Increased Carotid Artery Lesion Inflammation Upon Treatment With the CD137 Agonistic Antibody 2A

Leif Å. Söderström, MD, PhD; Hong Jin, MD, PhD; April S. Caravaca, MS; Maria L. Klement, PhD; Yuhuang Li, MD, PhD; Anton Gisterå, MD, PhD; Ulf’ Hedin, MD, PhD; Lars Maegdefessel, MD, PhD; Göran K. Hansson, MD, PhD; Peder S. Olofsson, MD, PhD

Background: Increased inflammatory activity destabilizes the atherosclerotic lesion and may lead to atherothrombosis and symptomatic cardiovascular disease. Co-stimulatory molecules, such as CD137, are key regulators of inflammation, and CD137 activity regulates inflammation in experimental atherosclerosis. Here, we hypothesized that CD137 activation promotes carotid artery inflammation and atherothrombosis.

Methods and Results: In a model of inducible atherothrombosis with surgical ligation of the right carotid artery and a subsequent placement of a polyethylene cuff, elevated levels of CD137 and CD137 ligand mRNA in atherothrombotic vs. non-atherothrombotic murine carotid lesions was observed. Mice treated with the CD137 agonistic antibody 2A showed signs of increased inflammation in the aorta and a higher proportion of CD8+ T cells in spleen and blood. In carotid lesions of 2A-treated mice, significantly higher counts of CD8+ and major histocompatibility (MHC)-class II molecule I-A
d cells were observed. Treatment with the CD137 agonistic antibody 2A did not significantly affect the atherothrombosis frequency in 16-week-old mice in this model.

Conclusions: Levels of CD137 and CD137 ligand mRNA were higher in advanced atherosclerotic disease compared to control vessels, and treatment with the CD137 agonistic antibody 2A, in a murine model for inducible atherothrombosis promoted vascular inflammation, but had no significant effect on atherothrombosis frequency at this early disease stage.

Key Words: 4-1BB; Atherosclerosis; Co-stimulation; Cytokines; Leukocytes

Increased inflammatory activity within the atherosclerotic plaque increases the risk for precipitation of cardiovascular disease.\(^1\)\(^4\) Inflammation is now considered a potential treatment target in atherothrombosis, the event responsible for most clinical manifestations of atherosclerosis, and anti-cytokine therapy is in clinical trials for prevention of cardiovascular disease.\(^5\)\(^6\)

The inflammatory process in the atherosclerotic lesion is orchestrated by T cells that require signaling from the T-cell receptor as well as co-stimulatory molecules for activation.\(^2\)\(^7\) The stability of the atherosclerotic lesion depends on the interaction between many different cell types in the lesion,\(^8\) of which several express CD137 (alternative names are TNFRSF\(^9\) or 4-1BB), a potent co-stimulatory receptor-molecule in the tumor necrosis factor superfamily (TNFSF).\(^9\)

We have previously shown that CD137 is expressed on T cells and endothelial cells in human atherosclerotic lesions, and that CD137 mRNA levels are increased in atherosclerotic lesions compared to control arteries free of atherosclerosis.\(^9\) Stimulation of CD137 in experimental atherosclerosis exacerbates inflammation in the vessel wall,\(^9\) and atherosclerosis-prone mice deficient in CD137 show a more stable atherosclerosis lesion phenotype.\(^9\) In addition, activation of the CD137 associated signaling has been reported to promote lesion calcification and angiogenesis, two features of advanced atherosclerotic lesions.\(^11\)\(^12\)

In humans, a genotype linked with CD137 mRNA levels in leukocytes, is associated with increased intima media thickness in carotid arteries and with increased non-cardiac
vascular clinical events in individuals with risk factors of cardiovascular disease. It is important to understand the implications of CD137/CD137L activation in the pathophysiology of atherothrombosis, particularly as CD137 agonists are currently in clinical trials for treatment of cancer (https://clinicaltrials.gov).

In light of the available evidence on CD137 activity in atherosclerotic lesions, we hypothesized that activation of CD137 promotes atherothrombosis, and investigated the effects of the CD137 agonistic antibody, designated 2A, in a murine model.

Methods

Studies were approved by the regional ethics committee for animal studies.

Mouse Transcriptome Analysis

For the mouse transcriptome analysis, the right ligated carotid arteries of a total of 18 Apolipoprotein E deficient (ApoE−/−) mice were harvested and analyzed accordingly. Samples were divided into atherothrombotic or non-atherothrombotic based on macroscopic features and histomorphology (hematoxylin and eosin staining, immunohistochemistry (IHC) for α-smooth muscle actin (αSMA) and cross-linked fibrin, as described below). Eleven non-ligated left arteries were used as controls. Tissue was prepared and analysis was performed, as previously described. In brief, plaques and corresponding left carotid artery (as controls) were harvested for RNA extraction using RNeasy Micro Kit (QIAGEN, Düsseldorf, Germany). A RNA Mouse Transcriptome Assay (MTA, Affymetrix 902514) and differential expression analysis (Affymetrix transcriptome analysis console v3.0) was performed according to the manufacturer’s instructions. Nine atherothrombotic, 9 non-atherothrombotic and 11 non-ligated controls were analyzed using Affymetrix GeneChip Mouse Transcriptome Array 1.0. In this study, the dataset was queried for values of a select number of transcripts. Results are given as relative mRNA expression in arbitrary units (au) log₂ transformed fold changes, compared with background intensity.

Real Time-Polymerase Chain Reaction

Murine mRNA from spleen and abdominal aortic tissue was prepared using Qiagen RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Subsequently, cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368814) according to the manufacturer’s instructions. Results from murine tissue were normalized to values of hypoxantine-guanine phosphoribosyl transferase (Hprt). Analysis was performed on a 7900HT FAST Real-Time PCR (Perkin-Elmer, Applied Biosystems) using Taqman Assay-on-demand (Applied Biosystems). For real-time PCR, the 2^−∆∆CT method was used and results are expressed as arbitrary units (au) unless otherwise specified.

Antibody Production and Injections

CD137 agonistic antibody, 2A, and IgG2a control antibodies of the same subclass were produced using hybridomas grown in 2-compartment bioreactors (CELLLine CL1000; Integra Biosciences AG, Switzerland). The 2A hybridoma was a gift from Professor Lieping Chen (Yale Cancer Center, Yale University, CT, USA). Antibodies were purified using a HiTrap Protein G column (GE #17-0405-01) according to the manufacturer’s instructions. Antibodies were dialyzed with phosphate buffered saline (PBS) and diluted to a final concentration of 1 mg/mL before injection. In total, 100 μL (equal to 100 μg) of either 2A or IgG2a was injected intra-peritoneally on a weekly basis for 4 weeks; a total of 5 injections before harvest. Mice were weighed at the time of injection, starting from the second injection.

Cell Proliferation Experiments

To assess the efficacy of the present batch of the 2A antibody, we exposed murine splenocytes from C57BL/6 mice in culture to 2A (200 ng/μL), or an equal amount of phosphate buffered saline (PBS) in 96-well plates pre-coated with anti-CD3 antibody (clone C363.29B; Southern Biotech, AL, USA) at a dilution of 1:1,000. Proliferation was measured as 3H-thymidine incorporation using a scintillation counter (Wallac, Finland). Results are expressed as counts per minute (CPM).

Mice and Animal Surgery

The mouse model of atherothrombosis used in this study has been previously described and basically characterized. Forty-three male ApoE−/− mice were bought from Taconic (Taconic Europe A/S, Denmark). Four of the 43 mice died during the experimental atherothrombosis induction. Deaths were not specific to any group, and two of the mice were euthanized due to fighting injuries according to the regulations specified in the ethical permit. After adaptation, surgery was started at 12 weeks of age (Figure 1). Surgery was performed as previously described. In brief, the right carotid artery was partially ligated during general isoflurane anesthesia. Mice received 0.1 mg/kg buprenorfin subcutaneously before wakening and recovery. After 4 weeks, a conical polyethylene cuff (Promolding B.V., The Netherlands) was surgically placed around the ligated vessel proximal to the ligation to provoke atherothrombosis. Four days after cuff placement mice were euthanized and plaques were assessed (see below). One person performed...
all surgical procedures.

**Mouse Tissue and Blood Collection**

Mouse tissue was harvested according to standard procedures. Mice were euthanized using CO₂. After blood was drawn, mice were perfused with normal saline at a 100-cm H₂O pressure. Biopsies from carotid arteries on both the ligated right side and the untreated left side were collected, and the right side was characterized for the presence of atherothrombosis. After visual assessment of atherothrombosis (see below), the carotid arteries from both left and right sides were collected and mounted in Tissue-tek OCT (Sakura, Netherlands) for IHC. The ascending aorta, aortic arch and first part of the descending aorta was dissected and placed in 4% formaldehyde for en face staining. The rest of the aorta, including the abdominal aorta, was placed in RNAlater (Qiagen, Hilden, Germany). Blood was put in EDTA tubes for blood count analysis using a scil Vet abc Animal Blood Counter (scil animal care company, IL, USA) according to the manufacturer’s instructions, or used for fluorescence activated cell sorting (FACS) analysis (described below).

**Measurement of Atherothrombosis Frequency**

A blinded investigator performed an assessment of carotid atherothrombosis by visual inspection at the time of tissue collection. Plaques were considered positive for atherothrombosis if an obvious thrombus in the plaque or in the vessel was visualized after perfusion of cold normal saline (0.9% NaCl). Perfusion through the left ventricle was performed under pressure infusion of 100 cm H₂O to reach equal perfusion pressures between all mice. In cases of uncertainty of atherothrombosis, plaques were sectioned and stained with hematoxylin and eosin, or in selected cases, a fluorescent staining of cross-linked fibrinogen was performed to determine if atherothrombosis had occurred. One mouse was excluded because the presence of atherothrombosis could not be determined clearly.

**IHC**

Histological staining after harvesting was performed in the vulnerable lesion region of the carotid artery, which was assessed for gene expression analysis using a standardized protocol. In brief, arteries were sectioned into 7-μm-thick serial sections. A biotin-avidin-immunoperoxidase reaction, in combination with the Vectastain ABC kit (Vector Laboratories, USA), using primary antibodies against αSMA (Abcam ab5694, dilution 1:100) and cross-linked fibrin (internal, dilution 1:100), was performed. For negative control, mouse IgG (Abcam, Cambridge, UK) was used. For hematoxylin and eosin stainings, frozen sections were thawed before incubation with eosin. After washing with water, sections were incubated with Mayer’s hematoxylin. Sections were then dehydrated in ethanol and subsequently in xylene before mounting with Pertex mounting media. Analyses were obtained at room temperature using a Zeiss Axioplan 2 (Carl Zeiss MicroImaging, Germany) with Zeiss Achromplan and Zeiss Plan-Neofluar lenses, a Nikon Digital Sight (DS) R1 camera, and the NIS-Elements F 3.00 software (Nikon, Japan). Acetone-fixed cryosections of carotid arteries embedded in Tissue-tek OCT (Sakura, Netherlands) were incubated with 5% normal horse serum, Tris-buffered saline, 0.1% Tween 20, and stained after quenching in a 3% hydrogen peroxide solution. Samples were stained with CD3 (500 A2, eBioscience, 1:75), CD4 (H129.19, BD Pharmingen, 1:100), CD8 (53-6.7, BD Pharmingen, 1:100), CD25 (KH74, BD Pharmingen, 1:300) primary antibodies overnight at 4°C. Binding was detected using a biotinylated secondary antibody (1:200) and stained using 3,3’-diaminobenzidine (DAB) (Vector Laboratories) followed by hematoxylin staining. Images were captured with a Leica Microsystems DMRB microscope. Positively stained cells were counted manually by a blinded investigator in an area restricted by the internal elastic lamina. Immunofluorescent stainings were performed to confirm atherothrombosis. Briefly, 5-μm-thick frozen carotid in-cuff sections were blocked for 1 h with Mouse IgG Blocking Reagent (Mouse on Mouse Basic Kit, Vector Laboratories, Petersborough, UK), and subsequently with 2.5% normal horse serum after washing. Sections were then incubated for 30 min with an in-house manufactured monoclonal antibody against cross-linked fibrin. Binding was detected using a goat anti-mouse secondary antibody (Alexa Fluor 488, Thermo Fisher Scientific, Waltham, USA). After washing, sections were counterstained with Hoechst (bisbenzimide H 33258, Sigma-Aldrich, St. Louis, USA). Fluorescent staining was detected by using a Leica TCS SP5 confocal microscope, with accompanying (LAS AF) software.

**En Face Staining**

The aortic arches from the root to approximately mid heart level were preserved in 4% formaldehyde at harvest. Aortas were subsequently pinned with Austerlitz insect pins and stained with Sudan IV (Sigma). Micrographs were acquired using a Leica MZ6 microscope with a Leica DC480 camera, and a Leica DC Camera Twain V5.1.1 was used to import images into Photoshop version 5 (Adobe, CA, USA). En face lipid accumulation was calculated as the percentage plaque area of the total surface area of the aortic arch, using ImageJ software (https://imagej.nih.gov).

**Flow Cytometry**

Single cell suspensions of spleen and blood cells were prepared and stained with primary labeled antibodies, according to the manufacturer’s instructions. Surface markers used were: live dead (Invitrogen, CA, USA), anti-CD3-Brilliant violet 421 (Biolegend, CA, USA), CD4 APC-H7 (Becton Dickinson), CD8 PerCP (PharMingen), and CD19 PE-Cy7 (Biolegend, CA, USA). Samples were analysed using a CyAn ADP flow cytometer (Dako) and data was subsequently analyzed using FlowJo software, version 10 (FlowJo, OR, USA).

**Isolation and Culture of Primary Mouse Aortic Smooth Muscle Cells (mAoSMCs)**

Aortas from 6- to 8-week-old mice were isolated, as previously described. Briefly, 3 aortas from Apoe−/− mice were pooled. Cells isolated from smooth muscle cells (SMC) were passaged 3-fold before undergoing further studies. Cells were allowed to grow to ~70% confluence in DF10 (DMEM-F12 medium; Gibco, NY, USA) containing 10% FBS (Gibco) and 100 U/mL penicillin/streptomycin (Gibco). Cells were plated at 40–50% confluence in 24-well plates. Cells were grown in DMEM-F12 (Thermo Fisher, CA, USA) supplemented with 10% FBS.
with 2% FBS and further supplemented with 5% FBS. Plasma from either 2A- or IgG2a-treated mice were added to the culture media to a concentration of 10%, and proliferation was then analyzed using the IncuCyte™ Live Cell ZOOM Imaging System for 72 h. Proliferation was measured as a percentage of confluence.

Statistical Analysis
Data from experiments are reported as mean ± standard error of mean (SEM) unless otherwise specified. Differences between groups were analyzed using Chi-square test for atherothrombosis. For analysis of IHC and for analysis of aortic cytokine mRNA levels, the Mann-Whitney U-test was used. Student’s t-test was used for splenocyte proliferation, blood and spleen analysis, except for analysis of eosinophils where Fisher’s exact test was used. One-way ANOVA followed by Dunnet’s post-hoc test and Tukey’s HSD (honest significant difference) multiple comparisons test was used for mRNA analyses and mAoSMC proliferation analysis. The Tukey’s HSD test was used for multiple comparisons within groups; that is, for adjustment for the 3 different groups within CD137 and CD137L mRNA levels. Bonferroni adjustment for the number of cytokines tested was deemed overly conservative and, therefore, inappropriate in this setting. P < 0.05 was considered significant. Outliers were identified using the robust regression followed by outlier identification (ROUT) method with the Q-value set to 0.1%. All calculations were performed using Prism 6 (GraphPad software, CA, USA).

Results
CD137 and CD137 Ligand mRNA Levels Are Elevated in Murine Thrombogenic Carotid Atherosclerotic Lesions
CD137 and CD137 ligand (CD137L) are expressed in human carotid atherosclerosis. To investigate mRNA expression of CD137 and CD137L in carotid atherosclerotic lesions in mouse carotid atherothrombosis, Apoe^{-/-} mice were subjected to carotid artery ligation, as previously described.17,19 and carotid lesions from experimental mice were analyzed using Affymetrix GeneChip arrays. CD137 and CD137L mRNA levels were significantly higher in biopsies from ligated arteries (“atherothrombotic”) than in non-ligated carotid arteries from the contralateral side (“control”) (Figure 2A, B). CD137 and CD137L mRNA levels were also significantly elevated in the adenoid area of murine carotid atherothrombosis. In the adenoid area, CD137 and CD137L mRNA levels were significantly higher than in the non-ligated carotid arteries. (Figure 2A, B). CD137 and CD137L mRNA levels were also significantly elevated in the adenoid area of murine carotid atherothrombosis. In the adenoid area, CD137 and CD137L mRNA levels were significantly higher than in the non-ligated carotid arteries.
higher in atherothrombotic compared to non-atherothrombotic lesions (Figure 2A, B).

Next, we studied cytokine mRNA levels in this model of atherothrombosis using the gene array data. mRNA levels of tumor necrosis factor (TnF), interleukin (Il)-1β and Il-6 were significantly higher in atherothrombotic and non-atherothrombotic lesions compared to arteries from the non-ligated contralateral side used as controls, and levels of TnF and Il-1β, but not Il-6, were further significantly increased in thrombotic compared to non-atherothrombotic lesions (Figure 2C, D).

### Table. Leukocytes in Blood and Spleen From 2A- and IgG2a-Treated Mice

| Analysis                        | n   | Treatment   | P-value |
|---------------------------------|-----|-------------|---------|
|                                 |     | IgG2a       | 2A      |
| Blood count                     |     |             |         |
| Red blood cell count (x10⁹/L)   | 13+15| 9.53±0.22   | 9.25±0.24 | 0.4132 |
| White blood cell count (x10⁹/L)| 13+15| 9.53±0.52   | 11.91±1.00 | 0.0537 |
| Platelet count (x10⁹/L)         | 13+15| 1,322±80.54 | 1,019±91.41 | 0.0179 |
| Proportion granulocytes (%)     | 13+15| 36.37±3.51  | 46.00±2.39 | 0.0285 |
| Granulocyte count (x10⁹/L)      | 13+15| 3.55±0.40   | 5.71±0.64 | 0.0108 |
| Proportion monocytes (%)        | 13+15| 4.27±0.17   | 5.33±0.14 | <0.0001 |
| Monocyte count (x10⁹/L)         | 13+15| 0.38±0.02   | 0.58±0.05 | 0.0026 |
| Proportion lymphocytes (%)      | 13+15| 53.90±5.04  | 48.67±2.37 | 0.3358 |
| Lymphocyte count (x10⁹/L)       | 13+15| 5.62±0.50   | 5.63±0.46 | 0.9869 |
| # Mice with eosinophil count >5%| 2+12| 2           | 12       | 0.0018 |
| Blood (FCM)                     |     |             |         |
| CD3 (% of gated cells)          | 13+15| 0.34±0.10   | 0.58±0.18 | 0.2689 |
| CD4 (% of lymphocyte gate)      | 14+16| 46.42±1.72  | 42.29±1.93 | 0.1249 |
| CD6 (% of lymphocyte gate)      | 14+16| 33.01±1.41  | 38.26±1.55 | 0.0196 |
| Spleen (FCM)                    |     |             |         |
| CD4 (%) of splenocyte gate      | 18+19| 46.63±1.93  | 40.58±2.01 | 0.0368 |
| CD8 (%) of splenocyte gate      | 18+19| 44.14±1.85  | 51.17±1.90 | 0.0121 |
| CD8/CD4 (ratio)                 | 18+19| 1.01±0.09   | 1.35±0.10 | 0.0159 |

Student’s t-test was used for analysis, except for eosinophils where Fisher’s exact test was used. FCM, flow cytometry.

2A Treatment Increases Plaque Infiltration of CD8+ Cells

In light of the increase in CD137, CD137L and pro-inflammatory cytokine levels in murine thrombotic carotid lesions observed here, we proceeded to investigate the effects of CD137 activation on carotid atherothrombosis. First, we tested the biological effect of the CD137 agonistic antibody, 2A, in cultures of murine spleenocytes. The addition of 2A significantly increased splenocyte proliferation in vitro compared to vehicle controls, as measured by ³H-Thymidine incorporation (CPM, 3,238±829 vs. 2,012±580, P=0.03, n=5).

Next, 12-week-old Apoe−/− mice were subjected to carotid artery ligation and treated with weekly injections of 2A or control IgG2a until euthanasia at 16 weeks of age (Figure 1). There was no significant difference in weight development between 2A- and IgG2a-treated mice over the experimental period (data not shown). Blood from 2A-treated mice showed significantly increased counts and proportions of granulocytes and monocytes and an increased proportion of CD8+ cells compared to blood from IgG2a-treated mice (Table). No other significant differences in investigated red or white blood cell counts were observed (Table). Spleens from 2A-treated animals had a significantly increased proportion of CD8+ cells, an increased CD8/CD4 ratio, and a decreased proportion of CD4+ compared IgG2a-treated mice (Table).

In aortas collected from the 2A-treated mice, mRNA levels of TnF, interferon gamma (Ifnγ) and Il-10 were higher, and levels of Il-6 were lower, than in aortas from IgG2a-treated mice in this experiment (Figure 3A–D). There were no significant differences in mRNA levels of Il-1β, Il-5 or transforming growth factor (Tgf)-β between groups (data not shown). In carotid artery atherosclerotic lesions, the number of CD8+ and MHC class II molecule I-Aβ+ (I-Aβ+) cells was significantly higher in 2A-treated than in IgG2a-treated mice (Figures 3E–F and 4A–H). Of note, plaques in the mouse model used in this study show an inherent morphological heterogeneity, which was reflected in the histological analysis. There were no significant differences in CD3 or CD4 counts in the sections from the carotid artery atherosclerotic lesions (data not shown).

No Significant Effect of 2A Treatment on Atherothrombosis

To investigate whether the 2A treatment influenced atherosclerosis development at this relatively early stage of disease development in Apoe−/− mice, aortic arches from 2A- and IgG2a-treated Apoe−/− mice were pinned, stained with Sudan IV, and the stained atherosclerotic area measured. There was no significant difference in stained area (percent of total area) between 2A- and IgG2a-treated mice (2.78±0.31 vs. 3.10±0.35%, P=0.49, n=38).

The lesion development was pronounced in the ligated carotid artery, as expected, in both 2A- and IgG2a-treated mice. Thrombosis frequency was lower in 2A- compared to IgG2a-treated mice, but there was no significant difference between groups (Figure 3G).
atherothrombosis and precipitation of clinical disease has been lacking. CD137 agonists, such as urelumab, are currently considered in clinical trials for cancer treatment and potential adverse cardiovascular effects should be considered.

The association between carotid atherothrombosis and risk for potentially devastating stroke necessitates a better understanding of the effects of CD137 stimulation in carotid atherosclerosis.

We have previously shown that treatment of atherosclerosis-prone Apoe<sup>−/−</sup> mice with the CD137 agonist, 2A, significantly increased vascular inflammation, but did not increase the frequency of local atherothrombosis in hypercholesterolemic Apoe<sup>−/−</sup> mice.

No Significant Effect of 2A Treatment on mAoSNC

To investigate whether the 2A treatment influenced proliferation of mAoSNCs from Apoe<sup>−/−</sup> mice, cultured cells were incubated in media supplemented with 10% plasma from either 2A- or IgG<sub>2a</sub>-treated Apoe<sup>−/−</sup> mice. There was no significant difference in proliferation between mAoSNCs cultured with plasma from 2A- or IgG<sub>2a</sub>-treated mice (Figure 3H) (n=11).

Discussion

Levels of CD137 and CD137L mRNA were elevated in murine carotid atherosclerotic lesions, and were even higher in atherothrombotic lesions, suggesting increased local lymphocyte co-stimulation. Administration of the CD137-agonistic antibody, 2A, promoted vascular inflammation, but did not increase the frequency of local atherothrombosis in hypercholesterolemic Apoe<sup>−/−</sup> mice.

Activation of CD137 promotes plaque inflammation and atherosclerosis development in mice, but data on atherothrombosis and precipitation of clinical disease has been lacking. CD137 agonists, such as urelumab, are currently considered in clinical trials for cancer treatment and potential adverse cardiovascular effects should be considered. The association between carotid atherothrombosis and risk for potentially devastating stroke necessitates a better understanding of the effects of CD137 stimulation in carotid atherosclerosis.

We have previously shown that treatment of atherosclerosis-prone Apoe<sup>−/−</sup> mice with the CD137 agonist, 2A, significantly increased aortic inflammation, as evidenced by higher mRNA levels of pro-inflammatory cytokines, and increased counts of CD8<sup>+</sup> and I-A<sup>b</sup> cells in atherosclerotic lesions in aortic roots. Our findings were confirmed by Jeon et al and Jung et al in other mouse models of atherosclerosis, together with additional data supporting the significance of CD137 for atherosclerosis development. Furthermore, others reported that CD137 stimulation in Apoe<sup>−/−</sup> mice promotes plaque calcification, angiogenesis, and vascular remodeling. However, the effect of CD137 activation on carotid atherosclerosis is not
Aortic mRNA levels of cytokines were measured as a proxy for cytokine levels in the carotid artery because the carotic artery was in its entirety used for evaluation of atherothrombosis. mRNA levels of pro-inflammatory cytokines in the aortic biopsies were significantly higher in 2A-treated mice, indicating an augmented pro-inflammatory state in the vasculature. In line with this, we observed an increased infiltration of CD8+ cells with T cell-like morphology and an increased number of I-A\textsuperscript{b}+ cells in the carotid atherosclerotic lesions, providing further evidence that CD137-stimulation promotes inflammation in atherosclerotic plaques. The findings here of increased I-A\textsuperscript{b} expression in 2A-treated mice suggest increased activation of antigen presenting cells (APC). The most abundant APC in atherosclerotic plaques is the macrophage,\textsuperscript{9} and previous studies did not show increased macrophage infiltration in plaques upon 2A stimulation.\textsuperscript{9} In light of this, we speculate that the increased I-A\textsuperscript{b} expression observed in the present study reflects increased macrophage activation rather than infiltration.

Interestingly, a population of tissue-resident dendritic cells in mice can reportedly express CD8+. We find it unlikely that changes in this population are the underlying cause of the increase in CD8+ cells in atherosclerotic lesions observed here because dendritic cell morphology is distinctly different compared to T cell morphology, and the migrating CD8+ dendritic cells in mice reportedly show low CD8 expression.\textsuperscript{26}

Published data suggest that CD137 activity promotes factors associated with plaque rupture. CD137 activation has been reported to augment atherosclerotic lesion growth, increase plaque inflammation and increase vascular immune cell infiltration.\textsuperscript{9,16} In addition, a CD137 agonist inhibits smooth muscle cell proliferation in vitro.\textsuperscript{9} Surprisingly, there was no significant effect by 2A treatment on atherothrombosis frequency, and plasma from mice treated with 2A or IgG\textsubscript{2a} did not significantly change the proliferation rate of mAoSMCs in vitro in this study. Accordingly, based on the available evidence taken together, the effect of CD137 agonists in atherosclerosis may promote lesion growth rather than precipitate atherothrombosis. However, it cannot be ruled out that CD137 activation promotes weakening of the atherosclerotic lesion cap, particularly over longer periods of time. It might therefore be possible that therapeutic activation of CD137 may carry less risk for adverse cardiovascular events in the short term, while long-term treatment may require more caution, but this is quite speculative because the experimental evidence is currently inconclusive.

In the experimental model for atherothrombosis used in this study, the atherosclerotic carotid lesions develop over a relatively short period of time compared to the life-long process of atherosclerosis development in humans. Naturally, the tissue composition in terms of cellular and interstitial content, enzymatic activity and physical properties of the atherosclerotic lesions in this model may be significantly different from that of lesions that developed over months, years and decades. In addition, advanced atherosclerosis occurs at older age in male Apoe\textsuperscript{−/−} mice.\textsuperscript{27} Accordingly, the inflammatory phenotype of advanced atherosclerosis was not fully developed in the 16-week-old mice studied here, possibly masking effects of CD137 activation. Furthermore, the mechanism for atherothrombosis development in this model\textsuperscript{17} is not fully understood. Changes in shear stress and activation of the endothelium have been proposed to play a prominent role, while the contribution of inflammation in precipitating atherothrombosis has not been elucidated in this model. In light of this, it is possible that inflammation plays a less prominent role in atherothrombosis in the model used in this study than in advanced human lesions.

These are important weaknesses of this study, but there is a lack of relevant models for atherothrombosis that are validated to fully represent human pathophysiology. The model used here represents one of the available approaches for investigating murine carotid atherothrombosis.

We were surprised to observe a lower rupture frequency in the 2A-treated group, albeit not statistically significant. Should this finding represent a verifiable effect, it would suggest the potential for a protective role of CD137 activation. The notion is supported by our previous findings on
the minor allele of rs2453021, which was associated with increased adverse non-cardiac events and decreased CD137 mRNA levels in an immortalized cell population in separate cohorts. Furthermore, Perisic et al found reduced expression of CD137 protein in ruptured human atherosclerotic lesions. Hence, the effects of CD137 activation in plaque pathophysiology are likely complex, and a definitive understanding of CD137 and CD137L in cardiovascular disease is currently lacking.

In conclusion, this study shows that mRNA levels of CD137 and CD137L are higher in atherothrombotic than non-atherothrombotic murine atherosclerotic lesions. 2A stimulation of CD137 in a model of inducible atherothrombosis led to increased vascular inflammation, increased infiltration of CD8+ cells and inflammatory activation in carotid lesions from ligated Apoe−/− mice, but there was no significant effect on atherothrombosis frequency at this early disease stage. Further studies are warranted to delineate the physiological role of CD137/CD137L in the development of atherothrombosis and clinical disease.

Acknowledgments
The authors wish to thank Professor Lieping Chen for the 2A hybridoma. We are grateful to Konstantinos Polyzos, Daniel Kethelthu, Marcello Petri and Roland Baumgartner for help with the dissections and to Annetl Olsson and Ingrid Törnberg for assistance with tissue preparation and cell culture. We thank Ronja J. Strawbridge for statistical advice.

This study was supported by Knut och Alice Wallenberg foundation (Knut och Alice Wallenbergs stifteelse) (Grant number 2014-0212), Swedish Medical Society (Svenska Läkaresällskapet), Center of Excellence for Research on Inflammation and Cardiovascular disease (CERIC), Stockholm County Council (Stockholms läns landsting) (ALF), Heart-lung Foundation (Hjärt-lungfonden, grant number 20150767), The Swedish Research Council (Vetenskapsrådet), Ragnar Söderberg Foundation, Karolinska Institutet (KID), the European Research Council (ERC Starting Grant NORVAS), and the European Union’s Seventh Framework Programme [FP7/2007-2013] under grant agreement Athero-Flux (no 602222) and VIA (no 603131).

Disclosures
The authors report no conflicts of interest.

References
1. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of arterial disease. Circulation Journal Vol.81, December 2017
2. hansson GK, Hermansson A. The immune system in atherosclerosis. Nature Rev Immunol 2011; 12: 291 – 301
3. Olofsson PS, Soderstrom LA, Wagsater D, Sheikine Y, Ocaya P, Lang F, et al. CD137 is expressed in human atherosclerosis and promotes development of plaque inflammation in hypercholesterolemic mice. Circulation 2008; 117: 1292 – 1301
4. Jung HI, Choi JH, Jin J, Jeong SJ, Jeon S, Lim C, et al. CD137-destabilizing factors from T cells and macrophages accelerate the destabilization of atherosclerotic plaques in hyperlipidemic mice. FASEB J 2014; 28: 4779 – 4791.
5. Ridker PM, Thuren T, Zalewski A, Libby P. Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: The Athero-Flux (no 602222) and VIA (no 603131). Circulation Journal Vol.81, December 2017
6. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nature Rev Immunol 2011; 12: 291 – 301
7. Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol 2005; 23: 23 – 68.
8. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nature Rev Immunol 2011; 12: 291 – 301
9. Olofsson PS, Soderstrom LA, Wagsater D, Sheikine Y, Ocaya P, Lang F, et al. CD137 is expressed in human atherosclerosis and promotes development of plaque inflammation in hypercholesterolemic mice. Circulation 2008; 117: 1292 – 1301
10. Jung HI, Choi JH, Jin J, Jeong SJ, Jeon S, Lim C, et al. CD137-destabilizing factors from T cells and macrophages accelerate the destabilization of atherosclerotic plaques in hyperlipidemic mice. FASEB J 2014; 28: 4779 – 4791.
11. Chen Y, Bangash AB, Song J, Zhong W, Wang C, Shao C, et al. Activation of CD137 signaling accelerates vascular calcification in vivo and vitro. Int J Cardiol 2017; 230: 198 – 203.
12. Weng J, Wang C, Zhong W, Li B, Wang Z, Shao C, et al. Activation of CD137 signaling promotes angiogenesis in atherosclerosis via modulating endothelial Smad1/5-NAF1c1 pathway. J Am Heart Assoc 2017; 6: e004756.
13. Soderstrom LA, Gertow K, Folkersen L, Sabater-Lleal M, Sundman E, Sheikine Y, et al. Human genetic evidence for involvement of CD137 in atherosclerosis. Mol Med 2014; 20: 456 – 465.
14. Vinay DS, Kwon BS. Therapeutic potential of anti-CD137 (4-1BB) monoclonal antibodies. Expert Opin Ther Targets 2016; 20: 361 – 373.
15. Colwell J. Shelved 4-1BB antibodies make comeback. Cancer Discov 2015; 5: 1118.
16. Jeon HI, Choi JH, Jung HI, Park JG, Lee MR, Lee MN, et al. CD137 (4-1BB) deficiency reduces atherosclerosis in hyperlipidemic mice. Circulation 2010; 121: 1124 – 1133.
17. Sasaki T, Kuzuya M, Nakamura K, Cheng XW, Shibata T, Sato K, et al. A simple method of plaque rupture induction in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2006; 26: 1304 – 1309.
18. Loven J, Orlando DA, Sigova AA, Lin CY, Rahi PB, Burge CB, et al. Revisiting global gene expression analysis. Cell 2012; 151: 476 – 482.
19. Eken SM, Jin H, Chernogubova E, Li Y, Simon N, Sun C, et al. MicroRNA-210 enhances fibrous cap stability in advanced atherosclerotic lesions. Circ Res 2017; 120: 633 – 644.
20. V. Andres BDE. Methods in mouse atherosclerosis. Methods in Molecular Biology 1: Humana Press, 2015. doi:10.1007/978-1-4939-2929-0.
21. MaegdelesfuL, Azuma J, Toh R, Deng A, Merk DR, Raiesdana A, et al. MicroRNA-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion. Sci Transl Med 2012; 4: 222ra22.
22. Larsson M, Rayzman V, Nolte MW, Nickel KF, Bjorkqvist J, Jamsa A, et al. A factor Xla inhibitor antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk. Sci Transl Med 2014; 6: 222ra17.
23. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KL4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat Med 2015; 21: 628 – 637.
24. Wilcox RA, Flies DB, Zhu G, Johnson AJ, Tamada K, Chapoval AI, et al. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. J Clin Invest 2002; 109: 651 – 659.
25. Li Y, Yan J, Wu C, Wang Z, Yuan W, Wang D. CD137-CD137L interaction regulates atherosclerosis via cyclophilin A in apolipoprotein E-deficient mice. PLoS One 2014; 9: e88563.
26. Shortman K, Heath WR. The CD8+ dendritic cell subset. Immuno Mol Med 2010; 234: 18 – 31.
27. Caligiuri G, Nicoletti A, Zhou X, Tornberg I, Hansson GK. Effects of sex and age on atherosclerosis and autoimmunity in apoE-deficient mice. Atherosclerosis 1999; 145: 301 – 308.
28. Perisic L, Hedin E, Razuvaev A, Lengquist M, Osterholm C, Folkersen L, et al. Profiling of atherosclerotic lesions by gene and tissue microarrays reveals PCSK6 as a novel protease in unstable carotid atherosclerosis. Arterioscler Thromb Vasc Biol 2013; 33: 2432 – 2443.

Circulation Journal Vol.81, December 2017