Atheroprotective Effects of Alum Are Associated With Capture of Oxidized LDL Antigens and Activation of Regulatory T Cells

Maria Wigren, Daniel Bengtsson, Pontus Dunér, Katarina Olofsson, Harry Björkbacka, Eva Bengtsson, Gunilla Nordin Fredrikson, Jan Nilsson

Abstract—The immune system represents a promising novel target for prevention of atherosclerosis. Several pilot vaccines that reduce atherosclerosis in experimental animals have been developed. The aluminum hydroxide adjuvant Alum has been shown to have antiatherogenic properties in itself, suggesting that it may be a suitable adjuvant in possible future atherosclerosis vaccines. To characterize the immune pathways mediating this protection, we treated wild-type C57BL/6 and Apoe−/− mice with Alum or PBS. Analyses of splenocytes isolated from 12-week-old mice demonstrated that Alum increased the presence of CD4+CD25+FoxP3+ regulatory T cells and downregulated the expression of T cell activation markers CD28 and ICOS in Apoe−/− mice but not in C57BL/6 wild-type mice. A similar immunosuppressive phenotype was found also in 25-week-old Apoe−/− mice and was associated with reduced atherosclerosis. Alum precipitates recovered from the injection site of Apoe−/− mice contained antigens derived from oxidized LDL. These findings demonstrate that treatment of Apoe−/− mice with Alum results in an increase of regulatory T cells and suggest that these are activated by tolerogenic antigen-presenting cells presenting oxidized LDL antigens. Our findings provide improved mechanistic understanding of the atheroprotective properties of aluminum hydroxide adjuvants but also point to the importance of determining if hypercholesterolemia may compromise the efficacy of Alum-containing vaccines used clinically today. (Circ Res. 2009;104:00-00.)

Key Words: atherosclerosis ■ lipids ■ regulatory T cells ■ vaccine

The possibility of treating autoimmune diseases by immune modulation with autoantigens has been gaining increasing attention. Ongoing clinical trials in this area include vaccines for type 1 diabetes, rheumatoid arthritis, multiple sclerosis, and Alzheimer’s disease.1 A vaccine against atherosclerosis based on modulation of autoimmune responses against antigens in oxidized LDL is also in late preclinical development.2 Adjuvants enhancing the immunogenicity of vaccine antigens are critical components of almost all vaccines and play an important role in modulating the characteristics of the subsequent immune response. Aluminum-containing adjuvants are the most widely used adjuvants in clinical vaccines, and their safety is well documented.3 Interestingly, it has recently been demonstrated that the aluminum hydroxide adjuvant Alum has atheroprotective properties in itself, suggesting that it could be a particularly suitable adjuvant in a potential future atherosclerosis vaccine.4 Aluminum-containing adjuvants primarily induce humoral immunity but are less effective in activating T cell–mediated delayed-type hypersensitivity immune responses.5 Their mechanism of action is generally considered to involve delayed clearance of the antigen from the injection site,6 induction of a local inflammatory response stimulating the recruitment and activation of antigen-presenting cells (APCs),7–10 and conversion of soluble antigens into a particulate form facilitating their uptake by phagocytosis in APCs.11,12 However, it is unclear how these mechanisms can contribute to a protection against atherosclerosis when Alum is administered in absence of an antigen. In the present study, we investigated the possibility that Alum, when injected into a hypercholesterolemic environment, could adsorb self antigens and activate immune responses against these in a manner that contributes to protection against atherosclerosis.

Materials and Methods

Mice, Immunization, and Tissue Preparation

Male apolipoprotein (apo)E-deficient and wild-type C57BL/6 mice were from Taconic Laboratory. Food and tap water were administered ad libitum. Mice were given subcutaneous injections (100 μL) with Alum (aluminum hydroxide; Pierce) in PBS (1:1) at 6, 9, and 11 weeks of age. Injections of only PBS served as controls. Mice were euthanized at 12 or 25 weeks of age by intraperitoneal injection of ketamine and xylazine. Spleens were harvested and stored in PBS on
ice. Plasma was collected by cardiac puncture and stored at −80°C until analyzed. The mice in the 25 weeks were given high-cholesterol diet (0.15% cholesterol, 21% fat) from 10 weeks of age. At euthanasia, mice were whole-body perfused with PBS followed by Histochoice (Amresco) and the descending aorta was then dissected free of connective tissue and fat, cut longitudinally, mounted en face, and stored in Histochoice. The Animal Care and Use Committee approved the experimental protocols used in this study.

**Cell Preparation and Cultures**

Splenocytes in single cell suspension were prepared by pressing spleens through a 70-µm cell strainer (BD Falcon). Erythrocytes were removed using red blood cell lysing buffer (Sigma). Cells were cultured in culture media (RPMI 1640 media containing 10% heat-inactivated FCS, 1 mmol/L sodium pyruvate, 10 mmol/L Heps, 50 U of penicillin, 50 µg/mL streptomycin, 0.05 mmol/L β-mercaptoethanol, and 2 mmol/L L-glutamine; Gibco) in 96-well round bottom plates (Sarstedt). For proliferation assay, $2 \times 10^5$ splenocytes/well were cultured alone or with 2.5 µg/mL concanavalin A (Con A) (Sigma) during 90 hours. To measure DNA synthesis, the cells were pulsed with 1 µCi [methyl-3H]-thymidine (Amersham); during the last 16 hours, macromolecular material was harvested on glass fiber filters using a Filter Mate Harvester (Perkin Elmer) and analyzed using a liquid scintillation counter (Wallac). For cytokine analysis, $3 \times 10^5$ splenocytes were cultured alone or with 2.5 µg/mL Con A for 72 hours, and cytokine concentrations were measured in the cell culture supernatant using a Th1/Th2 9-plex (Meso Scale Discovery) according to the instructions of the manufacturer. The lower detection limit for all cytokines in this assay is ~0.5 pg/mL.

**Flow Cytometry**

Splenocytes were stained with fluorochrome-conjugated antibodies and analyzed with a CyaN ADP flow cytometer (Beckman Coulter). The antibodies used in these experiments were Pacific Blue-anti CD4, allophycocyanin-anti-CD25, phycoerythrin/Cy7-anti-CD4, phycoerythrin/Cy5-anti-ICOS, fluorescein isothiocyanate-anti-CD28, and allophycocyanin/Cy7-anti-CD8 (all from BioLegend) and pacific blue-anti-Foxp3 (eBioscience).

**Staining of the Descending Aorta**

En face preparations of the descending aorta were washed in distilled water, dipped in 78% methanol, and stained for 40 minutes in 0.16% oil red O dissolved in 78% methanol/0.2 mol/L NaOH. The stained plaque areas were quantified blindly using Image pro plus 4.5 software (Media Cybernetics).

**Western Blot and Malondialdehyde-Linked Fluorescence**

The white gelatinous Alum precipitate formed at the immunization site was removed after 15 minutes or 1 week after injection. The collected precipitate was then homogenized and dissolved in a protein isolation buffer (1 mol/L Tris, 0.5 mol/L EDTA, 5 mol/L NaCl, 10% Brij96, 10% NP40, and 20% protease inhibitory cocktail from Sigma). Homogenized Alum precipitate (100 mg) and native and copper-oxidized LDL (5 µg) were separated by SDS-PAGE (7.5%, Tris-HCl gel, Bio-Rad). The separated proteins were then transferred into a poly(vinylidene difluoride) membrane (Bio-Rad). Nonspecific binding was blocked by incubation of the membrane in blocking buffer (0.05% Tween-20 and 5% nonfat milk in PBS) over night. The protein content was analyzed using a oxidized LDL-specific mouse antibody (2D03) generated against the malondialdehyde (MDA)-modified 661 to 680 amino acid sequence of human apoB (1 µg/mL in blocking buffer, kindly provide by Biovent International, Lund, Sweden). After incubation with rabbit anti-mouse horseradish peroxidase antibody (Dako, 1:1000 in blocking buffer) the bands were detected using Alphalmager imaging system (Alpha Innotech). Average spot densities were analyzed with ImageJ (http://rsb.info.nih.gov/ij/). Homogenized precipitate was diluted in PBS, and the MDA fluorescence intensity (excitation and emission wavelength, 390 nm and 460 nm, respectively) was measured with Infinite M200 (Tecan). The buffer background value was subtracted and the fluorescence intensity was normalized to mg precipitate.

**Immunohistochemistry and Oil Red O Staining of Tissue Sections**

Tissue retrieved from the subcutaneous injection site was cut in 10-µm-thick sections. The slides were dehydrated in xylene and ethanol, incubated with 0.5% Triton-X in PBS for 5 minutes and 3% H$_2$O$_2$ for another 10 minutes at room temperature. Subsequently, they were blocked with 10% goat serum in PBS for 5 minutes and incubated overnight at 4°C with primary rabbit anti-mouse apoB (Abcam) or anti-human MDA-apoB peptide (2D03, Biovent) antibodies diluted in 10% goat serum in PBS. Biotinylated goat anti-rabbit IgG antibodies (Vector Laboratories) diluted in PBS were used as secondary antibody. Antibody localization was visualized using an avidin/DH/biotinylated peroxidase complex (Vector Laboratories), followed by a diaminobenzidine-H$_2$O$_2$ substrate solution (Vector Laboratories). Omission of the primary or secondary antibody was used as control.

For immunohistochemical and oil red O staining of atherosclerotic plaque, 10-µm sections were cut from the aortic root. Each series included up to 6 sections per mouse. The sections were refixed in Histochoice for 10 minutes, washed in dH$_2$O$_2$, and dipped in 60% isopropanol. For lipid staining, the sections were put in 0.24% oil red O diluted in 60% isopropanol for 10 minutes, rinsed with tap water to remove redundant oil red O, and dipped in 60% isopropanol followed by washing with dH$_2$O$_2$ before nuclear staining with hematoxylin for 15 seconds. Slides used for staining with rat anti-mouse M2A-2 antibodies (monocyte/macrophage, BMA Biomedicals, Augst, Switzerland) and rabbit anti-human CD3 (cross-reacts with mouse CD3$:^b$; Dako, Solna, Sweden) were first fixed in ice-cold acetone for 10 minutes, washed in PBS for 5 minutes, and then blocked with 10% mouse serum in PBS for 30 minutes and quickly dipped in PBS. Biotinylated rabbit anti-rat IgG and goat anti-rabbit IgG (Vector Laboratories, Burlingame, California) were used as secondary antibodies and DAB detection kit for color development (Vector). Omissions of the primary or secondary antibodies were used as controls. Lipid deposition, immunostained, and plaque area were quantified with Image-Pro-Plus 4.5 software (Media Cybernetics).

**Peptide ELISA**

Microtiter plates (Nunc MaxiSorp, Nunc, Roskilde, Denmark) were coated with either native LDL, copper-oxidized LDL (200 µg/mL), or the apoB-100–derived peptide 210 (amino acids 3136 to 3155, 20 µg/mL) overnight at 4°C. Coated plates were washed with PBS—0.05% Tween-20 and then blocked with SuperBlock in Tris-buffered saline (TBS, Pierce) for 10 minutes at room temperature, followed by an incubation of mouse plasma diluted 1:50 in TBS—0.1% Tween-20 for 2 hours at room temperature and overnight at 4°C. After washing, depositions of IgM or IgG were detected by using biotinylated goat anti-mouse IgM or IgG (Jackson ImmunoResearch) diluted 1:5000 in SuperBlock 1:10 in TBS for 2 hours at room temperature followed by another wash. The plates were then incubated with streptavidin conjugated with alkaline phosphatase (Dako) for 2 hours at room temperature. The color reaction was developed using n-pitrophenyl phosphate (Pierce), and the plates were incubated for 30 minutes in the dark before the reaction was stopped with 2 mol/L NaOH. IgG subclasses were detected with alkaline phosphatase conjugated rat anti-mouse IgG1 (diluted 1:2000) or IgG2a (diluted 1:1500) antibodies (Pharmingen) in SuperBlock 1:10 in TBS after 3 hours incubation at room temperature. The color reaction was performed as above. The absorbance was measured at 405 nm.

**Plasma Cholesterol and Triglyceride**

Total plasma cholesterol and plasma triglycerides were quantified with the colorimetric assays Infinity Cholesterol and Triglyceride (INT), respectively (Sigma).
Statistics
Data are presented as means±SE. In normally distributed material, Student’s 2-tailed t test was used, and for skewed material, the Mann–Whitney rank sum test was performed. Statistical significance was considered at the level of $P<0.05$.

Results
In a first set of experiments, Apoe$^{-/-}$ mice received 3 subcutaneous injections of Alum or PBS at 6, 9, and 11 weeks of age. (These dosing intervals were used to allow comparisons with previous studies using apoB peptide-based vaccines containing Alum as adjuvant.) The mice were euthanized 1 week after the last injection, and the effect on immune activation was assessed by flow cytometric analyses of splenocytes. A parallel group of wild-type C57BL/6 wild-type mice received the same type of treatment to determine the possible influence of hypercholesterolemia on Alum-induced immune responses. The cholesterol and triglyceride levels were 17.32±3.62 versus 14.15±2.38 mmol/L ($P<0.05$) and 0.67±0.32 versus 0.52±0.12 mmol/L in control and Alum-treated Apoe$^{-/-}$ mice and 3.81±0.38 versus 3.77±0.51 mmol/L and 0.32±0.13 versus 0.41±0.17 mmol/L in control and Alum-treated wild-type mice. Treatment with Alum increased the presence of CD4$^{+}$CD25$^{+}$FoxP3$^{+}$ regulatory T cells (Tregs) in the spleen of Apoe$^{-/-}$ mice (7.35±0.38% versus 5.22±0.44% in mice given PBS, $P<0.005$) but not in wild-type mice (Figure 1A). There was also a decreased
expression of the T-cell activation markers ICOS and CD28 on CD4 cells from
Apoe<sup>−/−</sup> mice treated with Alum (Figure 1B and 1C). Alum did not influence the expression of ICOS and CD28 in wild-type mice. There was no difference in spleen CD4 or CD8 cells (expressed as percentage of total lymphocytes) between Apoe<sup>−/−</sup> and wild-type mice, and the relative expression of these cells did not change in response to Alum (Figure 2). Interestingly, when comparing PBS-treated control mice, the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs constituted a significantly higher proportion of the total spleen CD4 T-cell population in Apoe<sup>−/−</sup> mice than in wild-type mice (5.22±0.23% versus 2.44±0.44%, P<0.001; Figure 1A).

To further characterize the effect of Alum on immune activation we analyzed cytokine release from cultured splenocytes with or without stimulation with the polyclonal T cell activator Con A. The basal cytokine expression did not differ between Apoe<sup>−/−</sup> and wild-type splenocytes (data not shown). Stimulation with Con A resulted in an increased release of interferon (INF)γ and tumor necrosis factor (TNF)α in cultured splenocytes isolated from Alum-treated wild-type mice, whereas no effect was seen in splenocytes from Apoe<sup>−/−</sup> mice (Figure 3A and 3B). Con A–induced release of interleukin (IL)-4 and IL-10 was not affected by Alum treatment (Figure 3C and 3D). Plasma levels of INFγ and TNFα were not elevated in Alum-treated wild-type mice, but the levels of IL-4 and IL-10 were reduced (Figure 4). The plasma level of IL-10 was higher in Apoe<sup>−/−</sup> mice than in wild-type mice.

Collectively, the results described above demonstrate that the immune response to Alum is markedly different in normo- and hypercholesterolemic mice. In wild-type mice, Alum exerts an anticipated modest proinflammatory effect, whereas in the hypercholesterolemic Apoe<sup>−/−</sup> mice expansion of the Treg pool is the predominant response. We have previously shown that immunization with apoB peptide vaccines inhibits atherosclerosis in Apoe<sup>−/−</sup> mice. ApoB fragments similar to those used in these vaccines are likely to be generated during oxidation of LDL in vivo and are typically modified by MDA and other aldehydes released from oxidized fatty acids. Immunohistochemical analyses of subcutaneous tissue using antibodies against mouse apoB demonstrated accumulation of LDL in Apoe<sup>−/−</sup> but not in wild-type mice (Figure 5). Accordingly, we tested the possibility that Alum would precipitate apoB peptide antigens generated by oxidation of LDL at the injection site in these mice. Generation of oxidized LDL was assessed by measur-
ing MDA-specific fluorescence and staining tissue with an oxidized LDL-specific antibody generated against the MDA-modified 661- to 680-aa sequence of apoB.\textsuperscript{20} Alum was injected subcutaneously in Apoe\textsuperscript{−/−}/H11002 mice and the administered material (which is a clearly identifiable gelatinous precipitate) was recovered after 15 minutes or 7 days. Western blot analysis demonstrated that oxidized LDL was present in the Alum precipitate 7 days after injection, whereas no or only minute oxidized LDL immunoreactivity could be detected after 15 minutes (Figure 6A and 6B). This observation was confirmed by the presence of MDA fluorescence in the precipitate at 7 days but not after 15 minutes (Figure 6C), as well as by immunohistochemical staining of tissue from the injection site, demonstrating presence of oxidized LDL in the Alum deposit (Figure 6D). Collectively, these findings suggest that Alum can capture oxidized LDL antigens present at the injection site in hypercholesterolemic animals. To determine whether this is associated with activation of an antibody response against oxidized LDL, we measured plasma levels of antibodies against apoB peptide p210 in mice that had received 3 injections of Alum or PBS as described above. IgG against p210 was present in plasma of Apoe\textsuperscript{−/−}/H11002 mice whereas the levels in wild-type mice were low (Figure 7). Total IgG levels against the p210 apoB antigen did not change in response to Alum injections, but there was a trend toward an increase in Th2-specific p210 IgG1 in Alum-treated Apoe\textsuperscript{−/−}/H11002 mice (0.72±0.12 versus 0.54±0.17 absorbance units in mice given PBS, \(P=0.06\)).

In a second set of experiments, we studied whether the immune responses induced by Alum at 12 weeks were observed also in older animals and associated with an inhibition of atherosclerosis. Apoe\textsuperscript{−/−}/H11002 mice received 3 subcutaneous injections of Alum or PBS at 6, 9, and 11 weeks of age as before and were fed a high-fat diet from 10 weeks age until euthanasia. A final treatment with Alum was given 1 week before euthanasia to boost immune responses. Plasma cholesterol levels were higher in Alum-treated mice than in PBS mice (29.70±12.49 versus 18.47±2.70 mmol/L, \(P<0.005\)), whereas there were no difference in triglycerides (0.60±0.09 versus 0.57±0.12 mmol/L). To determine whether the suppressive immune responses evoked by the initial Alum immunizations was transient or remained unchanged in older animals, we studied spleen cells from mice euthanized at the 25-week time point. The pattern of immunologic activation induced by Alum in these older mice was found to be similar to that observed at 12 weeks. Treatment with Alum was associated with a relative expansion of the CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} Treg population, a decreased expression of the activation marker

\textbf{Figure 4.} Effect of Alum on plasma cytokine levels. Quantification of INF-\(\gamma\) (A), TNF-\(\alpha\) (B), IL-4 (C), and IL-10 (D) in plasma from 12-week-old wild-type (WT) and Apoe\textsuperscript{−/−}/H11002 mice treated with Alum or PBS. The cytokine levels were analyzed by multiplex technology (MesoScale).

\textbf{Figure 5.} Subcutaneous accumulation of LDL in Apoe\textsuperscript{−/−}/H11002 mice. Subcutaneous tissue from WT (A) and Apoe\textsuperscript{−/−}/H11002 (B) mice was stained with an antibody against mouse apoB (brown; original magnification, ×20). Arrows indicate positive staining.
Antibody levels were determined by ELISA. CD28 on CD4 cells and at this time point also with a reduction of CD4 cells (assessed as percentage of total spleen lymphocytes; Figure 8). To determine whether the expansion of spleen Treg population was associated with a functional suppression of T cell proliferation, we exposed cultured spleen to the polyclonal activator Con A. Alum treatment was found to reduce Con A-induced T-cell proliferation by >50% (Figure 9). Analysis of atherosclerosis, as assessed by oil red O staining of the aorta, revealed a 35% reduction of atherosclerosis in animals given Alum (Figure 10), demonstrating that the immune responses activated by Alum were associated with atheroprotection. Effect of Alum on plaque lipid accumulation and inflammatory activity was determined by oil red O staining and immunohistochemistry on sections from the aortic root. There was no difference in total aortic root plaque size (2.74±1.16×10^5 versus 2.75±1.16×10^5 μm²) or percentage of oil red O–stained area (11.9±3.0 versus 13.4±6.3%) between the control and Alum groups. However, plaques from Alum-treated mice had less T cells, as assessed by staining with an antibody against CD3 (1.0±0.7 versus 2.9±1.3% of total plaque area, P<0.05), and more macrophages, as assessed by staining with the MOMA-2 antibody (27.0±2.7 versus 17.7±7.1% of total plaque area, P<0.05).

**Discussion**

The present observations demonstrate that the immune responses activated by the aluminum hydroxide adjuvant Alum differ markedly in wild-type and ApoE−/− mice. In normocholesterolemic wild-type mice, Alum produced a modest proinflammatory effect, as assessed by an increased release of TNFα and INFγ from Con A–stimulated splenocytes and reduced levels of the antiinflammatory cytokine IL-10 in plasma. Alum did not influence Tregs or the expression of activation markers on CD4 T cells in wild-type mice. The immune response induced by Alum in hypercholesterolemic ApoE−/− mice was markedly different and was characterized by expansion of immunosuppressive natural Tregs, inhibition of T-cell proliferation, and downregulation of the activation markers CD28 and ICOS on CD4 cells. In older animals, Alum treatment was also associated with a relative reduction of the CD4 population in the spleen. The mechanisms by which Alum activates immune suppression in hypercholesterolemic ApoE−/− mice remain to be fully characterized. However, Alum may boost an immune reaction against self antigens at the injection site by facilitating the uptake of such antigens by local APCs or by the recruitment of inflammatory APC. Antigens derived from oxidized LDL were shown to be present in the Alum deposits 1 week after injection. Accordingly, a possible explanation for our findings is that Alum captures apoB antigens released from oxidized LDL at the injection site, delivers these self antigens to APCs that migrate to peripheral lymphatic tissues where they activate antigen-specific Tregs to protect against oxidized LDL autoimmunity. One can speculate that if such oxidized LDL specific Tregs subsequently encountered oxidized LDL in the artery wall, they would dampen ongoing local inflammation and inhibit atherosclerosis development.23 If this were correct, it would explain why activation of Tregs by Alum occurs only in hypercholesterolemic animals because no or only minor amounts of oxidized LDL antigens are likely to be present at the injection site in wild-type mice. It also suggests that the atheroprotective effect of Alum could be attributable to its ability to partially mimic the effect of apoB peptide vaccines14–17 and enhance a natural protection against oxidized LDL autoimmunity.22 The present results further showed that the atheroprotective effect of Alum differed between the subvalvular and descending part of the aorta. Differences in effects between these sites have previously
been observed also in mice immunized with oxidized LDL and apoB peptides. In the present study, Alum-treated mice had higher cholesterol levels than controls given PBS alone, which may have contributed to an increased macrophage accumulation in the subvalvular plaques of these mice.

Another possible mechanism by which Alum may activate Tregs is by capturing antigens from apoptotic cells. Presentation of antigens from apoptotic cells by dendritic cells is known to induce tolerance.23 Interestingly, Ait-Oufella et al24 have recently reported that mice lacking the lactadherin receptor that mediates uptake of apoptotic membrane fragments in macrophages are characterized by an accelerated atherosclerosis and reduced Treg activity. However, it appears less likely that a mechanism involving presentation of apoptotic cell antigens can account for the clear difference in Alum-induced immune responses between Apoe−/− and wild-type mice. The present study provides no direct evidence that the atheroprotective effect of Alum is mediated by Tregs. However, there is considerable support for the notion that an expansion of the Treg population such as that induced by Alum would have an inhibitory effect on atherosclerosis. Deletion of costimulatory molecules required for Treg acti-

Figure 8. Effect of Alum on spleen T cells in 25-week-old Apoe−/− mice. Splenocytes from 25-week-old Apoe−/− mice treated with Alum or PBS were analyzed with flow cytometry to determine percentage CD25+FoxP3−-expressing (A) and CD28-expressing (B) spleen CD4 cells. The total CD4 cell fraction in the spleen (C) is expressed as a percentage of total lymphocytes. Representative dot plots are shown for each experiment with cells from PBS-treated mice to the left and cells from Alum-treated mice to the right. The percentages shown are of the total CD4 T-cell population in A and B and the total lymphocyte population in C.

Figure 9. Alum inhibits Con A–induced splenocytes proliferation. Cultured splenocytes from Apoe−/− mice, treated with Alum or PBS, were stimulated with Con A. T-cell proliferation index is expressed as thymidine incorporation ratio between stimulated and nonstimulated cells.

Figure 10. Reduced atherosclerosis in Alum-treated Apoe−/− mice. Plaque areas in descending aortas from 25-week-old Apoe−/− mice treated with Alum or PBS were assessed by en face oil Red O staining, and the percentage stained area of total aortic area was determined by computerized image analysis.
vation, as well as Treg depletion by anti-CD25 antibody treatment, significantly increases plaque formation.\textsuperscript{25,26} Similarly, inhibition of Th3 cells through deletion of the T-cell receptor for transforming growth factor-\(\beta\) markedly enhances the progression of the disease,\textsuperscript{27} whereas administration of a clone of ovalbumin-specific Tr1 cells together with its cognate antigen inhibited plaque development in apoE\textsuperscript{\textminus\textminus} mice.\textsuperscript{28}

Because Apoe\textsuperscript{\textminus\textminus} and wild-type mice may differ in other ways than plasma cholesterol levels, it cannot be excluded that the immune response to Alum in Apoe\textsuperscript{\textminus\textminus} mice is influenced by other factors than the presence of hypercholesterolemia. ApoE mediates the presentation of serum-borne lipid antigens and can be secreted by APCs as a mechanism to survey the local environment to capture antigens.\textsuperscript{29} Accordingly, it is possible that Alum may mimic apoE in delivering lipid antigens to APCs restoring a better balance of immunoreactivity against modified lipid autoantigens. In this context, it is also interesting to note the different pattern of basal immune activation between Apoe\textsuperscript{\textminus\textminus} and wild-type mice. Whereas Apoe\textsuperscript{\textminus\textminus} mice had higher levels of autoantibodies against the apoB antigen, p210 they also had significantly more natural Tregs in the spleen and higher plasma levels of the antiinflammatory cytokine IL-10. Collectively, these observations suggest that apoE\textsuperscript{\textminus\textminus} mice have a loss of tolerance against modified self antigens, such as oxidized LDL, that is counteracted by the suppressive effect of an expanded natural Treg population.

Even though the present observations may help to explain the atheroprotective properties of Alum and support its usefulness as part of a possible future atherosclerosis vaccine, they may have more important implications for the understanding of the efficacy of Alum-containing vaccines in a wider perspective. It should be kept in mind that, from a clinical point of view, Apoe\textsuperscript{\textminus\textminus} and wild-type mice are not ideal models to study the influence of plasma lipid levels on Alum-induced immune responses. The plasma cholesterol levels of wild-type mice are considerably lower than in humans and that of Apoe\textsuperscript{\textminus\textminus} mice is markedly higher (\(\approx\)4 to 6 times in this study). However, it will be important to determine whether hypercholesterolemia tends to shift the immune response induced by Alum toward activation of Tregs also in humans. If this were the case, it would be likely that hypercholesterolemic individuals may develop a lower level of protection against infection when immunized with vaccines containing aluminum hydroxide adjuvants. There may even be a potential risk for development of tolerance against the vaccine antigen. We have not been able to identify of any clinical studies specifically addressing the question how hypercholesterolemia affects the efficacy of Alum-containing vaccines. The present observations strongly argue that such studies should be performed. The observation that the immune responses to Alum are altered in presence of hypercholesterolemia also focuses attention on the possibility that this may be associated with an increased risk of adverse events. However, review of the evidence of adverse events after exposure to aluminum-containing vaccines by Jefferson et al found no evidence that aluminum adjuvants cause any serious or long-lasting adverse events.\textsuperscript{30}

In conclusion, we have shown that although Alum activates an anticipated modest proinflammatory response in normcholesterolemic wild-type C57BL/6 mice, its effect on hypercholesterolemic Apoe\textsuperscript{\textminus\textminus} mice is markedly different and characterized by activation of immune suppressive natural Tregs and downregulation of CD4 cell activation markers. Alum was also shown to precipitate oxidized LDL antigens at the injection site, suggesting that activation of Tregs could occur through presentation of these antigens by APCs in a tolerogenic fashion. Our findings provide improved mechanistic understanding of the atheroprotective properties of aluminum hydroxide adjuvants but also point to the importance of determining whether hypercholesterolemia may compromise the clinical efficacy of Alum-containing vaccines.

Acknowledgments

We thank Ingrid Söderberg, Irena Ljungcrantz, and Ragnar Alm for expert technical assistance.

Sources of Funding

This study was supported by grants from the Swedish Medical Research Council, the Swedish Heart-Lung Foundation, the Crafoord Foundation, the Knut and Alice Wallenberg Foundation, the Bergvall Foundation, the Swedish Society of Medicine, the Royal Physiographic Society, the European Community’s Sixth Framework Programme, the Albert Pählsson Foundation, the Malmö University Hospital Foundation, and the Lundström Foundation.

Disclosures

None.

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Disclosures

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References


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Circ Res. published online May 28, 2009;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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