (5 x 10⁶ cells). For four consecutive wk, the mice were intraperitoneally injected without any adjuvants once a week. All mice were killed at 16 wk of age, and atherosclerotic lesions of the aortic roots were assessed.

Blood leukocyte counts and plasma lipid analysis. After the mice were fasted overnight, they were anesthetized with pentobarbital sodium. Immediately after euthanasia, blood was collected by cardiac puncture. Blood leukocytes were counted using an automated blood cell counter. Differential leukocyte counts were determined under a microscope by using smears stained with Wright’s stain solution (Muto Pure Chemicals, Tokyo, Japan). Plasma lipid levels were determined using an automated clinical chemistry analyzer and a specific kit for lipids (Bethyl Laboratories, Montgomery, TX).

Histological analysis of atherosclerotic lesions. The proximal aortas and sections of hearts containing the aortic root were removed, equilibrated in 30% sucrose, and embedded in OCT compounds and sections of hearts containing the aortic root were removed, equilibrated in 30% sucrose, and embedded in OCT compounds. For quantitative analysis of the atherosclerosis, the average lesion area of five sections (every 10th section, each separated by 100 μm) from each mouse was measured according to a previously described method (17, 28). From each mouse, two sections were stained with an anti-mouse monocyte/macrophage monoclonal rat Ab (MOMA-2; BioSource International, Camarillo, CA; 1:50 dilution) or an anti-human T-cell polyclonal rabbit Ab (CD3; Dako, Glostrup, Denmark; 1:100 dilution), followed by detection with biotinylated secondary Abs and streptavidin-horseradish peroxidase. The stained area was quantitatively analyzed, and the percentage of the stained area (the stained area per total atherosclerotic lesion area) was calculated as described previously (28).

Macrophage extraction from ApoE-KO mice. Peritoneal macrophages were collected from the thioglycollate-injected apoE-KO mice by washing the peritoneal cavity as described previously (9). These cells were washed twice with PBS and used for in vitro experiments or protein extraction. Mononuclear cells from the spleen were isolated and plated onto culture dishes with RPMI medium containing 10% FBS and incubated in a 5% CO₂ chamber for 30 min at 37°C. The adhesive cells were used as macrophages for cytokine production assays and immunofluorescence studies.

Immunoblotting using mouse plasma and protein analysis by two-dimensional gel electrophoresis. PMA-stimulated THP-1 cells were collected and homogenized in a homogenizing buffer comprising 40 mM Tris-aminomethane (Tris-base), 1 mM EDTA, and 10% sucrose. The crude homogenates were ultracentrifuged at 100,000 g for 4 h to separate the cytosolic fractions. The precipitated pellets were solubilized in another homogenizing buffer containing 8 M urea, 40 mM Tris-base, and 4% 3-dimethylammonio-1-propanesulfonate and ultracentrifuged to extract the particulate fractions. Similarly, membrane proteins were extracted from mouse macrophages. First, 20 μg of proteins from THP-1 or macrophages were analyzed using Western blotting by using the plasma from PBS- or THP-1-treated apoE-KO mice as the primary Abs. Furthermore, 1 μg of HSA or 20 μg of HepG2 proteins were immunoblotted using the plasma from the HSA- and HepG2-treated apoE-KO mice, respectively. Moreover, 80 and 250 μg of THP-1 proteins were analyzed using two-dimensional (2-D) gel electrophoresis with silver staining and immunoblotting, respectively. Isoelectric focusing was performed in the immobilized pl (isoelectric point) gradient tube gel (pl 6–8 or pl 8–10; Daiichi Pure Chemicals, Tokyo, Japan). The proteins in the tube gels were then equilibrated in sample buffer and transferred to slab gels. They were resolved by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes, and immunoblotted. Briefly, the membranes were incubated at 4°C overnight in a blocking buffer with the plasma of the corresponding apoE-KO mice. For quantitative analysis of the atherosclerosis, the average lesion area of five sections (every 10th section, each separated by 100 μm) from each mouse was measured according to a previously described method (17, 28). From each mouse, two sections were stained with an anti-mouse monocyte/macrophage monoclonal rat Ab (MOMA-2; BioSource International, Camarillo, CA; 1:50 dilution) or an anti-human T-cell polyclonal rabbit Ab (CD3; Dako, Glostrup, Denmark; 1:100 dilution), followed by detection with biotinylated secondary Abs and streptavidin-horseradish peroxidase. The stained area was quantitatively analyzed, and the percentage of the stained area (the stained area per total atherosclerotic lesion area) was calculated as described previously (28).

Table 1. Effect of THP-1 immunization on lipid profile, immunoglobulin levels, and peripheral leukocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBS</th>
<th>THP-1 Immunization</th>
<th>HSA Immunization</th>
<th>HepG2 Immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (male), g</td>
<td>28.5±0.6 (8)</td>
<td>27.6±0.9 (7)</td>
<td>27.9±1.4 (6)</td>
<td>27.2±1.5 (6)</td>
</tr>
<tr>
<td>Body weight (female), g</td>
<td>23.8±0.6 (10)</td>
<td>23.4±0.4 (7)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>12.6±1.6 (18)</td>
<td>11.6±0.6 (14)</td>
<td>11.5±1.4 (6)</td>
<td>12.1±1.9 (6)</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>0.48±0.08 (18)</td>
<td>0.54±0.12 (14)</td>
<td>0.52±0.18 (6)</td>
<td>0.51±0.21 (6)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>0.66±0.01 (18)</td>
<td>0.66±0.10 (14)</td>
<td>0.63±0.19 (6)</td>
<td>0.68±0.21 (6)</td>
</tr>
<tr>
<td>IgG, mg/ml</td>
<td>15.39±2.94 (10)</td>
<td>16.29±3.60 (7)</td>
<td>15.68±3.92 (6)</td>
<td>16.78±4.71 (6)</td>
</tr>
<tr>
<td>White blood cell count, 10⁶ cells/μl</td>
<td>5.2±0.4 (8)</td>
<td>4.8±0.4 (7)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Granulocytes, %</td>
<td>18.0±2.9</td>
<td>18.0±3.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Monocytes, %</td>
<td>9.8±2.1</td>
<td>12.0±2.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>72.1±2.5</td>
<td>67.8±3.4</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>0.1±0.3</td>
<td>0.2±0.5</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; numbers in parentheses are number of mice. Body weight and plasma lipid levels were not significantly different among the corresponding groups at the age of 16 wk. Blood cell count was performed using an automated clinical blood analyzer. The percentage of white blood cells was counted in 300 cells (×200 magnification) in each smear. There were no differences in the number and percentage of white blood cells between the two examined groups. THP-1, human monocyte cell line; HSA, human serum albumin (control protein); HepG2, human nonmacrophage cell line (control cells).
mice (1:1,000 dilution). Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-mouse IgG (Amer- sham, Little Chalfont, UK; 1:3,000 dilution) and enhanced ECL
detection kit (Amersham).

Titration of anti-THP-1 protein antibody by ELISA. Microplates
(Iwaki Glass, Tokyo, Japan) were coated with 5 μg/ml homoge-
nized THP-1 protein. After the plate was blocked with 1% bovine
serum albumin, it was treated with diluted mouse plasma (1:1,000)
and incubated at room temperature for 1 h. After the solution
in the plate was aspirated and the plate was washed three times
with PBS, alkaline phosphatase-conjugated affinity-purified sec-
dary Abs (anti-mouse IgG, 1:10,000 dilution; Chemicon Interna-
tional, Temecula, CA; or anti-mouse IgM, 1:30,000; Stressgen
Biotechnologies, Victoria, BC, Canada) and 50 μl of alkaline
phosphatase substrate liquid were added for color development.
The absorbance was measured using a microplate reader (at a
wavelength of 405 nm).

Immunofluorescence and immunohistochemical staining for char-
acterization of the immunoglobulins induced by THP-1 immunization.
Mouse macrophages were extracted from the spleen as described in
Macrophage extraction from ApoE-KO mice and plated on the collagen-
coated chamber glass slides (Nalge Nunc International, Naperville, IL).
After the nonadhesive cells were removed, the adhesive macrophages
were fixed with 10% neutralized formaldehyde for 15 min (with or
without 0.2% Triton X for permeabilizing the cells) and washed four
times with PBS. We performed the immunofluorescence staining with
mouse plasma obtained from THP-1-immunized or PBS-injected nonim-
munized mice (1:100 dilution) and Texas red-conjugated goat anti-mouse
immunoglobulin secondary Ab (Amersham; 1:500 dilution). Microscopic
investigations were performed using a confocal laser scanning micro-
scope. Atherosclerotic lesions of the apoE-KO mice were immunostained
using mouse plasma obtained from THP-1-immunized or nonimmunized
mice (1:100 dilution), followed by detection with biotinylated secondary
anti-mouse Abs and streptavidin-horseradish peroxidase.

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**Fig. 1.** Representative photos of the aortic root from apolipoprotein E knockout (apoE-KO) mice at the age of 16 wk. A: nonimmunized male control mouse. B: human serum albumin (HSA)-immunized male mouse. C: HepG2-immunized male mouse. D: THP-1-immunized male mouse. Original magnification, ×80; bar, 200 μm. E: quantitative analysis of atherosclerotic lesions in the nonimmunized group (solid circles), control-immunized group (shaded circles), and THP-1-immunized group (open circles). Mean lesion area of 5 sections in the aortic root from each mouse was estimated. Values are means ± SE in each group. *P < 0.01 vs. corresponding PBS-treated control groups.
Thioglycollate-induced infiltration of macrophages in vivo. To estimate the infiltration of macrophages in vivo, we assessed the thioglycollate-induced cell infiltration as described previously (9, 28).

In vitro macrophage adhesion assay. Peritoneal macrophages collected from the thioglycollate-injected apoE-KO mice were used in this assay. These cells were incubated for 15 min at 37°C in RPMI medium containing 10% mouse plasma, which was obtained from the THP-1-immunized or nonimmunized apoE-KO mice. During incubation time, the tubes were shaken on a shaker. After this treatment, the cells were plated onto culture dishes at a density of 10^7 cells/ml, and 30 min after the seeding, the plate was washed twice with PBS and fixed with neutralized 10% formaldehyde. Adhesive cells were counted using an inverted phase-contrast microscope (Nikon, Tokyo, Japan) at ×200 magnification. At least five areas per dish were counted and averaged.

Cytokine production from macrophages. For ELISA, we used commercially available kits (BioSource International), and the detection was according to the indicated protocols. Macrophages were stimulated with 2 μM PMA in RPMI medium containing 10% FBS for 16 h in 5% CO2 at 37°C. The medium was then collected and used for the measurement of monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-12, and IL-10.

Statistics. Data are means ± SE. The nonparametric Mann-Whitney U-test was used to detect significant differences when two groups were compared. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Blood analyses and general appearance of mice. There were no differences in plasma lipid levels and mean body weights among the four groups of apoE-KO mice at the age of 16 wk (Table 1). Compared with the nonimmunized control group, THP-1 or other control protein or cell immunization did not affect plasma IgG levels. Moreover, THP-1 immunization did not change the peripheral white blood cell count or the proportion of monocytes. No other abnormal findings were observed in any group.

THP-1 immunization reduced atherosclerotic lesions in ApoE-KO mice. Representative hematoxylin- and eosin-stained aortic root sections from 16-wk-old apoE-KO mice are shown in Fig. 1, A–D. The mean lesion areas were 0.16 ± 0.03, 0.16 ± 0.04, and 0.16 ± 0.02 mm² in the nonimmunized, HSA-immunized, and HepG2-immunized apoE-KO male mice, respectively (Fig. 1E). In contrast, THP-1 immunization significantly reduced atherosclerotic lesion formation (0.03 ± 0.01 mm²; P < 0.01). As shown in Fig. 1E, a similar finding was obtained in the case of female mice (0.25 ± 0.04 mm² in the nonimmunized group vs. 0.12 ± 0.02 mm² in the THP-1-immunized group; P < 0.01), although the lesions were more extensive in the females than in the males.

THP-1 immunization reduced macrophage infiltration to atherosclerotic lesions. Macrophages were abundant in the subendothelial area of the atherosclerotic lesions in the 16-wk-old nonimmunized apoE-KO mice (Fig. 2A). In contrast, the number of macrophages in the lesions was decreased in the THP-1-immunized apoE-KO mice (Fig. 2B). Quantitative analysis revealed that the percentage of the MOMA-2-stained area was significantly decreased in the THP-1-immunized group (Fig. 2C), but the percentage of the CD3-stained area remained unchanged (6.6 ± 2.1% in THP-1-immunized group vs. 5.2 ± 3.6% in the nonimmunized group; not significant).

THP-1 immunization induced macrophage-reactive antibodies. For detection of antibodies that were induced by the THP-1 immunization and reacted with macrophages, the particulate fractions of THP-1 proteins were resolved by SDS-PAGE and analyzed using immunoblotting with the plasma obtained from the THP-1-immunized or nonimmunized mice. No bands were detected when the plasma from the PBS-treated nonimmunized mice was applied. In contrast, several bands were recognized in the immunoblots when the plasma from the THP-1-immunized mice was used (Fig. 3A). Further analysis using 2-D electrophoresis revealed that some (not all) macrophage membrane proteins could be the targets of the THP-1-induced Abs (Fig. 3B). Moreover, these Abs could react with proteins from mouse macrophages (Fig. 3C). To assess the time course of the Abs titer in THP-1-immunized mice, we performed ELISA using mouse plasma (Fig. 4A). Anti-THP-1 protein Abs were induced and maintained at significantly higher levels throughout the experimental period in the immunized mice than in the nonimmunized control mice. As shown in Fig. 3D, control HSA and HepG2 immunization induced the Abs that reacted with the corresponding immunized protein.

Characterization of Abs induced by THP-1 immunization. To characterize the THP-1 immunization-induced Abs, we performed immunofluorescent and immunohistochemical
staining using mouse plasma. The cell surfaces of nonpermeabilized and permeabilized macrophages were visualized with the Abs (Fig. 4B), suggesting that membrane associated proteins were immunoreactive with the Abs present in the THP-1-immunized mouse plasma. Moreover, the atherosclerotic lesions, particularly those rich in foam cells, were stained with the Abs (Fig. 4C).

THP-1 immunization reduced thioglycollate-induced macrophage infiltration to the abdominal cavity. We investigated whether THP-1 immunization could affect the infiltration of macrophages in vivo by using the thioglycollate-induced inflammatory cell infiltration model. In this model, >80% of the infiltrated cells at 4 days following the drug injection are reported to be macrophages (9). THP-1 immunization markedly reduced the number of cells infiltrating to the abdominal cavity at 4 days after the thioglycollate injection (Fig. 5A). However, the number of peritoneal cells under the basal conditions (mainly innate macrophages) and the number of infiltrated cells at 1 day after thioglycollate injection (mainly granulocytes) did not differ between the THP-1-immunized and nonimmunized groups (Fig. 5A). These findings indicate that THP-1 immunization inhibits the infiltration of macrophages in vivo.

Pretreatment with plasma from THP-1-immunized mice reduced in vitro macrophage adhesion in vitro. The cell adhesion of mouse macrophages collected from the abdominal cavity was assessed as described in MATERIALS AND METHODS. Treatment with plasma from the THP-1-immunized mice significantly reduced the number of adhesive macrophages in vitro (Fig. 5B).

THP-1 immunization changed cytokine production by macrophages. IL-10 production was markedly increased and IL-12 production was decreased in macrophages from THP-1-immunized mice compared with those from nonimmunized mice (Fig. 5C). However, MCP-1 production by the macrophages was not affected by THP-1 immunization.

DISCUSSION

Macrophage infiltration to the atherosclerotic lesions plays a central role in the initiation and progression of atherosclerosis (4, 6, 22). However, therapeutic interventions to modify the
functions of monocytes and macrophages have not yet been clinically established. In the present study, we demonstrated that the xenogenic macrophage immunization induced macrophage-reactive Abs, inhibited macrophage infiltration, and reduced atherosclerosis in apoE-KO mice (Figs. 1–4). Our data indicate the feasibility of cell-mediated immunotherapy for atherosclerosis with the use of xenogenic macrophages. Xenogenic macrophage immunization induces antibodies that react with self-macrophages and inhibits their function, which is similar to the cases of immunotherapy for tumor angiogenesis (26) and vascular remodeling (24).

Increasing evidence demonstrates the importance of the immune system in atherogenesis (1, 4, 5, 6, 22). Intervention toward the inflammatory process could be a therapeutic strategy for atherosclerosis. Several interesting findings have been reported with regard to the role of immunoglobulins and B lymphocytes in atherogenesis. Nicoletti et al. (15) reported that the injection of polyclonal nonspecific immunoglobulin reduced atherosclerosis in apoE-KO mice, although the underlying mechanisms have not completely been elucidated. Moreover, they also revealed that B lymphocytes inhibit atherosclerotic lesion formation in apoE-KO mice (1). These data indicate the possibility that B lymphocyte-associated immune responses are antiatherogenic (5, 6). Palinski et al. (18) reported that the continuous immunization of hyperlipidemic rabbits with malondialdehyde (MDA)-modified LDL reduced the progression of atherosclerosis by inducing autoantibodies against MDA-LDL. Zhou et al. (30) obtained similar data in mice and showed that the induction of the immune response against homogenates of atherosclerotic plaques inhibited atherosclerotic lesion formation in apoE-KO mice. In their experiment, not only the oxidized-LDL but also some components of macrophages in the atherosclerotic lesions would serve as antigens to induce Abs. On the other hand, immunization with heat shock protein 65, one of the atherosclerotic plaque proteins, induced atherosclerosis, and the anti-heat shock protein 65 Abs were thought to be proatherogenic (27). It is likely that the atherosclerotic plaques contain various antigens that are targeted by the immune system, and the role of each antigen and its specific Ab in atherogenesis must be clarified in the future. Nonetheless, some specific immunoglobulins, such as the anti-MDA or anti-oxidized LDL Abs, exhibit antiatherogenic properties and are expected to serve as therapeutic tools for the treatment of atherosclerosis (18, 23, 29). In our present study, the plasma from THP-1-immunized mice, but not from nonimmunized mice, was immunoreactive against the membrane proteins of mouse macrophages, suggesting the induction of Abs by the THP-1 immunization. It is likely that several Abs rather than one specific singular Ab may cooperatively inhibit the atherosclerosis in apoE-KO mice.

Recently, several lines of evidence have indicated that the modulation of macrophage functions by Abs could be a therapeutic strategy for preventing atherosclerosis. Murayama et al. (13) reported that administration of the anti-c-fms (the receptor of macrophage colony-stimulating factor) Ab prevented early atherogenesis in apoE-KO mice. Fraser et al. (2) reported that anti-scavenger receptor Ab inhibited the adhesion of macrophages. Anti-intracellular adhesion molecule-1 and anti-α5-integrin Abs also have been shown to inhibit macrophage homing to atherosclerotic plaques in mice (20). These studies indicate that some specific Abs, which target macrophage-related proteins, act as blocking Abs, modify the functions of macrophages, and might inhibit atherosclerosis. However, there are difficulties, particularly procedural ones, in the application of these methods for the treatment or prevention of atherosclerosis in humans. Generally, repeated injections of xenogenic immunoglobulins were reported to induce blocking Abs against them. In contrast, immunization using xenogenic macrophages or specific antigens as immunogens utilizes the immune system and produces autoantibodies that probably function for comparatively long periods. Similarly to other bacterial or viral vaccines, the reinjection of a small amount of
antigens, i.e., xenogenic macrophages in this study, is sufficient to supply an adequate dose of Abs (Fig. 4A). As described in the present study, THP-1 immunization appears to induce anti-mouse macrophage Abs that modify the macrophage function (Figs. 4 and 5) and may be related to the prevention of atherosclerosis. Although immunization with the human control protein (HSA) and control cell (HepG2) could induce the Abs reacting with the immunized proteins, they did not affect the atherogenesis (Fig. 1). These data might imply that only some specific macrophage-reactive Abs played critical roles in the reduction of atherosclerosis in this study. The method of abolishing the B lymphocyte immune tolerance toward self-proteins by immunization with xenogenic cell proteins might be a new therapeutic approach to prevent several diseases involving the target cells (24, 26). Although deleterious effects appear possible, THP-1 immunization did not affect the number and the percentage of peripheral leukocytes (Table 1) or induce any deleterious phenotypes.

In the present study, we demonstrated that THP-1 immunization affected in vivo macrophage infiltration, in vitro...
macrophage adhesion, and in vitro cytokine production from macrophages. Since the treatment of THP-1-immunized mouse plasma with macrophages inhibited the adhesion, some humoral factors must affect macrophage function. The production of IL-12, a potent inducer of atherosclerosis (8), by macrophages was significantly reduced, and that of IL-10, an anti-inflammatory and antiatherogenic molecule (11), was markedly increased by the THP-1 immunization (Fig. 5). The T helper (Th) phenotype of CD4-positive T lymphocytes is an important decisive factor for sensitivity to some immune diseases, including atherosclerosis (25a). IL-10 is one of the most important cytokines of the Th2 phenotype and is antiatherogenic (11). It has been demonstrated that changing the phenotype of T lymphocytes toward Th2 reduces atherosclerotic lesion formation in apoE-KO mice (14, 21). Several recent studies have reported that not only T lymphocytes but also macrophages exhibit different phenotypes (polarization) that probably affect the cell functions (12). Hypercholesterolemia is reported to induce a change in the phenotype of the peripheral blood monocyte subset (25). Furthermore, obesity induces a phenotypic switch in tissue macrophage polarization from M2, (alternatively activated macrophages that have low proinflammatory cytokine expression) to M1 (classically activated macrophages that produce proinflammatory cytokines and generate reactive oxygen species) (10, 12). Similar to the Th phenotype in T-lymphocytes, polarization of macrophages might be another factor associated with susceptibility to atherosclerosis (12). In this study, THP-1 immunization was observed to polarize M2 macrophages that may be antiatherogenic. Together, our findings suggest that THP-1 immunization at least affected the macrophage development or differentiation and resulted in inhibition of atherosclerosis.

One limitation of the present study may be the selection of controls. First, we thought that proteins or cells that are ubiquitous in the blood vessels and are exposed to plasma, such as macrophages, should be used as controls. Some human nonmacrophage cells such as human vascular endothelial cells (HUVEC) might be used as proper controls. However, HUVEC immunization has been shown to affect the angiogenesis (tumor angiogenesis) in mice (26) and could possibly affect the atherogenesis as previously reported in rabbits (7). Therefore, we selected PBS for the nonimmunization controls, HSA as a human control protein, and HepG2 as control xenogenic human cells for the targets of immunization. However, superior controls may exist. HAS and HepG2 immunization induced Abs that reacted with either proteins but did not affect the atherogenesis in apoE-KO mice. This finding indicates that the effects observed with THP-1 immunization were not nonspecific effects as a result of human cell or protein injection.

In conclusion, our study was the first to demonstrate that fixed xenogenic macrophage immunization (such as the injection of inactivated antigens for use as vaccines) reduces the atherosclerosis in apoE-KO mice via modulation of macrophage functions. Although further studies are required to clarify the underlying mechanisms, this method is a novel and potentially useful therapeutic immune-modulation technique for preventing atherosclerosis.

GRANTS

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