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HEMATOPOIETIC α7 NICOTINIC ACETYLCHOLINE RECEPTOR DEFICIENCY INCREASES INFLAMMATION AND PLATELET ACTIVATION STATUS, BUT DOES NOT AGGRAVATE ATHEROSCLEROSIS

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Abstract

The autonomic nervous system attenuates inflammation through activation of the α7 nicotinic acetylcholine receptor (α7nAChR), a pathway termed the cholinergic anti-inflammatory reflex. Interestingly, α7nAChR is expressed on immune cells and platelets, both of which play a crucial role in the development of atherosclerosis. To investigate the role of hematopoietic α7nAChR in inflammation and platelet function in atherosclerotic *ldlr*$^{-/-}$ mice and to identify its consequences for atherosclerotic lesion development. Bone marrow from *α7nAChR*$^{-/-}$ mice or wild-type littermates was transplanted into irradiated *ldlr*$^{-/-}$ mice. After a recovery period of 8 weeks, the mice were fed an atherogenic Western-type diet for 7 weeks. Hematopoietic α7nAChR deficiency clearly increased the number of leukocytes in the peritoneum (2.6-fold, $p<0.001$), blood (2.9-fold; $p<0.01$), mesenteric lymph nodes (2.0-fold; $p<0.001$) and spleen (2.2-fold; $p<0.01$), indicative of an increased inflammatory status. Additionally, expression of inflammatory mediators was increased in peritoneal leukocytes (TNFα, 1.6-fold, $p<0.01$; CRP, 1.8-fold; $p<0.01$) as well as in the spleen (TNFα, 1.6-fold, $p<0.01$). The lack of α7nAChR on platelets from these mice increased the expression of active integrin $\alpha_{IIb}\beta_3$ upon stimulation by ADP (1.9-fold, $p<0.01$), indicating increased activation status, while incubation of human platelets with an α7nAChR agonist decreased aggregation (-35%, $p<0.05$). Despite the large effects of hematopoietic α7nAChR deficiency on inflammatory status and platelet function, it did not affect atherosclerosis development or composition of lesions. Hematopoietic α7nAChR is important for attenuation of inflammatory responses and maintaining normal platelet reactivity, but loss of hematopoietic α7nAChR does not aggravate atherosclerosis development.

Introduction

Accumulating evidence indicates a prominent role of the autonomic nervous system in the regulation of inflammation via the so-called 'cholinergic anti-inflammatory pathway'. This pathway relies upon direct activation of the afferent vagus nerve by pro-inflammatory cytokines and subsequent activation of efferent nerve fibers resulting in the release of acetylcholine in peripheral organs (1-4). Acetylcholine stimulates α7 nicotinic acetylcholine receptors (α7AChR) which is highly expressed on immune cells, resulting in decreased NF-κB-mediated gene expression of pro-inflammatory cytokines (5,6). In addition to immune cells, α7nAChR is expressed on a variety of cells including neuronal cells (7), endothelial cells (8) and platelets (9).

Despite its widespread expression, mice deficient for α7nAChR are viable, develop normally and show no physical or neurological defects (10). However, upon endotoxin administration, *α7nAChR*$^{-/-}$ mice have significantly increased TNFα levels in the serum and spleen compared with wild-type mice (4). Serum IL-6 and C-reactive protein (CRP) are increased in *α7nAChR*$^{-/-}$ mice compared to control mice on an atherosclerosis-prone background (11). Accordingly, *in vitro* studies show that acetylcholine attenuates the release of pro-inflammatory cytokines (i.e. TNFα, IL-1ß, and IL-6) by macrophages conditioned by exposure to endotoxin (1). Additionally, cholinergic stimulation of α7nAChR inhibits endothelial cell activation and leukocyte recruitment during inflammation (8) and modulates platelet function (9). Together, these data suggest an attenuating effect for α7nAChR signaling on inflammation.

Atherosclerosis is a chronic inflammatory disease and the main cause of cardiovascular events. Atherosclerotic plaque development is initiated by trapping of lipids in the intima of the arterial wall, which stimulates vascular cells to produce inflammatory molecules and recruit monocytes and T cells to the vessel wall (12). Interestingly, the spleen is the most important source of acetylcholine (13) and splenic monocytes contribute largely to atherosclerotic lesion development (14). Additionally, circulating inflammatory markers that are under control of the cholinergic anti-inflammatory pathway, such as CRP and IL-6, have been implicated in the pathogenesis of atherosclerosis (15,16). Furthermore, recruitment, transmigration and activation of leukocytes, as well as platelet function contribute to atherogenesis (15-17).

We thus hypothesized that α7nAChR activity exhibits an inhibitory effect on atherosclerotic lesion development. As the cholinergic anti-inflammatory pathway exerts its function through hematopoietic α7nAChR, the aim of this study was to assess the stimulatory effect of hematopoietic α7nAChR deficiency on inflammation and platelet function, and their consequences for atherosclerotic lesion development. Because wild-type (WT) mice do not develop atherosclerosis due to rapid hepatic (V)LDL clearance resulting in low (V)LDL-cholesterol levels (~2 mM), we used LDL

receptor deficient mice as recipients of α7nAChR-deficient or WT bone marrow. LDL receptor deficient mice have increased cholesterol levels on a chow diet that are further increased upon Western-type diet feeding, which allows the development of diet-induced atherosclerosis.

Material & Methods

Animals

α7nAChR$^{+/-}$ and *α7nAChR*$^{-/-}$ mice obtained from Charles River Laboratories (L'Arbresle, France) were used to generate *α7nAChR*$^{-/-}$ and *α7nAChR*$^{+/+}$ (wild-type, WT) mice (both C57Bl/6J background). Homozygous LDL receptor knockout (*ldlr*$^{-/-}$) mice (C57Bl/6J background) were obtained from The Jackson Laboratory (Bar Harbor, USA) as mating pairs and bred at the Gorlaeus Laboratories (Leiden, The Netherlands). Mice were housed in sterilized filter-top cages in a temperature-controlled room with a 12-h light/dark cycle and food and water were provided *ad libitum*. Mice were maintained on sterilized regular chow, containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK). To induce atherosclerosis, they were fed a Western-type diet (WTD), containing 15% (w/w) cacao butter, 1% (w/w) corn oil and 0.25% (w/w) cholesterol (Diet W, Abdiets, Woerden, The Netherlands). Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sucrose. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in accordance with the National Laws (ID 10161.1). All animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University and carried out in compliance with the Dutch government guidelines.

Bone marrow transplantation (BMT)

To induce bone marrow aplasia, female *ldlr*$^{-/-}$ recipient mice (10-12 weeks old), which represent an established model for the development of atherosclerosis, were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International, Hamburg, Germany) with a 6-mm aluminum filter 1 day before the transplantation. At the day of transplantation, bone marrow from female donor WT and *α7nAChR*$^{-/-}$ mice was isolated by flushing the femurs and tibias with phosphate-buffered saline (PBS) (n=3 per group). Single-cell suspensions were obtained by passing the cells through a 70 μm cell strainer (Falcon, The Netherlands). Irradiated *ldlr*$^{-/-}$ recipients received $5x10^6$ bone marrow cells by intravenous injection into the tail vein. After a recovery period of 8 weeks mice were fed the WTD for 7 weeks, after which animals were sacrificed. Body mass was recorded weekly throughout the study.

Assessment of successful bone marrow reconstitution

At the end of the study, bone marrow was isolated from the transplanted *ldlr*$^{-/-}$ mice, and hematologic chimerism was determined using genomic DNA by PCR at 15 weeks after BMT. The relative presence of the α7nAChR in bonemarrow was assessed using primers for murine α7nAChR (forward: 5'-CCTGGTCCTGCTGTGTTAAACTGCTTC-3'; reverse: 5'-CTGCTGGGAAATCCTAGGCACACTTGAG-3') and for the neomycin gene neocassette (forward: 5'-TGCTCCTGCCGAGAAAGTAT-3'; reverse: 5'-AATATCACGG GTAGCCAACG-3').

Plasma lipid and lipoprotein analysis

Blood was drawn after a 4 h fast at the start of WTD feeding and after 4 weeks of diet via tail-bleeding. At the end of the study, mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/mL), ketamine (40 mg/L) and atropine (0.05 mg/mL) before blood was collected by bleeding via the eye. Plasma was isolated by centrifugation and stored frozen at -80°C until further analyses. The concentrations of total cholesterol (TC) and triglycerides (TG) in plasma were determined using commercially available enzymatic colorimetric kits according to the manufacturer's protocols (236691 and 1488872; Roche Molecular Biochemicals, Indianapolis, IN, USA). The plasma concentration of phospholipids (PL) was determined using another enzymatic colorimetric kit (3009; Instruchemie, Delfzijl, The Netherlands). The distribution of lipids over the various lipoproteins in plasma was determined by fractionation of 50 µL of pooled plasma using a Superose 6 HR 10/30 column (Äkta System; Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Analysis of leukocytes

For analysis of leukocytes, at the end of the study, mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/mL), ketamine (40 mg/L) and atropine (0.05 mg/mL) before blood was collected by bleeding via the eye. The peritoneal cavities of the transplanted mice were lavaged with 10 mL ice-cold PBS to collect peritoneal leukocytes. Spleen and mesenteric lymphnodes were take out for quantification of cell composition, using an automated Sysmex XT-2000iV analyzer (Sysmex Europe GMBH, Norderstedt, Germany), and for gene expression analysis.

Gene expression analysis

Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. One microgram of total RNA was converted to cDNA with iScript cDNA Synthesis kit (Biorad) and purified with Nucleospin Extract II kit (Macherey-Nagel). RT-PCR was conducted on a IQ5 PCR

machine (Biorad) using Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of *Hprt*, *Gapdh* and *Cyclo*.

Analysis of murine platelet function

For analysis of murine platelet function, blood was collected into 0.1 volume 130 mmol/L trisodium citrate via cardiac puncture. Platelet count and mean platelet volume (MPV) were analyzed in whole blood using a Sysmex XT-2000iV Hematology Analyzer. The platelet count was corrected for the volume of anti-coagulant present.

To investigate platelet activation, we measured the ability of platelets to convert integrin $\alpha_{IIb}\beta_3$ to its active high-affinity open conformation ($\alpha_{IIb}\beta_3$*) upon stimulation by different agonists. For this purpose, 25 µL diluted whole blood (1:20 (v/v) in Hepes-Tyrode buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM Hepes, 5 mM D-glucose, pH 7.2)) was recalcified (1 mM $CaCl_2$) and stimulated by ADP (0.5 and 5 µM; Sigma, St. Louis, MO, USA), PAR-4 peptide (0.1 and 1.0 mM; Bachem, Weil am Rhein, Germany) or the Ca^{2+} ionophore A23187 (1 µM; Sigma) for 15 min at 20°C in the presence of 5 µL RPE-conjugated anti-active integrin $\alpha_{IIb}\beta_3$ (clone JON/A; Emfret Analytics, Eibelstadt, Germany). Samples were fixed in 1% formaldehyde in PBS, and integrin $\alpha_{IIb}\beta_3$ in its active high affinity open confirmation was determined by flow cytometry. The total expression level of integrin $\alpha_{IIb}\beta_3$ (both active and inactive integrin $\alpha_{IIb}\beta_3$) was also assessed by flow cytometry in whole blood. Whole blood (25 µL) was diluted (1:20 (v/v) in Hepes-Tyrode buffer (pH 7.2)), recalcified (1 mM $CaCl_2$), incubated with 5 µL RPE-conjugated anti-integrin $\alpha_{IIb}\beta_3$ (clone Leo.F2; Emfret Analytics) for 15 min at 20°C and analyzed by flow cytometry after fixation. The ratio of active integrin $\alpha_{IIb}\beta_3$* / total integrin $\alpha_{IIb}\beta_3$ protein was appointed as a marker for platelet activation.

Analysis of human platelet activation

For human platelet activation studies, platelets were isolated from freshly drawn venous blood from healthy volunteers, who claimed not to have taken any medication 10 days prior to blood collection. These studies were approved by the Medical Ethics Committee of University Medical Center Utrecht and informed consent was obtained for blood sampling from all donors. Blood was collected into 3.2% tri-sodium citrate and was centrifuged (156 x *g*, 15 min, 20°C) to prepare platelet-rich plasma. Tri-sodium citrate was used instead of EDTA to ensure presence of sufficient calcium for platelet activation. After addition of 0.1 volume ACD (2.5% (w/v) trisodium citrate, 1.5% (w/v) citric acid, 2% (w/v) D-glucose) to lower the pH of the plasma to pH 6.5, platelets were further purified by centrifugation (400 x *g*, 15 min, 20°C). After resuspension of the platelet pellet in Hepes-Tyrode buffer (pH 6.5), prostacyclin (PGI_2; Cayman Chemical, Ann Arbor, MI, USA) was added to a final concentration of 10 ng/mL and the washing procedure was repeated once. The platelet pellet was

resuspended in Hepes-Tyrode buffer (pH 7.2) and the platelet count was adjusted to $2.0x10^{11}$ platelets/L. Platelets were left for 30 min at 37°C to ensure a resting state.

For aggregation experiments, platelets were pre-incubated for 30 minutes at room temperature with the α7nAChR agonist PNU282987 (10 μM) or vehicle (DMSO). Platelet aggregation was initiated by addition of ADP (10 μM) in the presence of fibrinogen (0.5 mg/mL; Kordia Life Sciences, Leiden, The Netherlands) and was monitored in a Chrono-Log lumiaggregometer (Chrono-Log Corporation, Haverford, PA, USA) at 37°C and a stirring speed of 1000 rpm. Additionally, surface expression of P-selectin was determined as another marker of platelet reactivity and was measured by flow cytometry in citrated whole blood, treated with PNU282987 (10 μM) or vehicle prior to stimulation with ADP (serial dilutions ranging from 0-125 μM) for 20 min in the presence of RPE-conjugated anti-CD62P (BD Pharmingen, Franklin Lakes, NJ, USA).

Atherosclerosis quantification

Atherosclerotic mean lesion area (in μm^2) was quantified in cross-sections (5 μm) throughout the aortic root area starting from the appearance of open aortic valve leaflets. Per mouse, four sections with 50-μm intervals were used for atherosclerosis measurements. Sections were stained with hematoxylin-phloxine-saffron for histological analysis. Lesions were categorized for severity according to the guidelines of the American Heart Association adapted for mice(18,19). Various types of lesions were discerned: no lesions, mild lesions (types 1–3) and severe lesions (types 4–5). Immunohistochemistry for determination of lesion composition was performed as described previously(20). Rat anti-mouse antibody MAC3 (1:1000; BD Pharmingen, The Netherlands) was used to quantify macrophage area. Monoclonal mouse antibody M0851 (1:800; Dako, Heverlee, the Netherlands) against smooth muscle cell (SMC) actin was used to quantify SMC area. Sirius Red staining was used to quantify collagen area. Lesion area and composition were determined using ImageJ.

Statistical analysis

Data are presented as means ± SEM unless indicated otherwise. To compare differences among groups T-tests were performed. A P-value <0.05 was considered statistically significant.

Results

Hematopoietic α7nAChR deficiency does not affect body weight and plasma lipid levels

Ldlr$^{-/-}$ mice were transplanted with bone marrow from *α7nAchR*$^{-/-}$ mice or WT littermates. After a recovery period of 8 weeks on regular murine chow diet, the transplanted mice were challenged with an atherogenic Western-type diet (WTD) for 7 weeks. Mice transplanted with *α7nAchR*$^{-/-}$ bone marrow showed a modest temporary drop in body weight compared to mice transplanted with WT bone marrow at week 3 and 4 after BMT, but the weight curves were not different while feeding the WTD (**Figure 1A**). Reconstitution of the recipient *ldlr*$^{-/-}$ mice with bone marrow of donor mice was successful as assessed by α7nAChR transcript analysis in bone marrow (>90% chimerism; **Supplemental Figure 1**).

To evaluate whether hematopoietic α7nAChR deficiency affects lipid metabolism, plasma total cholesterol (TC), phospholipids (PL) and triglycerides (TG) were assessed at the start of WTD feeding and after 4 and 7 weeks of diet intervention. No differences in plasma lipid concentrations were found between *α7nAChR*$^{-/-}$ and WT transplanted mice at the start of the diet (not shown), after 4 weeks (not shown), nor after 7 weeks (**Figure 1B**). In addition, the distribution of cholesterol over lipoproteins of the *α7nAChR*$^{-/-}$ transplanted mice did not differ from that of the WT transplanted mice (**Figure 1C**).

Hematopoietic α7nAChR deficiency increases inflammatory status

As a measure of inflammatory status, the leukocyte count within the peritoneal cavity was quantified after lavage. Remarkably, disruption of hematopoietic α7nAChR significantly increased peritoneal leukocyte count by 2.6-fold (12.3±3.4 x10^9/L *vs.* 4.8±0.9 x10^9/L; p<0.05) (**Figure 2A**). This was due to a significant increase in multiple leukocyte cell types within the *α7nAChR*$^{-/-}$ *vs*. control transplanted mice, i.e. monocytes (6.3±1.6 x10^9/L *vs.* 2.9±0.5 x10^9/L; p<0.05) (**Figure 2B**), lymphocytes (3.9±1.3 x10^9/L *vs.* 1.7±0.4 x10^9/L; p<0.05) (**Figure 2C**), and neutrophils (0.41±0.14 x10^9/L *vs.* 0.16±0.04 x10^9/L; p<0.05) (**Figure 2D**), suggesting an increased pro-inflammatory status of the body. In line with these observations, in peritoneal leukocytes of *α7nAChR*$^{-/-}$ *vs*. control transplanted mice, mRNA expression of the pro-inflammatory cytokines TNFα (1.6-fold; p<0.01) and CRP (1.8-fold; p<0.01) was increased, while mRNA expression of IL-1β (1.6-fold; p=0.26) and IL-6 (1.2-fold; p=0.45) was not affected (**Figure 2E**). Interestingly, not only the number of leukocytes in the peritoneum was increased upon hematopoietic α7nAChR deficiency, but also in the blood (2.9-fold; p<0.01), mesenteric lymph nodes (2.0-fold; p<0.001) and spleen (2.2-fold; p<0.01) (**Figure 2F**). Analysis of subsets by flow cytometry revealed, comparable to the sysmex analysis on peritoneal leukocytes, an increase in all leukocyte subpopulations (not shown).

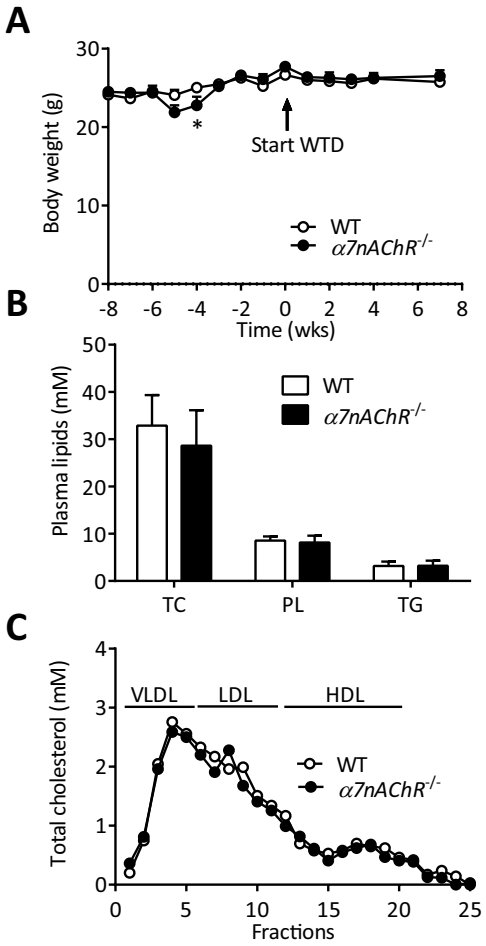

Figure 1 – Effect of hematopoietic α7nAChR deficiency on body weight, plasma lipid levels and cholesterol distribution over lipoproteins. *ldlr*$^{-/-}$ mice were transplanted with bone marrow of *α7nAChR*$^{-/-}$ (n=11) or WT mice (n=20) (t=-8 wks), allowed to recover for 8 weeks and subsequently (t=0 wks) fed a Western-type diet (WTD) during 7 weeks. Body weight was monitored during the whole experiment (**A**). After 7 wks of WTD feeding, blood samples were drawn and the concentrations of cholesterol, phospholipid and triglycerides in plasma were determined (**B**). The distribution of cholesterol over the different lipoproteins was determined by fractionation of pooled plasma by FPLC. VLDL, LDL and HDL were collected in fractions 1-6, 7-14, and 15-24, respectively (**C**). Values represent means ± SEM.

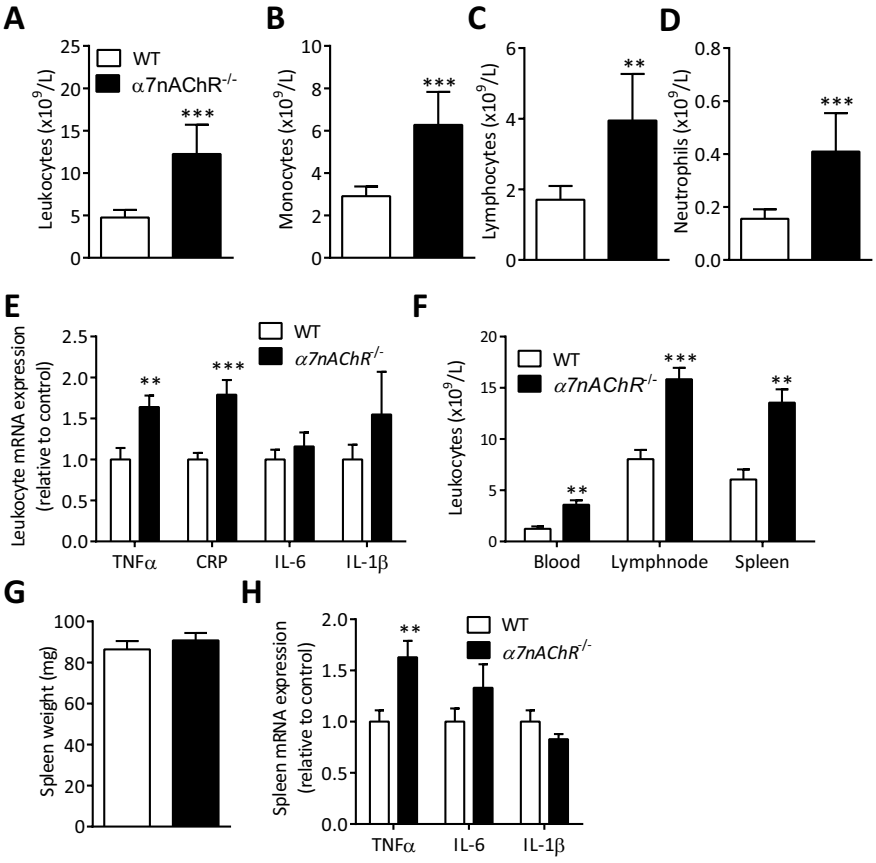

Figure 2 – Effect of hematopoietic α7nAChR deficiency on inflammation. After 7 weeks of WTD, the number of peritoneal leukocytes was determined (**A**) and their composition was analyzed, i.e. monocytes (**B**), lymphocytes (**C**) and neutrophils (**D**) in hematopoietic *α7nAChR*$^{-/-}$ (n=11) or WT mice (n=20). Total RNA was extracted from peritoneal leukocytes and mRNA expression of the pro-inflammatory cytokines TNFα, CRP, IL-6, and IL-1ß was determined (**E**). Leukocytes in blood, lymph nodes and spleen were counted (**F**). The spleen was isolated and weighed (**G**), after which mRNA expression of the pro-inflammatory cytokines TNFα, IL-6, and IL-1ß was determined (**H**) Values represent means ± SEM. **p<0.01 and ***p<0.001 denote statistical difference *vs.* controls.

As the cholinergic anti-inflammatory pathway is known to act via the spleen (13), the spleen was further analysed. The spleen of *α7nAChR*$^{-/-}$ and WT transplanted mice did not differ in weight (90±4 mg *vs.* 86±4 mg; p=0.47) (**Figure 2G**), but mRNA expression of TNFα was increased in the spleen of *α7nAChR*$^{-/-}$ transplanted mice compared to controls (1.6-fold; p<0.01) (**Figure 2H**), similar as observed for peritoneal leukocytes.

Overall, these findings show an increased inflammatory status of *α7nAChR*$^{-/-}$ transplanted mice compared to control transplanted mice.

Hematopoietic α7nAChR deficiency increases platelet reactive status

Functional α7nAChR is expressed on platelets and modulates activation of platelets (9), which may represent an important linkage between inflammation, thrombosis and atherosclerosis (17). Integrin $\alpha_{IIb}\beta_3$ is an integrin complex found on the surface of platelets, and is essential for platelet aggregation upon binding of fibrinogen. Upon platelet activation, integrin $\alpha_{IIb}\beta_3$ changes its conformation to an active high affinity state in which it is able to bind its ligands, including fibrinogen. Using flow cytometry, the expression of active integrin $\alpha_{IIb}\beta_3$ was measured after platelet activation by the agonists ADP and PAR-4 peptide. The lack of α7nAChR on platelets did not affect baseline expression of active integrin $\alpha_{IIb}\beta_3$ (not shown), but increased its expression upon stimulation by ADP compared to control platelets (2.0-fold for 0.5 μM, p<0.05 and 1.9-fold for 5 μM, p<0.01), and induced a non-significant increase of integrin $\alpha_{IIb}\beta_3$ after stimulation by PAR-4 peptide (1.5-fold for 0.1 mM, p=0.12 and 1.3-fold for 1 mM, p=0.19) (**Figure 3A**). The expression of total integrin $\alpha_{IIb}\beta_3$ (both active and inactive integrin $\alpha_{IIb}\beta_3$) on platelets did not differ between both groups (**Figure 3B,C**). These data indicate that α7nAChR signaling is not only involved in diminishing pro-inflammatory cytokine production by immune cells, but also in reducing the reactive state of circulating platelets.

For platelet activation, an increased intracellular Ca^{2+} concentration is essential. To study the contribution of platelet receptor signaling in activation of integrin $\alpha_{IIb}\beta_3$ in the absence of functional α7nAChR, the Ca^{2+} ionophore A23187 was used to directly induce Ca^{2+} entry without the activation of platelet receptors. After stimulation with A23187, no differences were observed in the expression of active integrin $\alpha_{IIb}\beta_3$ between both groups (**Figure 3D**). These results indicate that the differences in reactive state of the platelets are likely due to interactions between α7nAChR signaling and signaling upstream of Ca^{2+} mobilization. However, it should be noted that A23187 is a potent activator of platelets, which may have blunted the effects of α7nAChR deficiency.

As aggregation experiments were unfeasible to perform on platelets of these mice, human platelets from healthy individuals were incubated with the α7nAChR-selective agonist PNU-282987 to investigate the effects of α7nAChR signaling on platelet aggregation. Incubation with 10 μM PNU-282987, prior to stimulation with

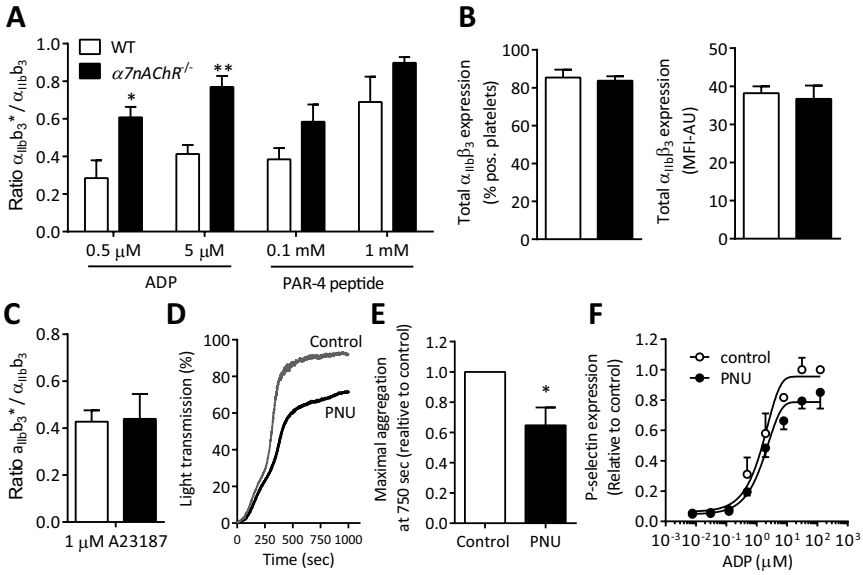

Figure 3 – Effect of hematopoietic α7nAChR deficiency on platelet reactivity. After 7 weeks of WTD, platelets were isolated from whole blood from hematopoietic *α7nAChR*$^{-/-}$ (n=4) or WT mice (n=4), stimulated with the platelet agonists ADP and PAR-4 peptide, and the ratio of the active form of integrin $\alpha_{IIb}\beta_3$* relative to total integrin $\alpha_{IIb}\beta_3$ protein was analyzed by flow cytometry (**A**). The expression of total integrin $\alpha_{IIb}\beta_3$ protein (both active and inactive form) was determined by flow cytometry (**B**). Integrin $\alpha_{IIb}\beta_3$ activation (*$\alpha_{IIb}\beta_3$ = active form of integrin) without activation of platelet receptors was determined by using the Ca^{2+} ionophore A23187 (**C**). In human platelets (n=3), aggregation was assessed upon ADP activation after pre-incubation with the α7nAChR agonist PNU-282987 (**D**) and maximal aggregation after 750 sec was determined (**E**). P-selectin expression, a marker for human platelet activation, was measured after activation by ADP (**F**). Values represent means ± SEM. *p<0.05 denotes statistical difference *vs*. controls.

ADP, inhibited platelet aggregation (**Figure 3E**), and reduced the maximal aggregation response by 35% (p<0.05) (**Figure 3F**). Additionally, the expression of P-selectin, another marker for platelet reactivity, tended to decrease after treatment with PNU-282987 prior to stimulation with increasing concentrations of ADP (**Figure 3G**). Pre-incubation with a physiological concentration of PNU-282987 (100 nM; **Supplemental Figure 2**) or shorter pre-incubation of 5 min instead of 30 min (**Supplemental Figure 3**) did not reduce P-selectin expression nor decrease platelet-bound fibrinogen, which is another marker for platelet aggregation. Together, these data indicate that stimulation of the α7nAChR reduces platelet activation and aggregation, while α7nAChR deficiency results in an increased platelet reactive state.

Hematopoietic α7nAChR deficiency does not affect atherosclerotic lesion development

An increased inflammatory status and increased platelet activation status, observed in hematopoietic α7nAChR deficient mice, may be expected to accelerate the development of atherosclerosis. To study the role of hematopoietic α7nAChR in atherosclerosis development, *α7nAChR*$^{-/-}$ and WT transplanted mice were sacrificed after 7 weeks of WTD feeding, and atherosclerotic lesion size and lesion severity were

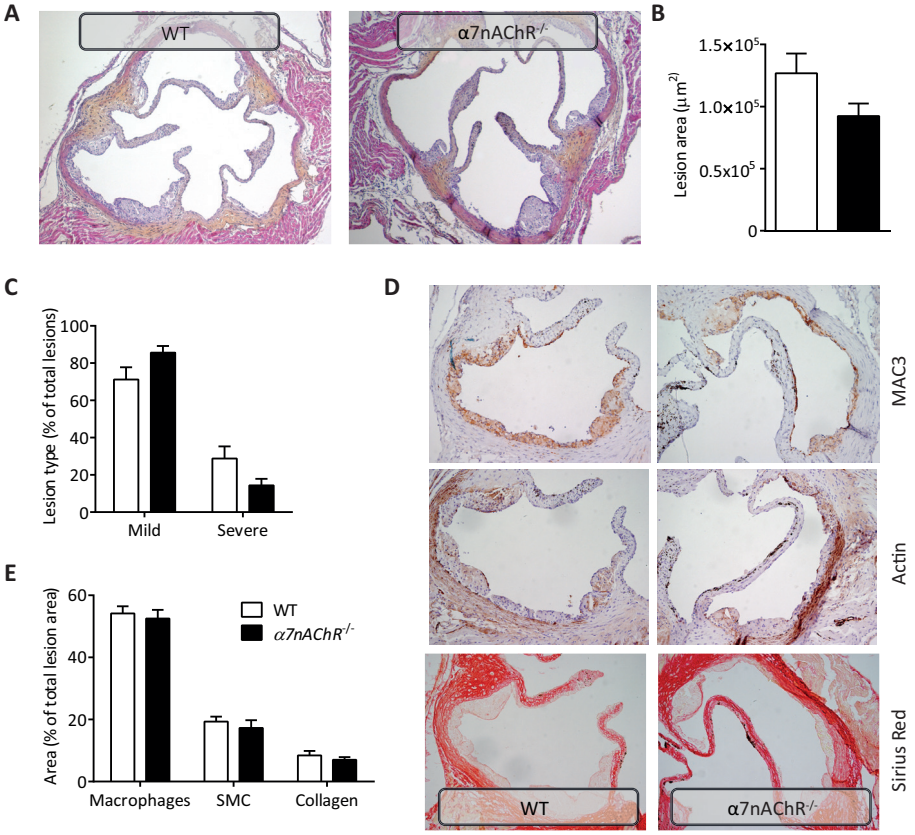

Figure 4 – Effect of hematopoietic α7nAChR deficiency on atherosclerotic lesion development. After 7 weeks of WTD, hearts were isolated of hematopoietic *α7nAChR*$^{-/-}$ (n=11) or WT mice (n=20), and cross-sections (5 µm) with 50-µm intervals throughout the aortic root area starting from the appearance of open aortic valve leaflets were used for atherosclerosis measurements. Sections were stained with hematoxylin-phloxine-saffron (HPS) for histological analysis. Representative images of HPS staining (**A**). Atherosclerotic mean lesion area (in µm^2) was quantified in four subsequent cross-sections (**B**). The same four sections per mouse were categorized according to lesion severity (**C**). Immunohistochemistry for determination of lesion composition; MAC3 for macrophages and actin for smooth muscle cells (SMC) or Sirius Red staining for collagen content (**D**). Comparable to lesion size, the composition of the lesions was determined in four subsequent cross-sections (**E**). Values represent means ± SEM per group.

determined in the valve area of the aortic root. However, α7nAChR deficiency in mice did not affect atherosclerotic lesion size (**Figure 4A,B**) and lesion severity, classified as mild (type 1-3) and severe (type 4-5) lesions (**Figure 4C**). In addition, no significant differences could be observed between the lesion composition of *α7nAChR*$^{-/-}$ and WT transplanted mice, with respect to the relative area of macrophages (MAC3 staining), smooth muscle cells (SMC; actin staining) and collagen (Sirius Red staining) (**Figure 4D,E**).

Discussion

In the present study, we determined the effect of the absence of hematopoietic α7nAChR on the inflammatory status and platelet function, and the consequences for atherosclerotic lesion development in hyperlipidemic *ldlr*$^{-/-}$ mice. We showed that hematopoietic disruption of α7nAChR clearly increased inflammatory status as indicated by an increased number of peritoneal leukocytes and an increased expression of inflammatory mediators by both peritoneal leukocytes (TNFα, CRP) and the spleen (TNFα). Apparently, hematopoietic α7nAChR deficiency recapitulates the pro-inflammatory phenotype of total body α7nAChR-deficient mice (4). Of note, in our study hematopoietic *α7nAChR*$^{-/-}$ mice were subjected to a WTD containing 16% fat and 0.25% cholesterol, a diet known to evoke only mild inflammation (21). Still the number of leukocytes in the blood, lymph nodes, spleen and peritoneum all increased by at least 2-fold, consistent with a role for the cholinergic anti-inflammatory pathway in prevention of inflammation during diet-induced hyperlipidemia. The increased number of leukocytes was explained by an increase in various leukocyte cell types (i.e., monocytes, lymphocytes and neutrophils).

In addition to leukocytes, the α7nAChR is also expressed on platelets (9). Therefore, we evaluated the effect of α7nAChR deficiency as well as specific α7nAChR activation on platelet reactivity by determining active integrin $\alpha_{IIb}\beta_3$ expression and platelet aggregation, respectively. Murine α7nAChR deficiency in platelets increased active integrin $\alpha_{IIb}\beta_3$ expression induced by classical platelet agonists. Accordingly, in human platelets, ADP-induced platelet aggregation was inhibited by the α7nAChR-selective agonist PNU-282987. As activation of platelets induces inflammation (17), these data are in line with the immunosuppressive function of the cholinergic anti-inflammatory pathway. Furthermore, these results indicate that activation of α7nAChR signaling is involved in normal platelet function as α7nAChR deficiency resulted in increased platelet reactivity in response to agonists. In seeming contrast to our findings, Schedel *et al.* (9) reported that stimulation of the α7nAChR with increasing concentrations of PNU-282987 (100 nM up to 10 μM) results in increased ADP-induced Ca^{2+} entry into human platelets, suggesting increased platelet activation. However, as platelet aggregation was not affected in these studies, the functional

relevance of those observations is unclear. Of note, PNU-282987 at a concentration of 1 μM also acts as an antagonist for 5-HT(3) receptors (22) that are expressed on platelets (23), which may thus have contributed to the observed effects in human platelets. Evaluation of the amount of platelet-leukocyte complexes *in vivo*, as well as direct platelet aggregation measurements, would have given valuable information on the enhanced platelet activation in response to ADP in the α7nAChR deficient mice and the diminished platelet response after α7nAChR activation on human platelets. Unfortunately, aggregation experiments were unfeasible to perform on platelets of these mice and therefore remain to be addressed.

In the present study the enhanced inflammation in hematopoietic *α7nAChR*$^{-/-}$ mice did not increase plasma lipids, although previous studies have shown that pro-inflammatory signaling in pre-clinical models does affect lipid metabolism (24). Furthermore, it has been shown that serum CRP and IL-6, which are markers in the pathogenesis of atherosclerosis (15,16), were increased in α7nAChR deficient mice on an apoE-deficient background, a highly inflammatory and oxidative stress mouse model (25), but not on a WT background (11). Together, these data indicate that the contribution of α7nAChR signaling to lipid metabolism may become apparent only under higher inflammatory conditions.

Hematopoietic α7nAChR deficiency increased the inflammatory status and caused increased platelet reactivity, both considered as important risk factors in atherosclerotic lesion development (16,17,26). Nonetheless, hematopoietic α7nAChR deficiency did not aggravate WTD-induced atherosclerotic lesion development in *ldlr*$^{-/-}$ mice. As the impact of the of circulating pro-inflammatory cells on plaque infiltration is best observed during early lesion development, we assessed atherosclerotic lesion development already at 7 weeks of Western-type diet feeding, which resulted in early lesions as reflected by the relatively small lesion area (~1x10^5 μm^2) and low lesion severity (~80% mild lesions), comparable to previous studies (27). The macrophage content of the plaque was not affected by hematopoietic α7nAChR deficiency, even in these early lesions, suggesting comparable leukocyte infiltration. It should be noted that the α7nAChR is also present on endothelial cells and that stimulation of the endothelial α7nAChR inhibits leukocyte recruitment during inflammation (8), which is a crucial step in atherosclerotic lesion development (12). Possibly, a potential aggravating effect of hematopoietic α7nAChR deficiency on inflammation and platelet activation may thus become apparent only when α7nAchR is also absent in endothelial cells. However, the contribution of endothelial cells to the cholinergic anti-inflammatory pathway is unknown, as well as the effects of whole-body α7nAchR deficiency on the development of atherosclerosis. Alternatively, it is possible that the diet used was too mild to evoke sufficient inflammation as addition of 0.25% cholesterol to the WTD only slightly increases liver inflammation in APOE*3-Leiden. CETP mice (21).

In conclusion, hematopoietic α7nAChR deficiency increases inflammatory status and platelet activation status, but does not affect atherosclerotic lesion development in *ldlr*$^{-/-}$ mice.

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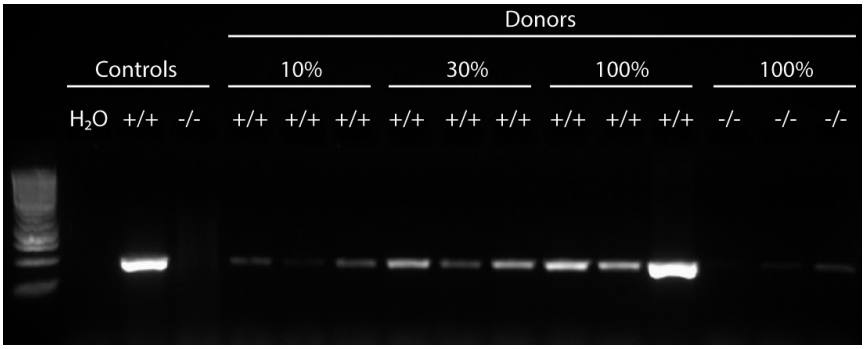
A



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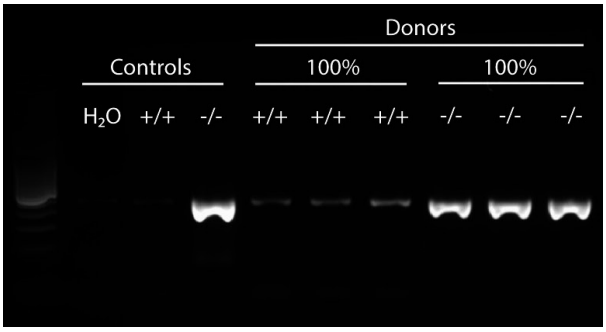
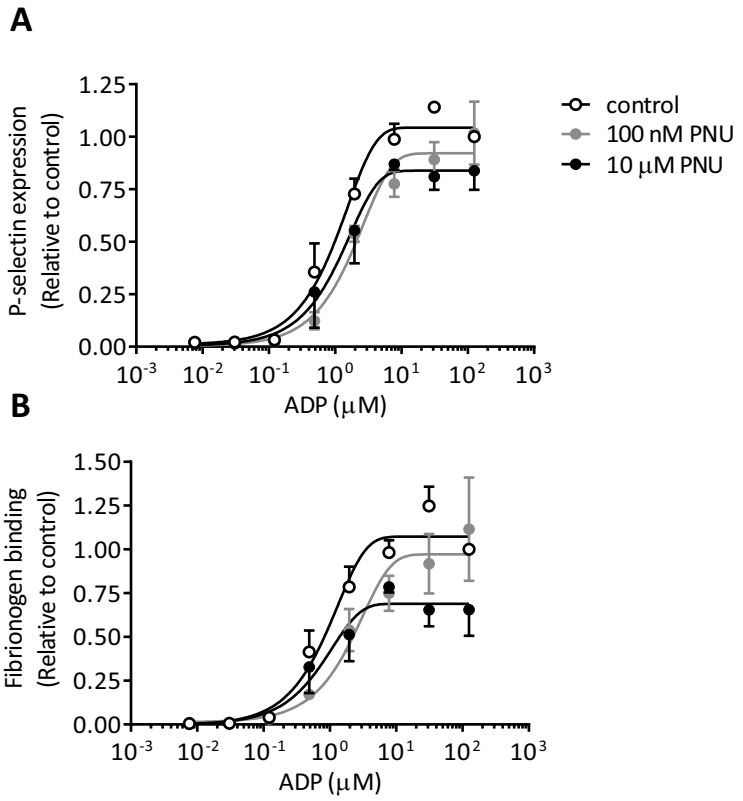
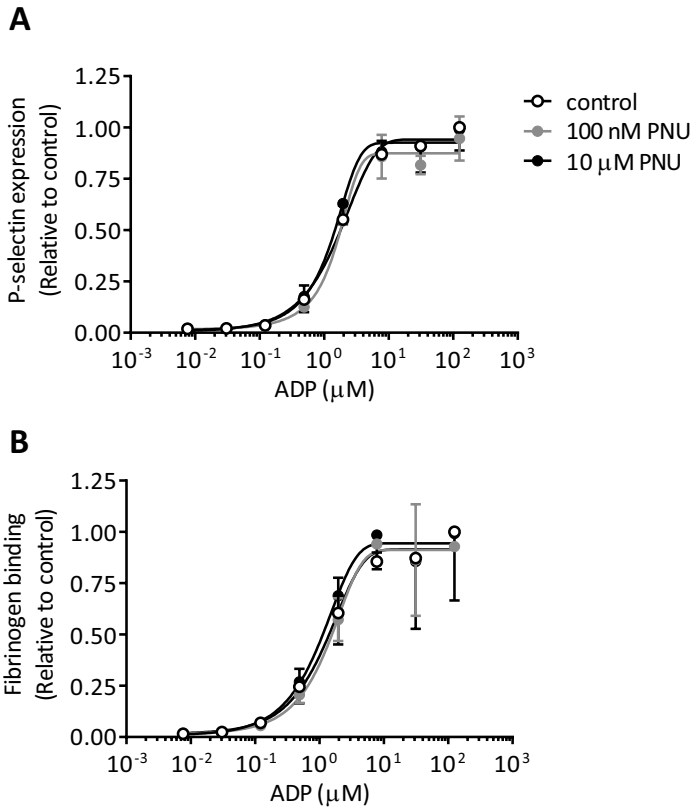


Supplemental Figure 1 – Hematologic chimerism of α7nAChR$^{-/-}$ mice. At the end of the study, bone marrow was isolated from the transplanted *ldlr*$^{-/-}$ mice, and hematologic chimerism was determined using genomic DNA by PCR at 15 weeks after BMT. The relative presence of the α7nAChR was assessed for murine α7nAChR, diluted up to 10% for hematopoietic α7nAChR$^{+/+}$ to determine chimerism (**A**), and for the neomycin gene neocassette (**B**). Figure include three representative hematopoietic α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice. Controls include DNA from α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice.



Supplemental Figure 2 – Effects of low dose α7nAChR agonist PNU282987 on human platelets. Human platelets were isolated from freshly drawn venous blood from healthy volunteers. Surface expression of P-selectin (**A**) and fibrinogen-binding (**B**) were determined by flow cytometry in citrated whole blood, treated with α7nAChR agonist PNU282987 (100 nM or 10 μM) or vehicle for 30 min prior to stimulation with ADP (serial dilutions ranging from 0-125 μM) for 20 min. Values represent means ± SEM.



Supplemental Figure 3 – Effects of short incubation with α7nAChR agonist PNU282987 on human platelets. Human platelets were isolated from freshly drawn venous blood from healthy volunteers. Surface expression of P-selectin (**A**) and fibrinogen-binding (**B**) were determined by flow cytometry in citrated whole blood, treated with α7nAChR agonist PNU282987 (100 nM or 10 μM) or vehicle for 5 min prior to stimulation with ADP (serial dilutions ranging from 0-125 μM) for 20 min. Values represent means ± SEM.