Objective—Cholinergic pathways of the autonomic nervous system are known to modulate inflammation. Because atherosclerosis is a chronic inflammatory condition, we tested whether cholinergic signaling operates in this disease. We have analyzed the expression of the α7 nicotinic acetylcholine receptor (α7nAChR) in human atherosclerotic plaques and studied its effects on the development of atherosclerosis in the hypercholesterolemic Ldlr<sup>−/−</sup> mouse model.

Approach and Results—α7nAChR protein was detected on T cells and macrophages in surgical specimens of human atherosclerotic plaques. To study the role of α7nAChR signaling in atherosclerosis, male Ldlr<sup>−/−</sup> mice were lethally irradiated and reconstituted with bone marrow from wild-type or α7nAChR-deficient animals. Ablation of hematopoietic cell α7nAChR increased aortic atherosclerosis by 72%. This was accompanied by increased aortic interferon-γ mRNA, implying increased Th1 activity in the absence of α7nAChR signaling.

Conclusions—The present study shows that signaling through hematopoietic α7nAChR inhibits atherosclerosis and suggests that it operates by modulating immune inflammation. Given the observation that α7nAChR is expressed by T cells and macrophages in human plaques, our findings support the notion that cholinergic regulation may act to inhibit disease development also in man. (Arterioscler Thromb Vasc Biol. 2014;34:2632-2636.)

Key Words: atherosclerosis ■ alpha7 nicotinic acetylcholine receptor ■ inflammation
revealed a significant population of cells present in inflamed regions of plaques (Figure 1). The protein expression pattern of α7nAChR coincided with that of both CD68+ and CD163+ macrophages, as well as T-cell marker CD3, but to a less extent with smooth muscle and endothelial cells (Figure 1A–1F). In inflamed areas of the plaque, where 39±5% of the cells were CD3+ T cells and 19±4% CD68+ macrophages, α7nAChR protein was expressed by 67±5% of all cells. This implies that in inflamed areas, the majority of α7nAChR+ cells were of immune origin. This was confirmed by double-staining, by which α7nAChR protein was identified on CD68+ macrophages (Figure 1G). α7nAChR was also detected on the CD163+ subset of macrophages, suggesting expression in alternatively activated M2 macrophages (Figure 1H). Furthermore, α7nAChR colocalized with a subset of CD3+ T cells in the lesions (Figure 1I). Single-channel micrographs of immunofluorescent staining are shown in Figure I in the online-only Data Supplement.

The global gene expression array database of the BiKE biobank was interrogated for α7nAChR mRNA expression pattern. We found a significant correlation between mRNA for α7nAChR, Chrna7, and M2 markers CD36 and CD163. The expression levels of Chrna7 and CD63 had a Pearson correlation coefficient of 0.346 (P=2.6e–04) and for Chrna7 and CD163 the Pearson correlation coefficient was 0.282 (P=0.0033). Although relatively weak correlations, this further supports that α7nAChR was expressed by alternatively activated M2 macrophages.

Increased Atherosclerosis in α7nAChR–/– BM Chimeric Ldlr–/– Mice

The finding of α7nAChR on immune cells in human atherosclerotic lesions prompted us to explore whether α7nAChR expression by hematopoietic cells affects atherosclerosis in an experimental model. BM was transplanted from Chrna7–/– mice lacking α7nAChR, or wild-type mice to irradiated Ldlr–/– mice. The first 72% increase in atherosclerosis in the aortic root (Figure 2A). When compared with lesions of mice transplanted with wild-type BM, those of mice receiving α7nAChR–/– BM were more advanced with large lipid deposits. Body weight was not influenced by the lack of α7nAChR in BM cells or did plasma cholesterol levels change (Table I in the online-only Data Supplement). Immunostaining for the macrophage marker CD68, the adhesion molecule vascular cell adhesion molecule 1 (VCAM-1), or the T-cell marker CD3 did not show any differences between groups (Figure 2B).

Aortic samples from α7nAChR–/–×Ldlr–/– chimeras exhibited increased mRNA expression of the proinflammatory Th1 cytokine interferon-γ (IFNγ; Figure 2C). In line with this, CD4 and CD8 mRNA levels were numerically increased in mice transplanted with α7nAChR–/– BM although this did not reach statistical significance. Aortic mRNA levels of the scavenger receptor CD36 were not altered, nor did we find any differences in expression of tumor necrosis factor-α (TNFα) or the macrophage marker CD68 (Figure 2C). Together, these data suggest that lack of α7nAChR signaling was associated with increased Th1 activity but not with significantly increased inflammatory cell infiltration into lesions.

Analysis of spleen mRNA did not show any difference between α7nAChR–/– and wild-type×Ldlr–/– chimeras with regard to CD68, CD4, CD8, or the mannose receptor, a marker for M2 macrophages (Figure IIA in the online-only Data Supplement). However, mRNA for the transcription factor FoxP3 that is expressed by regulatory T cells was significantly increased in α7nAChR–/–×Ldlr–/– chimeric mice. There were no differences in mRNA levels of the B-cell–specific CD19 marker, nor in antibody titers to oxidized low-density lipoprotein between the groups (Figure IIB in the online-only Data Supplement). Using flow cytometry, we investigated T-cell populations (CD3, CD4, CD8, and FoxP3), macrophages (F4/80 and CD11b), and granulocytes (Gr-1) in the spleen. There were no differences between the groups (data not shown). We further investigated the number of interferon γ positive (IFNγ+) cells in spleen and the coexpression of IFNγ with CD3, CD4, and F4/80. The total number of IFNγ+ cells, Th1 cells (CD3+CD4+IFNγ+ lymphocytes), and IFNγ+ macrophages (F4/80+IFNγ+) were not altered (Figure IIC in the online-only Data Supplement).

To investigate whether α7nAChR deficiency contributed to the increased T-cell activity, a proliferation assay was used to determine proliferative activity after stimulation with the T-cell mitogen, ConA. Splenocyte proliferation was significantly higher in animals lacking α7nAChR (Figure 2D). α7nAChR deficiency caused an increase in IFNγ secretion after ConA stimulation of splenocytes; however, this did not reach statistical significance (P=0.096; Figure 2D).

Discussion

In the current study, we demonstrate that the absence of α7nAChR from BM cells accelerates atherosclerosis in hypercholesterolemic Ldlr–/– mice. Therefore, a cholinergic signal mediated through this receptor inhibits the disease process. The fact that targeting α7nAChR in BM cells was sufficient to affect disease substantially indicates that hematopoietic cells are major intermediates in this disease-modifying axis. Because the lack of hematopoietic α7nAChR was associated with increased IFNγ expression in atherosclerotic arterial tissue, cholinergic inhibition of atherosclerosis likely involves Th1-type T cells. Furthermore, α7nAChR was identified in human lesions, where it colocalized with a subset of T cells and macrophages. α7nAChR modulation of adaptive and innate immunity likely contributes to the striking effect on atherosclerosis.

It has not been possible to identify α7nAChR protein in mouse lesions by immunostaining although mRNA was detected in lesions of ApoE knockout mice, both at 10 and 20 weeks (data not shown). In fact, all antibodies tested by us showed reactivity also in α7nAChR-targeted mice. This is in line with the experience of others.α7nAChR signaling has important effects on macrophage function.α4 Macrophages lacking α7nAChR exhibit increased...
expression of scavenger receptor CD36 and accumulate cholesterol intracellularly. However, CD36 expression was not altered in the α7nAChR−/−×Ldlr−/− chimeric mice of the current study. The cholinergic pathway also modulates adaptive immunity, with significant effects on T- and B-cell function. Vagotomized mice display increased CD4+ T-cell proliferation and cytokine release, suggesting that intact vagal nerve signaling dampens T-cell activation. In line with this, we observed an increased expression of the proatherogenic Th1 cytokine IFNγ in the aorta of α7nAChR−/−×Ldlr−/− chimeric mice and

Figure 1. α7R is expressed in human atherosclerotic plaques. A–F. Immunoperoxidase staining of serial sections for α7 nicotinic acetylcholine receptor (α7nAChR; A), macrophage markers CD68 (B) and CD163 (C), T-cell marker CD3 (D), smooth muscle cell marker α-actin (E), and endothelial cell marker von Willenbrand Factor (F). G–K. Immunofluorescent staining showing colocalization of α7nAChR and macrophage markers CD68 and CD163, as well as with T-cell marker CD3. Occasional endothelial and smooth muscle cells expressed α7nAChR although the majority of these cells were negative for α7nAChR. Colocalization of α7nAChR (green) and macrophage markers CD68 (red, G) and CD163 (red, H) and T-cell marker CD3 (red, I), respectively. Endothelial cell marker von Willenbrand Factor (red, J) and smooth muscle cell marker αSM-actin (red, K) did not colocalize with α7nAChR. Nuclei are stained blue with DAPI (4',6-diamidino-2-phenylindole). Magnification ×40. Scale bar, 1 mm and 200 μm, respectively.
Figure 2. α7 nicotinic acetylcholine receptor (α7nAChR) deficiency on hematopoietic cells accelerates atherosclerosis in Ldlr−/− mice. A, Irradiated mice were reconstituted with bone marrow from wild-type (WT, white boxes) or α7nAChR-deficient (α7nAChR−/−, black circles) mice. Atherosclerosis was quantified in serial sections of the aortic root, 100 to 800 μm from aortic sinus. Representative micrographs of Oil-red-O–stained lesions from mice reconstituted with WT or α7nAChR−/− bone marrow and mean lesion area (n=12–14). B, Immunostaining and quantification of representative cryosections from the aortic root for CD68, vascular cell adhesion molecule 1 (VCAM-1), and CD3. Graphs represents percentage staining for CD68, VCAM-1, and number of CD3+ cells per square millimeter of lesion area. n=12 to 14 per group. C, Aortic mRNA levels from WT and α7nAChR−/−×Ldlr−/− chimeras were analyzed with real-time polymerase chain reaction. Data expressed as arbitrary units (AU), n=15 per group. D, Spleen cells from α7nAChR−/− and WT mice were stimulated in vitro with T-cell mitogen ConA. Proliferation was measured by 3H-thymidine assay (left) and interferon (IFN)-γ was measured in supernatants after 48 hours by ELISA (right), n=7 to 9 mice per group. Data expressed as means±SEM. Lesion size (A, left) were analyzed using 2-way ANOVA (group P<0.0001, level P<0.0001, interaction P=0.8505) and mean lesion area (A, right) with Student t test after logarithmic transformation. D, Proliferation and ELISA data are expressed as estimated marginal mean±SEM. *P<0.05. Scale bar, 200 μm (A and B; CD68) and 100 μm (B; VCAM-1 and CD3), respectively.
an increased proliferative response in α7nAChR-deficient splenocytes. Surprisingly, splenic FoxP3 mRNA levels were increased in α7nAChR−/−Ldlr−/− chimeric mice; however, FoxP3 protein was not altered. Thus, α7nAChR signaling does not seem to influence the number of Th1 cells but rather exert an immunomodulating effect on T cells.

Because α7nAChR ablation in hematopoietic cells increased disease, an endogenous mediator operating through α7nAChR inhibits atherosclerosis. An obvious hypothesis is that this mediator is acetylcholine. In line with this notion, pharmacological inhibition of the enzyme responsible for acetylcholine degradation, cholinesterase, diminishes atherosclerosis in Apoe−/− mice.11 α7nAChR ligands include not only acetylcholine but also the antagonist kynurenic acid.12 Interestingly, metabolites of tryptophan degradation through the kynurenine pathway inhibit atherosclerosis.13 Whether such metabolites also could act as agonists and account for α7nAChR-mediated atheroprotection remains to be determined.

It should be mentioned that the nicotinic receptor family is a heterogeneous one with a large variety of nicotinic receptor subtypes. Previous reports show that nicotine, per se, is atherogenic.14,15 To our knowledge, the specific receptor mediating this proatherogenic effect has not been identified and it is possible that several different nicotinic receptors could contribute to this effect.

In conclusion, α7nAChR signaling operating via hematopoietic cells is an important modulator of atherosclerosis. As α7nAChR is expressed in human atherosclerotic lesions, this pathway likely operates also in human disease. Therefore, the α7nAChR pathway may be an interesting target for antiatherosclerotic therapy.

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Significance
Atherosclerosis is an inflammatory disease. Recently, the cholinergic part of the autonomic nervous system was shown to modulate inflammation in acute experimental models. This immune modulation is mediated via the α7 nicotinic acetylcholine receptor (α7nAChR). Here, we demonstrate that the α7nAChR exerts an important atheroprotective effect that is linked to immunomodulatory action. Furthermore, we demonstrate the expression of the α7nAChR in human atherosclerotic plaques. These findings suggest α7nAChR signaling as an important modulator of atherosclerosis and thus an interesting target for antiatherosclerotic therapy.
α7 Nicotinic Acetylcholine Receptor Is Expressed in Human Atherosclerosis and Inhibits Disease in Mice—Brief Report

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**SUPPLEMENTAL MATERIAL**

**Supplemental Table I. Body weight and plasma cholesterol**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT chimera (15)</td>
<td>23.2±0.9</td>
<td>21.9±1.2</td>
</tr>
<tr>
<td>α7R⁻/⁻ chimera (15)</td>
<td>23.1±0.8</td>
<td>21.4±0.6</td>
</tr>
</tbody>
</table>

p value n.s n.s
Supplemental Figures
Figure 1.
Supplemental Figure I. (A-L) Immunofluorescent staining, single channels, for α7nAChR (green, A-C) and macrophage markers CD68 (red, D) and CD163 (red, E), as well as with T cell marker CD3 (red, F). DAPI (blue, G-I) shows nuclear staining. Merged pictures shows co-localization of α7nAChR and CD68 (J), CD163 (K) and CD3 (L), respectively. Magnification X40. (M-N) Oil red o staining of aortic root, 200, 400, 600 and 800 µm from aortic sinus in WT (M) and α7nAChR−/− x Ldlr−/− (N) chimeras. Scalebar represents 200 µm.
Figure II.

A

\[ \text{CD4 mRNA (AU)} \]

\[ \begin{array}{c}
\text{WT} \\
\text{α7nACHR-/-}
\end{array} \]

\[ \begin{array}{c}
\text{CD8 mRNA (AU)} \\
\text{WT} \\
\text{α7nACHR-/-}
\end{array} \]

\[ \begin{array}{c}
\text{CD16 mRNA (AU)} \\
\text{WT} \\
\text{α7nACHR-/-}
\end{array} \]

B

\[ \text{OD (450 nm)} \]

\[ \begin{array}{c}
\text{IgM OXLDL} \\
\text{IgG OXLDL} \\
\text{IgM MDA-LDL} \\
\text{IgG MDA-LDL} \\
\text{IgG Phosphorylcholine-BSA} \\
\text{IgM Phosphorylcholine-BSA}
\end{array} \]

C

\[ \text{CD3+CD4+ IFN-γ+ cells (10^6)} \]

\[ \begin{array}{c}
\text{WT} \\
\text{α7nACHR-/-}
\end{array} \]

\[ \text{IFN-γ+ cells (10^6)} \]

\[ \begin{array}{c}
\text{WT} \\
\text{α7nACHR-/-}
\end{array} \]

\[ \text{Total IFN-γ+ cells (10^6)} \]

\[ \begin{array}{c}
\text{WT} \\
\text{α7nACHR-/-}
\end{array} \]
Supplemental Figure II. Splenocyte gene and protein expression and antibody titers. (A) Splenic mRNA levels from WT and α7nAChR<sup>-/-</sup> x Ldlr<sup>-/-</sup> chimeras were analyzed with real-time PCR. Data expressed as arbitrary units (AU), mean±SEM., MR=mannose receptor. n=15/group. (B) Plasma levels of IgMs and IgGs directed against Oxidized LDL (OxLDL), malondialdehyde-modified LDL (MDA-LDL) and phosphorylcholine from WT and α7nAChR<sup>-/-</sup> x Ldlr<sup>-/-</sup> chimeras were determined by chemiluminescent ELISA. n=15/group, data is expressed as mean±SEM. (C) Spleen cell populations from WT and α7nAChR<sup>-/-</sup> mice were counted and analyzed by flow cytometry. Absolute numbers of Th1 cells (CD3<sup>+</sup>CD4<sup>+</sup>IFN<sub>γ</sub><sup>+</sup>), macrophages expressing IFN<sub>γ</sub> (F480<sup>+</sup> IFN<sub>γ</sub><sup>+</sup>) and all IFN<sub>γ</sub><sup>+</sup> cells in spleen, were determined by multiplying cell frequencies with total spleen cellularity for each individual, n= 7-9 mice per group, data is expressed as estimated marginal mean ± SEM. (E) *p<0.05.
SUPPLEMENTAL METHODS

Immunostaining of α7nAChR receptors in human plaques

Human carotid artery plaques were collected from patients undergoing endarterectomy at Sahlgrenska University Hospital, Gothenburg, Sweden. Surgery was performed due to transitory ischemic attacks or ischemic stroke in combination with significant carotid artery stenosis. The investigation was approved by the Ethical Committee of Gothenburg and was in agreement with institutional guidelines and the principles that have been set forth in the Declaration of Helsinki. Informed consent was obtained from all subjects. Briefly, immunostaining was performed on acetone fixed cryosections. Following blocking with 3% H2O2 for 5 minutes, avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA, USA), 5% dry milk and 10% goat serum (Vector Laboratories) the sections were incubated with mouse anti-human monoclonal antibodies directed against CD68, Von Willebrand Factor, Smooth Muscle Actin (all from Dako Denmark, Glostrup, Denmark), CD163 (ABCAM, Cambridge, UK), or CD3 (Nordic BioSite AB, Täby, Sweden). Sections were subsequently blocked with goat serum prior to addition of a rabbit anti-human α7nAChR polyclonal antibody (#ab10096, Abcam, Cambridge, UK), diluted in goat serum, incubated for one hour at room temperature. Peroxidise staining were detected with ABC alkaline phosphatase kit (Vector Laboratories) and subsequently NovaRed (Vector Laboratories). Cells positive for CD3, CD68 and α7nAChR were quantified in serial sections of two different inflamed regions in each plaque (n=7) and normalized to the corresponding number of total cells. For immunofluorescens staining sections were incubated with Image-IT FX signal enhancer (Invitrogen-Molecular Probes, Eugene, OR, USA) for 30 minutes prior to incubation with H2O2. Immunofluorescens was detected by using DyLight 594 conjugated donkey anti-mouse IgG (Thermo Fisher Scientific) and for α7nAChR by using a secondary, biotinylated goat anti-rabbit IgG (Vector Laboratories) followed by incubation with an Alexa Fluor 488-streptavidin conjugate (Invitrogen). Sections were incubated with Sudan Black 0.1% for 10 minutes prior to mounting (DAKO fluorescent mounting medium, Dako, CA, USA). Samples without primary antibody, incubated with isotope rabbit polyclonal IgG isotype (Abcam) or with an irrelevant antibody, polyclonal rabbit-anti-cytokeratin-7 IgG that stains glandular epithelia but not arterial or immune tissues, served as negative controls. Images were produced with a Zeiss Axio Imager Z2 microscope with a 40X objective, connected with a Zeiss Axio Cam MRm camera, and captured using the Zeiss ZEN 1.0.1.0 software with deconvolution, and orthogonal projection.

Gene expression of α7nAChR in human plaques

Gene expression data was analyzed in the Biobank of Karolinska Endarterectomies (BiKE) at Karolinska University Hospital, Stockholm, Sweden, as previously described. Informed consent was obtained from all subjects. Briefly, carotid plaques from 107 patients undergoing carotid endarterectomy were profiled for gene expression using Affymetrix HG-U133 plus 2.0 microarrays according to manufacturer’s instruction. Data was pre-processed using the RMA algorithm including a log2-transformation step. The CHRNA7 was measured by the probe set 210123_s_at. The CD36 and CD163 genes were both measured by several probe sets on this array type. CD163 data are reported as 203645_s_at and CD36 data as 242197_x_at. However, the majority of the probe sets for each gene showed similar results (median CHRNA7-correlation of five CD36 probe sets is 0.28 and of three CD163 probe sets is 0.27).

Bone marrow transfer experiment
Low-density lipoprotein receptor deficient (Ldlr\(^{-/-}\)) mice, alpha 7 nicotinic acetylcholine receptor knockout, B6.129S7-Chrna7\(^{tm1Bay}\) (α7R\(^{-/-}\)), and their corresponding C57Bl/6J control mice (WT) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). α7nAChR\(^{-/-}\) mice were backcrossed by donating investigator to C57BL/6 for eight generations and then maintained at the Jackson Laboratory. All procedures were approved by the Stockholm North and the Gothenburg Committee for Experimental Animal Ethics.

Bone marrow transfer (BMT) was performed as previously described\(^2\). In brief, 6-9 weeks old Ldlr\(^{-/-}\) mice were irradiated with two doses of 700 rad, 3 h apart, and were randomly assigned to receive bone marrow derived from α7nAChR\(^{-/-}\) or C57Bl/6J donor mice at the age of 6-9 weeks old (n=15 recipients per group). The transplanted mice were allowed to recover from irradiation for 4 weeks, after which they were fed high-cholesterol atherogenic diet (HCD, D12108, Research Diets, New Brunswick, NJ) containing 1.25% cholesterol and 0% cholate for 8 weeks.

**Tissue preparation, immunohistochemistry and lesion analysis**

At sacrifice, mice were euthanized with carbon dioxide and organs were dissected. Blood from sacrificed mice was collected by cardiac puncture and vascular perfusion was performed with sterile RNase-free PBS. The heart and aortic root were frozen and cryosectioned for immunohistochemistry while the remaining aorta down to the bifurcation was dissected and snap-frozen for subsequent RNA isolation. Since there is a strong correlation between the extent of atherosclerosis in the aortic root and the entire aortic tree in murine atherosclerosis models\(^3\), we measured lesions in the aortic root as previously described\(^4\). Briefly, 10 μm sections were collected at 100 μm intervals starting at a 100-μm distance from the appearance of the aortic valves. Sections were air-dried and fixed with 4% formaldehyde in PBS. Lesions in the aortic sinus were analyzed after hematoxylin and Oil Red O staining of formaldehyde-fixed sections at 100 μm intervals. Images were captured for each section with a Leica DM-LB2 microscope (Leica, Wetzlar, Germany) equipped with a 20X/0.9 objective and a Leica DC300 camera. The surface area of the lesions and the circumference of the entire vessel were measured and lesion size was calculated using Leica QWin image analysis software.

Primary antibodies to CD68 (AbD Serotec, Düsseldorf, Germany), CD3 (Abcam, Cambridge, UK) and VCAM-1 (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).

**Cholesterol measurements and determination of specific OxLDL antibody titers**

Total serum cholesterol was determined using enzymatic colorimetric kit according to the manufacturer’s instructions (Randox Lab. Ltd. Crumlin, UK). Levels of IgMs and IgGs in plasma were determined by chemiluminescent ELISA as previously described\(^5\).

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

Total RNA was prepared from the aorta using the RNeasy Lipid Mini kit (Qiagen, Hilden, Germany) and analyzed by BioAnalyzer (Agilent Technologies, Waldbronn, Germany) capillary electrophoresis. Reverse transcription was performed with Superscript-II (Invitrogen) with random hexanucleotide primers (pdN6) in the presence of RNasin (Life Technologies, Cergy Pontoise, France), and cDNA
amplified by real-time PCR using primers and probes (Applied Biosystems, Foster City, CA, U.S.A.) for selected genes (see figure legends for details) in an ABI 7900HT Sequence Detector (Applied Biosystems). Reference mRNA species were evaluated using Reference Gene Panel Mouse (TATAA Biocenter, Gothenburg, Sweden) and analyzed using the GenEx Standard software (TATAA Biocenter). TATAA-box Binding Protein (Tbp) and Tubulin beta polypeptide (Tubb5) mRNA were suggested as references and subsequently used in the analysis of specific mRNA levels in aorta. For spleen samples Ywhaz mRNA was suggested and used in the subsequent analysis. Gene expression levels were analyzed using the GenEx Standard software.

**Preparation of spleen cells and flow cytometry**

Heterozygous α7nAChR+/− mice were bred with Ldlr−/− mice. From these litters α7nAChR−/− / Ldlr−/− and α7R+/+ / Ldlr−/− were used for flow cytometry analysis and splenocyte proliferation assay. All experiments included littermate controls. Mice were fed standard diet throughout the whole experiment and were sacrificed at 8-13 weeks of age.

Spleens were mashed through 70 μm cell strainers and suspended in Tris-buffered 0.83% NH₄Cl solution to lyse erythrocytes. The total number of spleen leukocytes was analyzed in an automated cell counter (Sysmex, Hamburg, Germany). Cells were suspended in complete medium (RPMI 1640 (PAA Laboratories) supplemented with 10% FCS (Sigma-Aldrich, St Louis, MO, USA), 1% 2-mercaptoethanol (Sigma-Aldrich) and 1% penicillin-streptomycin-L-glutamine solution (Sigma-Aldrich), and stimulated with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma) and Golgiplug® (BD, Franklin Lakes, NJ, USA) for 4 hours at 37°C and 5% CO₂. Fluorochrome-conjugated anti-mouse antibodies were used for intracellular staining of IFNγ (BD) in permeabilization buffer (eBioscience), and extracellular staining for CD3, CD4 (BD), F4/80 (Biolegend, San Diego, CA, USA). Samples were run on a BD FACS Canto II and data was processed using Flow Jo 10.0.4 (Three Star Inc, Ashland, USA). All analyses started with gates on singlet cells and lymphocytes, thereafter T helper 1 cells (Th1; CD3⁺CD4⁺IFNγ⁺) and IFNγ⁺F4/80⁺ macrophages were defined. Absolute numbers of cell populations were calculated by using total spleen cellularity obtained in cell counter and flow cytometric cell frequencies.

**Splenocyte proliferation and IFNγ ELISA**

Splenocytes were suspended in complete RPMI medium and seeded at 1x10⁶ cells/ml in triplicates in 96-well plates, the T cell mitogen concanavalin A (conA; Sigma) was used at 1,25 μg/ml and cells in medium only were used as controls. After 48 hrs of culture (37°C, 5% CO₂), supernatants were collected followed by addition of 1μCi[³H] thymidine (PerkinElmer, Waltham, MA, USA) for 18 hr, and then plates were stored in -20°C. Cells were harvested onto glassfibre filters and counted in a β-counter, proliferation is presented as counts per minute (cpm; median of cpm in conA-stimulated wells minus median of cpm in control wells).

Concentration of IFNγ in supernatants was measured using Enzyme-Linked Immunosorbent Assay ELISA kit (R&D Systems, MN, USA). Briefly, plates were coated with rat anti-mouse IFNγ antibodies, supernatants were added followed by biotinylated goat anti-mouse IFNγ antibodies, streptavidin-HRP and substrate solution (H₂O₂ and TMB). The reaction was stopped using 1M H₂SO₄. Plates were read at 450 nm, with wavelength correction at 540 nm, on a SpectraMax Plus (Molecular Devices, Sunnyvale, Calif).
Statistics

Results are expressed as mean ± SEM and differences between groups were considered significant at p<0.05. Normality was tested using the Shapiro-Wilk normality test and parametric or nonparametric tests were used accordingly. Atherosclerotic lesion area and mean lesion area was analyzed using 2-way ANOVA and Students’ t-test (GraphPad Prism, version 5.03, La Jolla, CA, USA) after logarithmic transformation. Pearson correlation was used for investigating associations between CHRNA7, CD36 and CD163, respectively. For flow cytometry and proliferation data, when the experiment was terminated on different days and to adjust for covariates, two-way ANOVA with experiment as nuisance factor were used. Proliferation, ELISA and flow cytometry data is expressed as estimated marginal mean ± SEM. All other data were analyzed using Mann-Whitney U test (IBM SPSS Statistics version 20.0, Chicago, IL, USA).

References