Genetic Depletion or Hyperresponsiveness of Natural Killer Cells Do Not Affect Atherosclerosis Development

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Running title: Natural Killer Cells and Atherosclerosis

Subject Terms:
Atherosclerosis

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In September 2017, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13 days.

ABSTRACT

***Rationale*:** Chronic inflammation is central in the development of atherosclerosis. Both innate and adaptive immunity are involved. Although several studies have evaluated the functions of NK cells in experimental animal models of atherosclerosis, it is not yet clear whether NK cells behave as protective or pro-atherogenic effectors. One of the main caveats of previous studies was the lack of specificity in targeting loss- or gain-of-function of NK cells.

Objectives We used two selective genetic approaches to investigate the role of NK cells in atherosclerosis: 1) *Ncr1*$^{iCre/+}$*R26*$^{lsl\text{-}DTA/+}$ mice in which NK cells were depleted, 2) *Noé* mice in which NK cells are hyperresponsive.

Methods and Results: No difference in atherosclerotic lesion size was found in *Ldlr*$^{-/-}$ mice transplanted with bone marrow cells from *Ncr1*^{iCre}*R26R*$^{lsl\text{-}DTA}$, *Noé* or WT mice. Also, no difference was observed in plaque composition in terms of collagen content, macrophage infiltration or the immune profile, although *Noé* chimera had more IFN-γ-producing NK cells, compared with WT mice. Then, we investigated the NK cell selectivity of anti-asialo GM1 anti-serum, which was previously used to conclude to the pro-atherogenicity of NK cells. Anti-asialo GM1 treatment decreased atherosclerosis in both *Ldlr*$^{-/-}$ mice transplanted with *Ncr1*^{iCre}*R26R*$^{lsl\text{-}DTA}$ or WT BM, indicating that its anti-atherogenic effects are unrelated to NK cell depletion, but to CD8+ T and NKT cells. Finally, to determine whether NK cells could contribute to the disease in conditions of pathological NK cell overactivation, we treated irradiated *Ldlr*$^{-/-}$ mice reconstituted with either WT or *Ncr1*^{iCre}*R26R*$^{lsl\text{-}DTA}$ BM with the viral mimic poly(I:C) and found a significant reduction of plaque size in NK-cell deficient chimeric mice.

Conclusion: Our findings, using state-of-the-art mouse models, demonstrate that NK cells have no direct effect on the natural development of hypercholesterolemia-induced atherosclerosis, but may play a role when an additional systemic NK cell overactivation occurs.

Keywords:
Atherosclerosis, immune system, inflammation, natural killer cells.

Nonstandard Abbreviations and Acronyms:

asialo-GM1	asialoganglioside M1
ATF2	activating transcription factor 2
BM	bone marrow
DTA	diphtheria toxin fragment A
Lsl	lox-stop-lox
Ncr1	natural cytotoxicity triggering receptor 1
NK	natural killer
GFP	green fluorescent protein
HFD	high-fat diet
Ldlr$^{-/-}$	low-density lipoprotein receptor-null
poly(I:C)	polyinosinic-polycytidylic acid
TLR	Toll-like receptor
WT	wild type

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of cholesterol, immune cells, and fibrous elements, forming atherosclerotic plaques in large and medium sized arteries [1]**.** Over the past 20 years, a large body of evidence supported the implication of innate and adaptive immunity in the development of the disease. Macrophages are the first immune cells to infiltrate developing lesions, as well as dendritic cells and T cells. However, less abundant cells such as NKT and NK cells are also present [2]. NK cells are bone marrow (BM)-derived innate lymphocytes characterized by their unique ability to kill aberrant cells without prior sensitization [3]. They act by means of direct cytotoxicity against their targets and by producing large array of cytokines and chemokines, the latter contributing also to the initiation of antigen-specific immune responses and thus shaping of adaptive immunity [4, 5]. Regardless of their activation status and their localization NK cells express the NKp46 marker encoded by the *Ncr1* gene. NK cells have been identified within atherosclerotic plaques in humans as well as in mice. In advanced atherosclerotic plaques, they have been localized to necrotic cores, deep within the plaques and in shoulder regions [6, 7]. Chemoattractants such as CCL2 and CX3CL1 may be involved in their recruitment to the developing lesions [8, 9]. Directly addressing the role of NK cells in immunity and atherosclerosis has been challenging due to the lack of a good animal model of NK cell-deficiency. Earlier studies used the *beige* mutant mice, an accepted model of defective NK cell functional activity [10], with opposing results. In the first study, *beige* mutant mice fed a high fat diet (HFD) containing cholate showed no differences in atherosclerotic lesions compared to controls [11]. However, in a more recent study by Schiller and his colleagues, NK cells were ascribed an athero-protective role when *beige* mutant mice were bred to $Ldlr^{-/-}$ mice [12]. *Beige* mutation, which involves the *Lyst* gene encoding a protein implicated in lysosomal trafficking, results in a complicated phenotype that goes beyond decreased NK cell activity [13, 14]. Defects in cell function may include, in addition to NK cells, neutrophils, macrophages, or smooth muscle cells (SMCs) [15], making it unclear whether the increase in atherosclerosis in $Lyst^{beige}$x$Ldlr^{-/-}$ mice was a consequence of defective NK cell function.

Subsequently, different results were observed using a Ly49A transgenic mouse model of NK cell deficiency [7]. In these transgenic mice, the Ly49A cDNA was expressed under control of the mouse granzyme A genomic sequence, inducing the expression of the receptor on all NK cells and one-half of T cells [16]. Ly49A is an inhibitory receptor, which recognizes MHC class-I molecules [17]. In this transgenic model, the number of NK ($NK1.1^{+}CD3^{-}$) cells was markedly reduced in the spleen and other peripheral tissues and the number of $NK1.1^{+}$ T cells was slightly reduced in the spleen [16]. However, this phenotype, which is not totally understood, is linked to the expression of the ubiquitous transcription factor ATF2, raising the possibility of additional defects [18]. Smaller size lesions were observed in $Ldlr^{-/-}$ recipients reconstituted with the BM of ly49A transgenic mice [7]. While the authors concluded that NK cell deficiency was responsible for the observed effect, they did not exclude the possibility that T cells, specifically NKT and CD8 subsets; which are known to express granzyme A and whose functions are influenced by Ly49A [19, 20] could contribute to this pro-atherogenicity. It is worth mentioning that NKT and CD8 T cells have been classified as proatherogenic cells [21 22].

Lately, Selathurai et al. performed loss- and gain-of-function studies to assess NK cell function in atherosclerosis [22]. In the loss-of-function experiments, treatment of $Apoe^{-/-}$ mice with anti-asialo-GM-1 antibody significantly reduced atherosclerotic lesion development. This study was supported by gain-of-function experiments where adoptive transfer of NK cells (WT, IFN-$\gamma^{-/-}$, or $perforin^{-/-}$/$granzyme^{-/-}$) into lymphocyte-deficient $Apoe^{-/-}$ x $Rag2^{-/-}$ x $Il2rg^{-/-}$ mice confirmed that its cytotoxic effects are proatherogenic and promote necrotic core development [23]. However, the glycolipid asialo-GM1 is also expressed by several cell types, including myeloid cells, epithelial cells, and T-cell subsets. Hence, the selectivity of NK cell depletion with anti-asialo-GM1 antiserum has been questioned [24].

Here, we used two state-of-the-art genetic approaches: 1) The $Ncr1^{iCre}R26R^{lsl\text{-}DTA}$ mice in which NK cells are selectively depleted [25] 2) The *Noé* mice in which NK cells are hyperresponsive [26]. BM from these mice were used to re-populate the hematopoietic system of lethally irradiated $Ldlr^{-/-}$ mice fed a high fat diet (HFD).

Using these two genetic models, we provide strong evidence that NK cells do not significantly affect atherosclerosis development unless an additional inflammatory environment is induced.

METHODS

Animals.
Experiments were conducted according to the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC) and were approved by the Ethical Committee of INSERM and the French Ministry of Agriculture (agreement A75-15-32). *Ncr1*^{iCre}*R26R*$^{lsl\text{-}DTA}$ mice (deficient in NK cells) [25] and *Noé* mice (high IFN-γ-producing- and CD107a-expressing- NK cells) [26] have been generated and characterized by Eric Vivier's laboratory (CIML, Marseille, France).

The *Ncr1*^{iCre} KI mice [27] were bred to *R26R*$^{lsl\text{-}DTA}$ mice to generate *NK46*$^{iCre/+}$*R26*$^{lsl\text{-}DTA/+}$ mice referred as to *Ncr1*^{iCre}*R26R*$^{lsl\text{-}DTA}$ mice thereafter. *Ncr1*^{iCre} KI mice were generated from C57BL/6 ES cells and *R26*$^{Rlsl\text{-}DTA}$ mice were obtained from Jackson. This line was backcrossed to C57BL/6 for 10 generations by the donating laboratory [27]. In this model, the *Cre*-mediated removal of the floxed STOP codon, leads to the expression of the diphtheria toxin fragment A and consequent cell death. A near to complete absence of NK cells in the bone marrow and every tested organ (including the blood, thymus, lymph nodes, spleen, liver) was observed in these mice [25]. Of note, a deficiency in ILC1 (which also express NKp46) in the liver and in NKp46^{+} ILC3 in the small intestine was also observed in these mice. Aside from NKp46^{+} cell deficiency, the immune status of the mice is strictly normal at steady state, in terms of cell populations and activation status [25].

The *Noé* mice were generated by random mutagenesis, in which a point mutation in the *Ncr1* gene impairs the cell surface expression of NKp46 receptor inducing higher capacity to produce IFN-γ and to degranulate. Once generated, *Noé* mice were back-crossed to C57BL/6J [26].

Ten-week-old male C57BL/6J *Ldlr*$^{-/-}$ mice were subjected to medullar aplasia by lethal total body irradiation (9.5 Gy). The mice were repopulated with an intravenous injection of BM cells isolated from femurs and tibias of sex-matched *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$, *Noé*, or control C57BL/6J mice. After four weeks of recovery, mice were fed a pro-atherogenic high fat diet (HFD) containing 15% fat, 1.25% cholesterol, and 0% cholate for 8, 12, or 15 weeks.

In some experiments, 14-week-old chimeric male *Ldlr*$^{-/-}$ mice, which received BM from *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ or control C57BL/6J mice, were treated every 5 days by intravenous injections of anti-asialo-GM-1 antibody (54 µg/injection), Wako chemicals, Richmond, VA, USA) or control serum (normal rabbit serum) for 8 weeks HFD. We used the same experimental protocol as previously described by Selathurai et al [23].

For NK cell-activation experiments, 14-week-old chimeric male *Ldlr*$^{-/-}$ mice, which received *Ncr1*^{iCre} *R26*$^{lsl\text{-}DTA}$ or control C57BL/6J BM, were treated twice a week by intra-peritoneal injection of 100 µg polyinosinic-polycytidylic acid [(poly(I:C)], a synthetic analogue of dsRNA, TLR3 ligand (Invivogen, SD, USA) or saline (as a control) during 8 weeks HFD.

For tracking NK cells in mouse lesions, lethally irradiated *Ldlr*$^{-/-}$ mice were reconstituted with BM of *Ncr-1*$^{gfp/gfp}$ mice and put on HFD for 8 weeks. The generation of *Ncr-1*$^{gfp/gfp}$ mice was previously described by Gazit et al [28].

Quantification of atherosclerotic lesions.
Mice were anesthetized with isoflurane before sacrifice. Plasma cholesterol was measured using a commercial kit (DiaSys® Cholesterol FS*, Germany). Quantification of lesion size and composition was performed as

previously described [29]**.** Briefly, the heart and ascending aorta were removed, perfusion-fixed in situ with 4% paraformaldehyde, then placed in phosphate-buffered saline (PBS)-30% sucrose solution overnight, before being embedded in frozen optimal cutting temperature compound and frozen at -70°C. Afterwards, 10-µm serial sections of aortic sinus were obtained (cut on cryostat). Lipids were detected using Oil Red O (Sigma-Aldrich, St. Louis, Missouri) coloration and quantified by a blinded operator using HistoLab software (Microvisions Instruments, Paris, France) [30], which was also used for morphometric studies. *En face* quantification was used for atherosclerotic plaques along thoraco-abdominal aorta, as previously described [29].

Collagen was detected using Sirius red staining. The presence of macrophages was determined using monoclonal rat anti-mouse macrophage/monocyte antibody (MOMA)-2 (specifically MAB1852).

At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used.

For NK cell tracking, frozen sections of spleen and aortic sinus of *Ncr-1$^{gfp/gfp}$ → Ldlr$^{-/-}$* mice were stained with rabbit anti-GFP antibody (NK cells) and counterstained with DAPI (nuclear dye) and MOMA-2 antibody (monocytes/macrophages).

To detect NK cells in human plaques, immunostaining studies were performed in atherosclerotic plaques from carotid arteries obtained after surgical thrombo-endaterectomy (Pathology department, HEGP hospital, Paris). We used a specific antibody against human NKp46 (generous gift of Innate Pharma, Marseille, France) in formalin fixed paraffin embedded tissues after antigen retrieval by heating in citrate buffer and ABC peroxidase technique as previously described [31]. Spleen was used as an internal control in the two separate experiments.

Spleen cell culture and cytokine assays.
Cells were cultured in RPMI 1640 medium supplemented with Glutamax (Thermo Fischer Scientific), 10% fetal calf serum, 0.02 mM β-mercapto-ethanol and antibiotics. For cytokine measurements, splenocytes were cultured at $5*10^5$ cells/well for 24 hours and stimulated with lipopolysaccharide (LPS) (1 µg/ml) and IFN-γ (100 UI/ml) (Sigma). IL-6, IL-12, TNF-α, IL-1β, IL-10, and IFN-γ productions in the supernatants were measured using specific ELISA (R&D Systems).

Flow cytometry.
NK cells were identified as $CD3^-NK1.1^+$ or $CD3^-NK1.1^+NKp46^+$. Monocytes were identified as $CD11b^+Ly6G^-$ or $CD11b^+CD115^{high}$. Among them, classical monocytes were $Gr1^{high}$ (or $Ly6C^{high}$) and non-classical monocytes were $Gr1^{low}$ (or $Ly6C^{low}$). Neutrophils were identified as $CD11b^+CD115^-Ly6G^+$. Regulatory T cells were considered as $CD3^+CD4^+CD25^{high}Foxp3^+$. $CD4^+$, $CD8^+$, and NKT ($CD3^+NK1.1^+$) lymphocyte subsets were also analyzed.

Cells were labelled with FITC or PE-Cy7-conjugated anti-NKp46 (29A1.4), APC-conjugated anti-NK1.1 (PK136), PerCp-Cy5.5-conjugated anti-CD3e (145-2C11), FITC-conjugated anti-CD4 (RM4-5), eFluor-450-conjugated anti-CD11b (M1/70), PE-conjugated anti-CD69 (H1.2F3), PE-Cy7-conjugated anti-CD115 (AFS98), PE-Cy7-conjugated anti-Foxp3 (FJK-16s), APC-conjugated anti-CD25 (PC61.5), V500-conjugated anti-B220 (RA3-6B2), PE-conjugated anti-IL-10 (JES5-16E3), PerCp-Cy5.5-conjugated anti-MHC II (M5/114.15.2) from eBiosciences, Alexa fluor 700-conjugated anti-CD8 (53-6.7), Brilliant Violet 421-conjugated anti-IFN-□ (XMG1.2) and FITC-conjugated anti-CD107a (1D4B) from Biolegend, FITC-conjugated anti-Ly6C (AL-21), PE-conjugated anti-Ly6G (1A8), PerCp-Cy5.5-conjugated Gr-1 (RB6-8C5), PE-Cy7-conjugated anti-CD11c (HL3), APC-conjugated anti-IgM (11/41) from BD Biosciences. For blood staining, erythrocytes were lysed using BD FACS lysing solution (BD Biosciences). For intracellular cytokine staining, lymphocytes were stimulated *in vitro* with leukocyte activation cocktail (BD Biosciences) according

to manufacturer's instructions for 4 h. Surface staining was performed before permeabilization using Foxp3 staining buffer kit (eBiosciences) and intracellular staining.

Forward scatter (FSC) and side scatter (SSC) were used to gate live cells excluding RBC, debris, and cell aggregates in total blood cells, splenocytes, and BM. Single cell suspensions stained with fluorophore-conjugated antibodies were acquired using an LSRII Fortessa (BD) flow cytometer and analyzed with FlowJo software (Miltenyi).

NK cell stimulation.

For specific NK cell stimulation, spleen cell suspensions were dispensed into 96-well 2HB Immulon plate previously coated with 25 μg/ml of purified anti-NK1.1 antibody or 10 μg/ml of anti-NKp46 antibody. Anti-CD107a antibody was added in the presence of monensin for 4 hours.

For IL-12/18 stimulation, spleen cells were incubated with 25 ng/ml IL-12 and 20 ng/ml IL-18 (R&D systems) for 4 hours in the presence of monensin. Cells were surface stained and intracellular IFN-γ was revealed. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fischer Scientific, L34957) was used as a viability dye and the percentage of IFN-γ-producing and CD107a-expressing NK cells was detected by Flow cytometry as mentioned above.

Quantitative real-time PCR.

Total ribonucleic acid (RNA) from spleen or abdominal aorta was extracted using Trizol reagent (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed on an ABI Prism 7700 Sequence Detection System (Thermo Fisher Scientific, Inc.) in duplicates. Cycle threshold for GAPDH was used to normalize gene expression of samples. Relative expression was calculated using the 2-delta-delta computed tomography (CT) method followed by geometric average, as recommended [32], [33]. The following primer sequences were used: IL-10 (F: 5' AAG TGA TGC CCC AGG CA 3'; R: 5' TCT CAC CCA GGG AAT TCA AA 3'), perforin (F: 5' ACA GTA GAG TGT CGC ATG TAC AGT TT 3'; R: 5' GAG GGC TCT GAG CGC CTT TTT GAA 3'), granzyme B (F: 5' ACT CTT GAC GCT GGG ACC TA; R: 5' AGT GGG GCT TGA CTT CAT GT 3'), IFN-γ (F: 5' TCA AGT GGC ATA GAT GTG GAA AGA A 3'; R: 5' TGG CTC TGC AGG ATT TTC ATG 3'), TGF-β (F: 5' CGG CCG GAA ATT CCC AGC TTC T 3'; R: 5' GTG AGA CGG GCT TCG GGG TCA T 3'), TNF-α (F: 5' GAT GGG GGG CTT CCA GAA CT 3'; R: 5' CGT GGG CTA CAG GCT TGT CAC 3'), IL-1β (F: 5' GAA GAG CCC ATC CTC TGT GA 3'; R: 5' GGG TGT GCC GTC TTT CAT TA 3'), and IL-6 (F: 5' AAA GAC AAA GCC AGA GTC CTT CAG AGA GAT 3'; R: 5' GGT CTT GGT CCT TAG CCA CTC CTT CTG T 3'). PCR conditions were 10 min at 95°C; 42 cycles of 95°C for 15 s, 60°C for 30s, and a final extension of 72°C for 30s.

Statistical analysis.

Values are expressed as mean +/- SEM. Differences between values were evaluated using non-parametric Mann-Whitney test or One-way ANOVA with Bonferroni correction to compare more than two groups. All these analyses were performed using GraphPad Prism version 5.0b for Mac (GraphPad Software) and values were considered significant at $P<0.05$.

RESULTS

Genetic NK cell depletion does not alter the development of atherosclerosis.

To assess the direct role of NK cells in the development of atherosclerosis, we performed BM transplantation experiments using either *control (WT)* or *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ BM cells to repopulate lethally irradiated male atherosclerosis prone-*Ldlr*$^{-/-}$ mice.

After 4 weeks of recovery, mice were put on high-fat diet (HFD) for 8, 12, or 15 weeks. Firstly, we confirmed NK cell depletion in spleen (Figure 1A-B), blood (Online Figure I), and BM (data not shown) of chimeric *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ → *Ldlr*$^{-/-}$ mice. As expected, the frequencies of other leukocyte populations in the spleen (Figure 1C), blood (Online Figure I), and BM (data not shown) were not different between groups of chimeric mice. Data shown in Figure 1 corresponds to 8-week HFD-fed mice. Data were similar in 12- and 15-week HFD fed-mice (data not shown). Circulating cholesterol levels and animal weights were similar between the two groups of mice (Online Table). Interestingly, NK cell deficiency did not alter lesion size in aortic root and thoracic aorta after 8 (Figure 2A-B), 12 (Figure 2C-D), or 15 weeks (Figure 2E-F) of HFD. Similarly, no effect was observed on atherosclerosis development in female *Ldlr*$^{-/-}$ mice reconstituted with BM of control or *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ mice and put on HFD for 8 weeks (Online Figure IIA, Figure IIB and Figure IIC). To determine whether plaque composition was affected by NK cell depletion, we analyzed macrophage content (Online Figure IID and Figure IIE), collagen content (Online Figure IIF and Figure IIG) and necrotic core (data not shown), but found no significant differences between groups.

Furthermore, we investigated the immune-inflammatory response in chimeric *Ldlr*$^{-/-}$ mice since NK cells are known to produce cytokines in response to infection or inflammation [34]. The expression levels of IL-6, IL-10, IL-1β, IL-12p70, TNF-α, and TGF-β mRNA measured in the spleen, were not different between control and NK cell-deficient chimeric mice (Online Figure IIIA). LPS and IFN-γ-stimulated splenocytes from chimeric *control or Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ mice also showed no difference in inflammatory phenotype with same levels of IL-6, IL-10, and TNF-α (Online Figure IIIB).

Hyperresponsive NK cells do not promote atherosclerosis development.

We next addressed the role of NK cell hyperresponsiveness in atherosclerosis by using *Noé* mice, in which a point mutation in the *Ncr1* gene impairs the cell surface expression of the NKp46 receptor inducing a higher capacity to produce IFN-γ and to degranulate [26]. We performed BM transplantation experiments using *Noé* or control (C57/B6J) mice to reconstitute the BM of male *Ldlr*$^{-/-}$ mice. After 8 weeks of HFD, an intact NK cell population (CD3^{-}NK1.1^{+}) was detected in *Noé* chimeric mice compared to controls (Figure 3 A, B, C). The frequencies of other leukocyte populations were also the same in spleen and blood of mice reconstituted with the WT or *Noé* BM (Figure 4S E and F). Interestingly, a higher frequency of IFN-γ-producing NK cells was observed in chimera mice reconstituted with the *Noé* BM. However, no difference in plaque size, aortic root and thoracic aorta (Figure 3 D to G) or plaque composition in terms of macrophage accumulation or collagen content (Online Figure IVA, Figure IVB, Figure IVC and Figure IVD) was observed between groups. No difference in immune-inflammatory response was detected either (data not shown). This series of experiments was repeated twice, and similar results were found. There were no differences in plasma cholesterol levels or mice body weight between *Noé* and control chimeric mice (Online Table).

These data show that NK cell hyperresponsiveness observed in *Noé* mice is not sufficient to affect the development of atherosclerosis.

Anti-asialo GM-1-mediated decrease in atherosclerosis is NK cell-independent.

The findings with the above two experiments were unexpected, given that the latest study on the role of NK cell in atherosclerosis came up with the conclusion that NK cells are pro-atherogenic through their production of perforin and granzymes [23]. Hence, we revisited the experimental model of Selathurai et al. [23], in which the authors used anti-asialo-GM1 antibody to deplete NK cells in *Apoe*$^{-/-}$ mice. We treated control (*WT*→ *Ldlr*$^{-/-}$) or NK cell-deficient (*Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ → *Ldlr*$^{-/-}$) chimeric mice either with anti-asialo-GM1 or control serum every other 5 days during the 8 weeks HFD.

Initially, we examined the effectiveness of anti-asialo-GM1 treatment for depleting NK cells in peripheral blood. A single dose of anti-asialo-GM1 antibody was sufficient to deplete NK cells within 5 days by greater than 80% in WT chimeric mice (Table). NK cells are absent in *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ chimeric mice. However, anti-asialo-GM1 treatment also depleted CD8+ T and NKT populations in both control and *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ chimeric mice compared to control groups treated with control serum (Table).

Similar effects in blood and spleen cells were observed in anti-asialo-GM1-treated chimeric mice after 8 weeks of HFD. Anti-asialo-GM1 treatment led to almost 80% reduction of NK cells in control mice, between 60% and 80% reduction in NKT cells in control and NK cell-deficient chimeric mice, respectively, and 30% reduction in CD8 T cells in both groups (Table).

Although anti-asialo-GM1 treatment seemed to be less effective in depleting NKT and CD8$^+$ T cell populations after 8 weeks HFD in the spleen, the trend towards a smaller size population persisted (Online Figure VA). There were no differences in the percentage of monocytes but we observed a trend of decrease in blood (Table) and spleen (data not shown) neutrophils.

Importantly, IFN-γ-producing CD8$^+$ T cell population was significantly decreased by almost 50% in anti-asialo-GM1-treated *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ chimeric mice with a trend also in control anti-asialo-GM1-treated chimeric mice (P=0.077), while no significant differences were observed in IFN-□-producing CD4$^+$ T cells (Online Figure VB).

Lesion size decreased significantly by 42% in anti-asialo-GM1-treated control chimeric mice compared to mice treated with control serum (Figure 4). However, more interestingly, similar effects were observed in NK cell-deficient chimeric mice with 68% reduction in aortic root lesion size in anti-asialo-GM1-treated *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ chimeric mice compared to mice treated with control serum (Figure 4), despite similar plasma cholesterol levels (Online Table). Therefore, we concluded that reduction of atherosclerotic lesions by anti-asialo-GM-1 treatment was NK cell-independent.

NK cells exacerbate atherosclerosis development in inflammatory conditions.

Finally, we addressed the role of NK cells on the development of atherosclerosis in inflammatory conditions in which these cells are known to be activated. To do so, we used poly(I:C), which mimics a viral infection. Control (*WT*→ *Ldlr*$^{-/-}$) or NK cell-deficient (*Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ → *Ldlr*$^{-/-}$) chimeric mice were treated repeatedly with 100 μg poly(I:C) twice a week, while another control group (*WT*→ *Ldlr*$^{-/-}$ mice) was treated in parallel with saline over 8 weeks HFD.

Interestingly, after 8 weeks of HFD, poly(I:C)-treatment of control mice significantly increased CD69 and CD107a expression on NK cells compared to control mice that received saline, although the increase in frequency of IFN-γ–producing NK cells was not significant. As expected, the percentage of NK cells significantly decreased by almost 50% in the spleen of poly(I:C)-treated compared to saline-treated chimeric mice reconstituted with WT BM (Figure 5 A). Moreover, the expression of perforin, granzyme B, and IFN-γ

mRNA was significantly enhanced in the spleen after poly(I:C) treatment, which also holds true for granzyme B in the aorta (Figure 5B). Interestingly, the expression of granzyme B mRNA, but not IFN-γ, in the spleen and aorta was reduced in poly(I:C) treated NK-deficient mice. This further confirm NK cell activation in response to poly(I:C) treatment.

Although no change in aortic root plaque size was observed in poly(I:C)-treated compared to saline-treated (*WT→ Ldlr*$^{-/-}$) chimeric mice, NK cell deficiency in poly(I:C) treated chimeric mice markedly decreased lesion development compared with poly(I:C) treated WT controls (Figure 6). Reduction in atherosclerosis was associated with reduction in macrophage infiltration in (*Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ *→ Ldlr*$^{-/-}$) poly(I:C)-treated mice compared to poly(I:C)-treated control mice (Online Figure VIA). No change in collagen content was detected (Online Figure VIB). No differences in body weight or serum cholesterol levels were observed between the 3 groups of mice (Online Table). Hence, NK cells might be proatherogenic when activated in a systemic inflammatory context.

NK cells are scarcely detected in human and mouse atherosclerotic lesions.

To specifically track NK cell homing to atherosclerotic lesions in hypercholesterolemic mice, we reconstituted lethally irradiated *Ldlr*$^{-/-}$ mice with BM from *Ncr-1*$^{gfp/gfp}$ mice. After 8 weeks of HFD, staining of atherosclerotic lesions with anti-GFP-antibody showed very low number of NK cells, 1 to 2 cells per lesion examined (Figure 7 A). In contrast, NK cells were abundant in the spleen.

In human, we also found very few NK cells in atherosclerotic plaques. Only one or two cells were detected in the plaques of uncomplicated atherosclerosis, while none were observed in advanced plaque lesions (Figure 7 B). Specificity of the anti-human NKp46 antibody was tested in human spleen samples.

DISCUSSION

Using two state-of-the-art genetic approaches, our study provides strong evidence that, in contrast to most previous studies, NK cells have no role in atherosclerosis development, unless a systemic inflammation is induced. Transfer of the BM of selectively depleted (*Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$) or hyper-responsive (*Noé*) mice into lethally irradiated *Ldlr*$^{-/-}$ mice had no effect on plaque size (aortic root and thoracic aorta) and composition, or immune-inflammatory response, as compared with control mice. However, treating NK cell-deficient chimeric mice with poly(I:C), in an attempt to mimic viral infection, significantly reduced lesion size, which suggests that NK cells might be proatherogenic only when activated by virus infection or inflammatory cytokines or in the context of tumor growth.

Earlier studies on the role of this innate immune cell population in atherosclerosis led to conflicting results. The very first studies to examine the role of NK cells in atherosclerosis used the *Beige* mutant mice. Accelerated atherosclerosis was observed in *beige Ldlr*$^{-/-}$ mice and NK cells were accredited to be anti-atherogenic [12]. However, an alternative NK-cell independent hypothesis is most likely since the beige mutation which leads to functional changes in lysosome trafficking may involve other cells such as lesion macrophages or SMCs leading to increased foam cell formation or apoptosis [35], [10]. In contrast to these studies, *Ldlr*$^{-/-}$ mice repopulated with Ly49A transgenic mice BM and which present a NK cell deficiency exhibited reduced atherosclerosis [7]. However, the expression of the inhibitory receptor Ly49A, which is regulated by granzyme A promoter in this model, is induced not only in NK cells, but also in subsets of $CD4^+$ and $CD8^+$ T cells [16]. Hence, this expression on T cells could be responsible for the decrease in lesion size in Ly49A transgenic BM recipients. Lately, experiments in *Apoe*$^{-/-}$ mice fed a HFD treated with anti-asialo-GM1 antibody to deplete NK cells attenuated atherosclerosis. In addition, adoptive transfer of *ex vivo* activated NK cells increased lesion size in lymphocyte deficient *Apoe*$^{-/-}$ mice, an effect that was prevented by transferring perforin or granzyme-

deficient NK cells, with suggests that NK cells augment atherosclerosis via cytotoxic dependent mechanisms [23]. However, the expression of asialo-GM1 is not strictly confined to NK cells among hematopoietic cells and is detected in subpopulations of NKT, CD8 T, and γδ T cells [36], [37] and in some activated forms of $CD4^+$ T cells, macrophages, eosinophils, and basophils under certain experimental conditions [38], [39], [40]. Our data suggest that the discrepancy observed in former studies was most likely due to effects on other cell populations targeted in addition to NK cells. Indeed, our study clearly demonstrates that NK cells have no role in atherosclerosis development.

NKp46 is also expressed in ILC1 and a subset of ILC3 (NKp46+ ILC3s). These cells are also deleted in the *Ncr1*^{iCre}*R26R*^{DTA} mice [25]. In our experimental protocol, recipient mice were irradiated before BM transplantation. Both NK cells and ILC1s are sensitive to irradiation and should thus originate from donor BM. Regarding the ILC3 population, $NKp46^-$ ILC3 have been shown to be resistant to irradiation [41], [42], but we have no information about the radioresistance of $NKp46^+$ ILC3. Therefore, in our model of BM transplantation of *Ncr1*^{iCre}*R26R*^{DTA} BM into irradiated *Ldlr*$^{-/-}$ mice, not only NK cells but also ILC1s and potentially NKp46+ ILC3s should have been depleted, while other lymphocytes and myeloid cells were unaffected suggesting that these other ILCs subset do not play a major role in atherosclerosis either.

Moreover, we questioned the specificity of using anti-asialo GM1 treatment to target NK cells in the context of atherosclerosis. We clearly demonstrated its effect was NK cell-independent as atherosclerosis was reduced by anti-asialo-GM1 treatment to the same extent in control and NK-deficient mice. We observed that the anti-asialo-GM1 treatment not only depleted NK cells, but also strongly reduced NKT and $CD8^+$ T cell population after 8 weeks of HFD. Among $CD8^+$ T cells, the IFN-γ-producing CD8 T cells was also reduced. This is consistent with a previous study showing that the asialo-$GM1^+$ CD8 population are high IFN-□-producing cells in response to stimulus [36]. These activated $CD8^+$ T cells which are targeted by anti-asialo-GM1 antibodies might thus be involved in the phenotype observed in atherosclerosis. This is consistent with the fact that $CD8^+$ T cell activation has been shown to exacerbate atherosclerosis [43]. Regarding the NKT cell population, although previous studies indicate that NK cell depletion with antibody against asialo-GM1 does not always drastically affect the size or function of NKT cell population [44], NKT cell population was significantly decreased in hypercholesterolemic mice treated with anti-asialo-GM1 antibody. NKT cells are considered pro-atherogenic [45]. Their depletion could thus also contribute to the protective phenotype in anti-asialo-GM1-treated mice.

We used two models in which NK cells functions are increased: the *Noé* model in which NK cells are known to be intrinsically hyperresponsive and the poly(I:C) model in which NK cells are activated due to an inflammatory environment.

Using the *Noé* mice, we showed that NK cell hyperresponsiveness in sterile conditions does not affect the atherosclerotic process, although hypercholesterolemia modestly increased IFN-γ-producing NK cell population. In contrast, a non-redundant role of NK cells was revealed in an inflammatory context induced by chronic poly(I:C) injection. Along this line, previous studies reported an aggravation of atherosclerosis in hypercholesterolemic mice infected with mouse Cytomegalovirus (MCMV) [46]. NK cells are one of the first innate immune cells to respond to and contain the MCMV infection through their direct perforin mediated cytotoxicity function and IFN-γ release [47]. In our study, the viral mimic poly(I:C) significantly augmented NK cell activation marker CD69 as well as CD107a degranulation marker. CD107a is a lysosomal associated membrane protein lining the membrane of cytotoxic granules [48]. Its expression is correlated with degranulation and target cell lysis [49]. In addition, in our experiments, we noticed an upregulation of the mRNA of perforin, granzyme B, and IFN-γ in the spleens of poly(I:C)-treated control mice, which highlights the marked activation of NK cells in response to poly(I:C). Interestingly, only the expression of granzyme B was significantly decreased in both spleen and aorta of poly(I:C) treated NK-deficient mice, which points out to a role of NK cytolysis in the proatherogenic effects of NK cells, in agreement with previous studies by Selathurai et al.[23] In

control mice, we observed no effect of poly(I:C) on atherosclerosis in the aortic root. The discrepancy observed could be attributed to the multiple effects of poly(I:C) in atherosclerosis context. Zimmer et al. demonstrated that intravenous administration of poly(I:C) induced endothelial dysfunction and increased atherosclerotic lesion development [50]. In contrast, Monaco's group described poly(I:C)-mediated atheroprotection. Intraperitoneal administration of poly(I:C) attenuated neointima formation and reduced injury induced media damage [51]. Further studies are required to fully elucidate how poly(I:C) influence atherosclerosis. Nonetheless, this series of experiments clearly establish that even though resting NK cells have no effects on atherosclerosis, NK cells might exacerbate atherosclerosis in case of activation by an inflammatory context like viral infections. The ability of activated NK cells to influence other immune cells in the atherosclerotic process should be considered. In immune competent mice, NK cell-derived IFN-γ promotes $CD4^+$ TH1 priming [52]. It is well established that IFN-γ-producing TH1 subset is a major proatherogenic subset of the adaptive immune system and may activate pro-atherogenic properties of both vascular and immune cells [53]. Moreover, perforin and granzymes, which are highly augmented after poly(I:C) treatment, lead to target cell apoptosis and can accelerate atherosclerosis by increasing necrosis [23].

In conclusion, our findings indicate that NK cells have no role in atherosclerosis development in hypercholesterolemic mice, unless a super-imposed inflammatory status mimicking viral infection is induced. Further studies are needed for detailed characterization of the mechanisms whereby NK cells' response to viral infection would influence the atherosclerotic process.

SOURCES OF FUNDING
This work was supported by Inserm, the Fondation pour la Recherche Medicale, CNRS (EV, SU), and Aix-Marseille University (EV, SU).

ACKNOWLEDGMENTS
WNE has received a scholarship from the Association of Specialization and Scientific Guidance (ASSG).

DISCLOSURES
None

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FIGURE LEGENDS

Figure 1. (A and B) Efficiency of NK cell (CD3-NK1.1$^+$NKp46$^+$) depletion in spleen (gated on total viable splenocytes) of male *Ldlr*$^{-/-}$mice reconstituted with the BM of NK cell-deficient (*Ncr1*^{iCre}*R26l*$^{sl\text{-}DTA}$ → *Ldlr*$^{-/-}$) (n=10) compared to control mice (WT → *Ldlr*$^{-/-}$) (n=8) after 8 weeks of high fat diet (HFD). (C) Representative quantitative analysis of the percentage of myeloid (neutrophils, monocytes (classical and non-classical), and dendritic cells) and lymphoid (CD4$^+$, CD8$^+$, and NKT cells) populations in the spleen of control and NK cell-deficient chimeric mice assessed by flow cytometry, after 8 weeks of HFD. ***p < 0.001

Figure 2. Representative photomicrographs of Oil red O-staining and quantification of lesion size in aortic sinus (A) or thoracic aorta (B) of male controls (WT → *Ldlr*$^{-/-}$) (n=8) and NK cell-deficient (*Ncr1*^{iCre}*R26l*$^{sl\text{-}DTA}$ → *Ldlr*$^{-/-}$) (n=10) mice after 8 weeks (A-B), 12 weeks (n=7 – 6, respectively) (C, D), or 15 weeks (n=5 – 3, respectively) HFD (E, F).

Figure 3. Representative flow cytometry plots (A) and quantitative (B) analysis of the percentage of (B) NK cells in the splenocytes of controls (WT → *Ldlr*$^{-/-}$) and (*Noé* → *Ldlr*$^{-/-}$)
chimeric mice gated on CD3-NK1.1$^+$ cells and (C) IFN-γ-producing NK cells gated on CD3$^-$NK1.1$^+$ cells. Representative photomicrographs of Oil red O-stained atherosclerotic lesions in the aortic sinus (D) and thoracic aorta (F) of male controls (n=8) and *Noé* chimeric mice (n=7), along with their corresponding quantifications (E and G, respectively) after 8 weeks HFD. **p < 0.01

Figure 4. Representative photomicrographs of Oil Red O-staining (A and B) and quantification of lesion size in left aortic sinus (C) after 8 weeks of HFD of male controls (A) and NK cell-deficient mice (B) treated with either α-Asialo-GM1 or control serum (n=10 per group). *p < 0.05, ***p < 0.001

Figure 5. (A) Representative quantitative analysis of flow cytometry-based staining of NK cells (gated on CD3-NK1.1$^+$), CD69$^+$ NK cells (gated on CD3$^-$NK1.1$^+$), IFN-□$^+$ NK cells (gated on CD3$^-$NK1.1$^+$), and CD107a$^+$ NK cells (gated on CD3$^-$NK1.1$^+$) in the three groups of mice: controls (WT → *Ldlr*$^{-/-}$) treated with saline (n=6) or poly(I:C) (n=11) and NK cell-deficient (*Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ → *Ldlr*$^{-/-}$) mice treated with poly(I:C) (n=13). (B) Quantification of perforin, granzyme B, and IFN-□ mRNA expression in the spleens and abdominal aorta of the 3 groups of chimeric mice. *p < 0.05, **p < 0.01, ***p < 0.001

Figure 6. Representative photomicrographs of Oil red O-staining (A) and quantification of lesion size in left aortic sinus (B) after 8 weeks of HFD in the three groups. **p < 0.01

Figure 7. (A) Photomicrographs of NK cells (anti-GFP$^+$) detected in spleen and aortic sinus of *Ncr-1*$^{gfp/gfp}$ → *Ldlr*$^{-/-}$ mice (n=3) at 8 weeks HFD, the dashed line marks the intima; L: lumen; A: adventitia. (B) Immunohistochemistry in human atherosclerotic plaques (n=17). Few NKp46$^+$ NK cells (brown) are detected in early fatty streak lesions (n=5), and none in advanced carotid artery plaques (n=12).

NOVELTY AND SIGNIFICANCE

What Is Known?

- Adaptive immunity contributes to atherosclerosis.
- The role of Natural Killer (NK) cells in atherosclerosis has been investigated in murine models in the past, but the results have been mixed.
- The previous mouse models are limited by a lack of selectivity with regard to deletion of NK cells.

What New Information Does This Article Contribute?

- Specific NK deletion or hyperresponsiveness does not affect atherosclerosis development in hypercholesterolemic mice.
- The absence of NK cells under simulated viral infection reduces atherosclerosis.

In general, previous studies suggested that NK cells play a proatherogenic role, however one recent study reported that NK cells are atheroprotective. These studies used loss of NK cell function in several mouse models, including Beige mutant mice, Ly49A transgenic mice, and antibody-mediated depletion (anti-asialo-GM1 antibody) in vivo approach. These models are limited by a lack of selectivity with regard to deletion of NK cells; they may involve loss of function for other immune cells, including neutrophils, macrophages or smooth muscles cells. In this study, we used two novel mouse models to investigate the role of NK cells in atherosclerosis: 1) *Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ model, where NK cells are specifically deleted; 2) the *Noé* mice in which NK cells are hyperresponsive. Our study provides novel evidence for the lack of a role for NK cells in atherosclerotic development in bone marrow transplantation studies in *Ldlr*$^{-/-}$ mice. However, data using the viral mimic Poly(I:C) demonstrates a significant reduction of plaque size in NK-cell deficient chimeric mice, supporting a proatherogenic role for NK cells when activated by inflammatory conditions.

Table: Quantitative analysis of the percentage of blood cells 5 days after starting treatment with anti-Asialo-GM1 and after 8 weeks HFD in controls (WT → *Ldlr*$^{-/-}$) and NK cell-deficient (*Ncr1*^{iCre}*R26*$^{lsl\text{-}DTA}$ → *Ldlr*$^{-/-}$) mice treated with either anti-Asialo-GM1 or control serum. (*p < 0.05; **p < 0.01; ***p < 0.001). Monocyte and neutrophil percentages were not assessed at 5 days post injection. N.D: not determined

	5-day HFD				8-week HFD			
	WT --> Ldlr$^{-/-}$		*Ncr1*^{iCre}*R26R*$^{lsl-DTA}$ --> Ldlr$^{-/-}$		WT --> Ldlr$^{-/-}$		*Ncr1*^{iCre}*R26R*$^{lsl-DTA}$ --> Ldlr$^{-/-}$	
	control Ab	a-asialo	control Ab	a-asialo	control Ab	a-asialo	control Ab	a-asialo
% NK	6.5±0.7	1.1±0.3***	N.D	N.D	3.6±0.5	0.8±0.2***	N.D	N.D
% NKT	0.7±0.1	0.3±0.05*	0.80±0.1	0.32±0.1**	0.4±0.07	0.2±0.04**	0.4±0.04	0.08±0.03***
% CD4	11.6±1.6	11.3±1.3	12±1.4	13.4±1.4	9.5±0.6	8.9±0.9	7.8±0.6	7.9±0.5
% CD8	6.1±0.4	3.4±0.2***	7.1±0.6	2.3±0.3***	4.5±0.3	3.1±0.3*	3.8±0.2	2.7±0.2*
% Neutrophils	-	-	-	-	16.3±4	12.6±2	18.8±3	14.1±1.7
% Monocytes	-	-	-	-	8.8±0.8	8.9±0.8	8.4±0.6	9.9±0.8

Figure 1 CIRCRES/2017/311743/R1

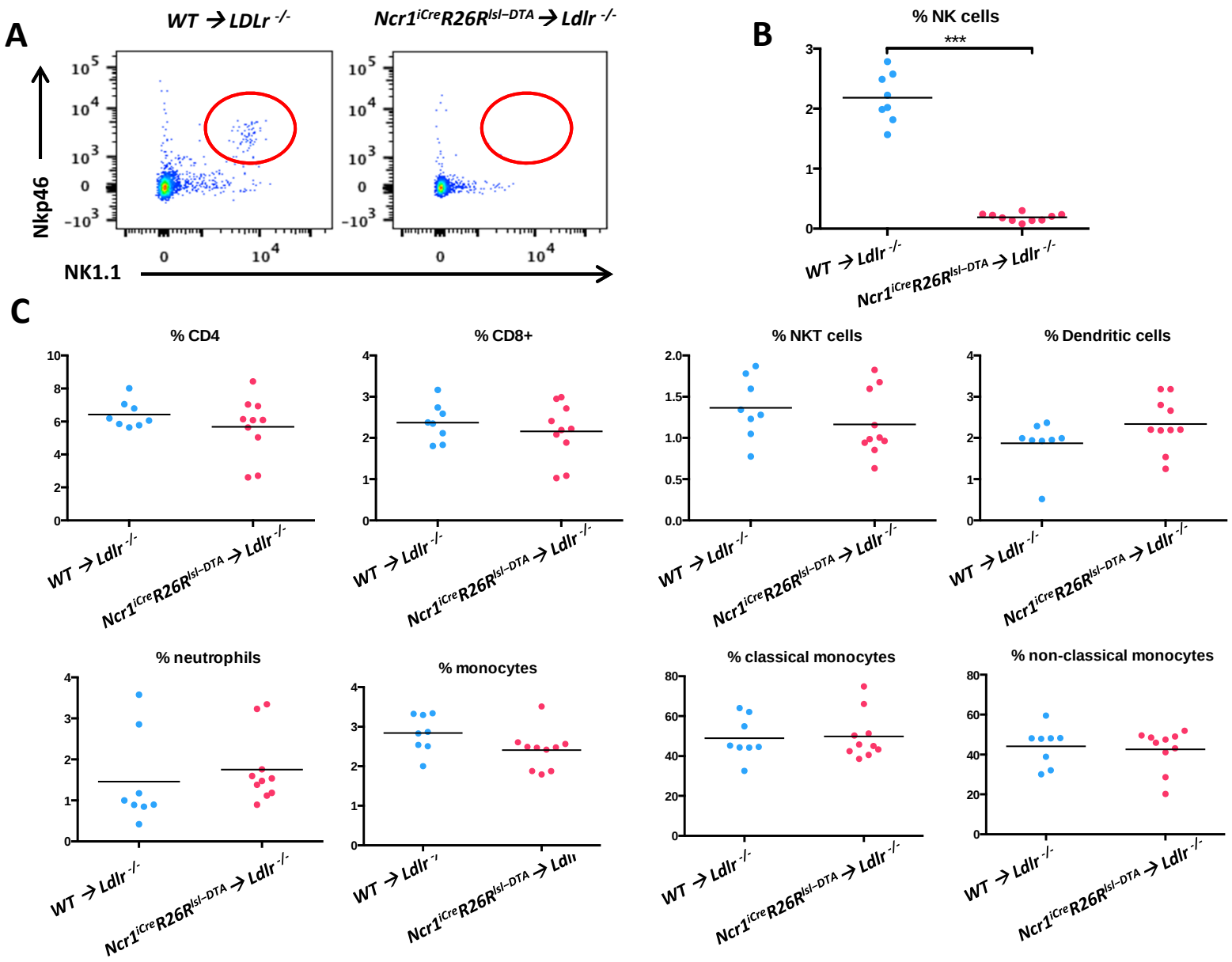
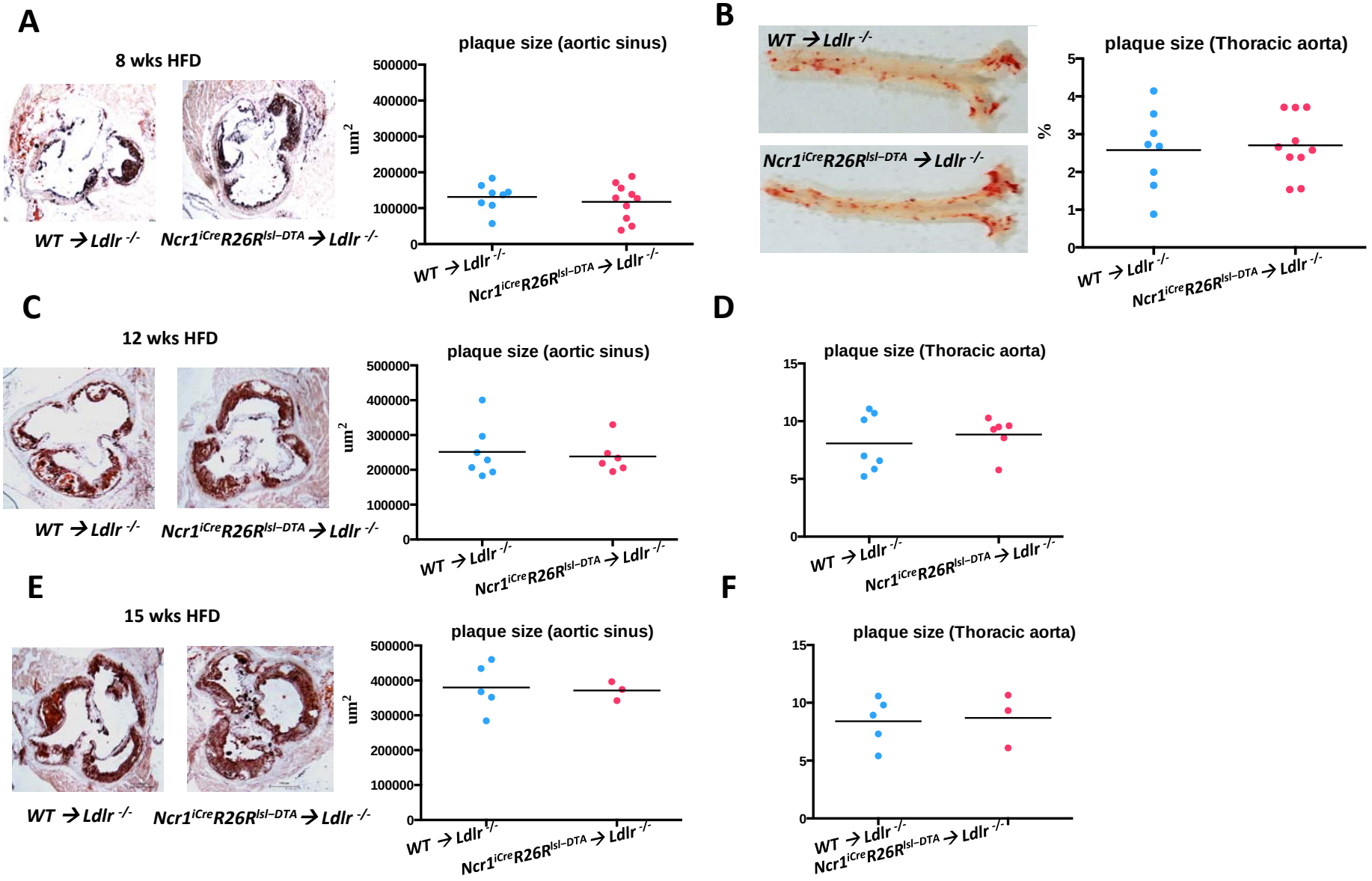


Figure 2 CIRCRES/2017/311743/R1



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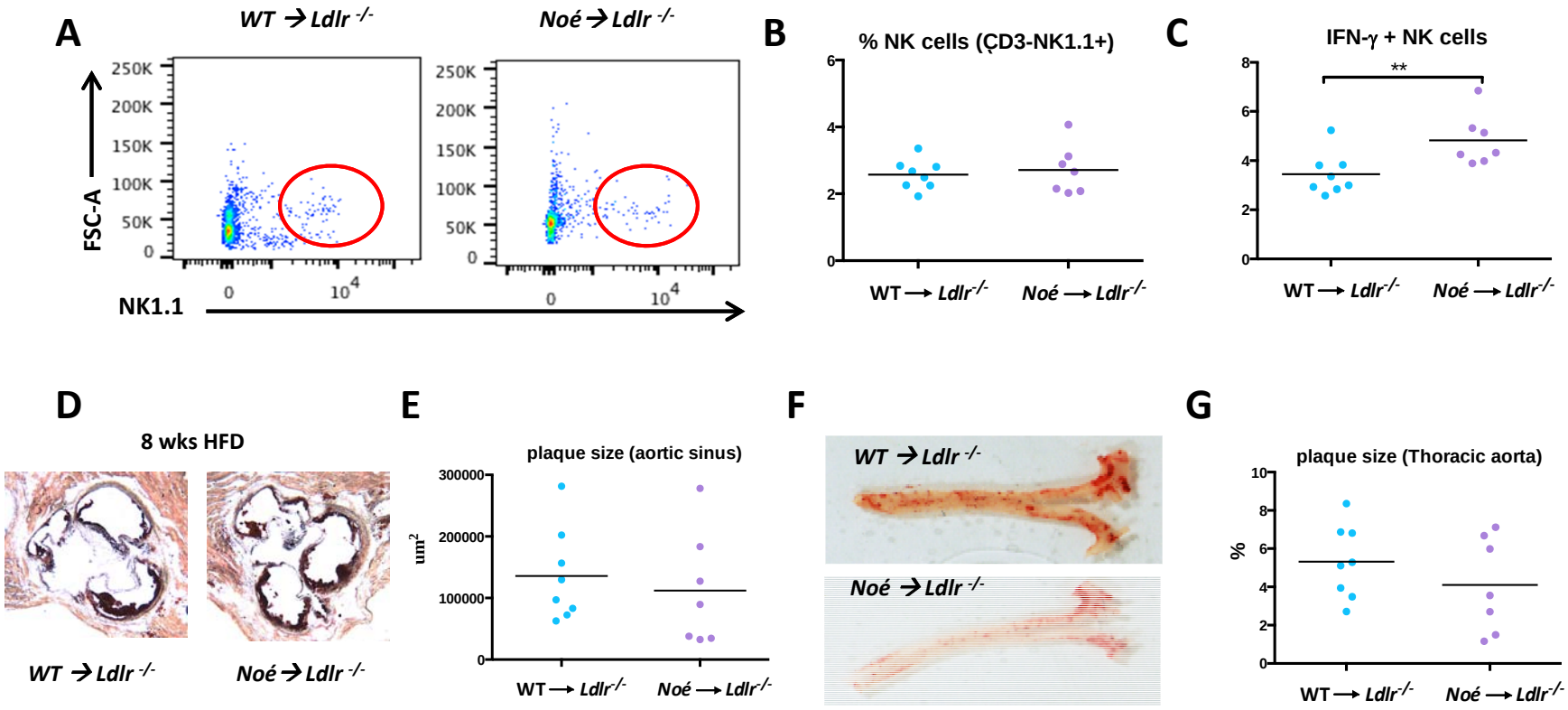

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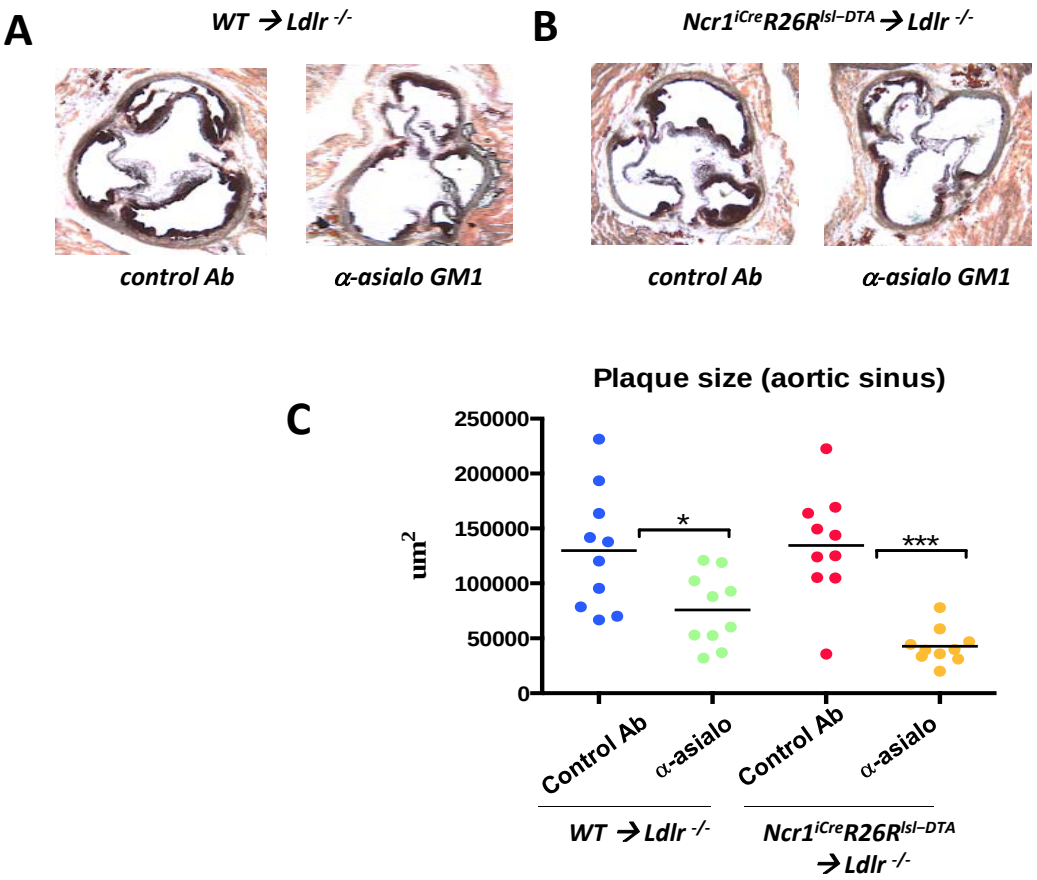


Figure 5 CIRCRES/2017/311743/R1

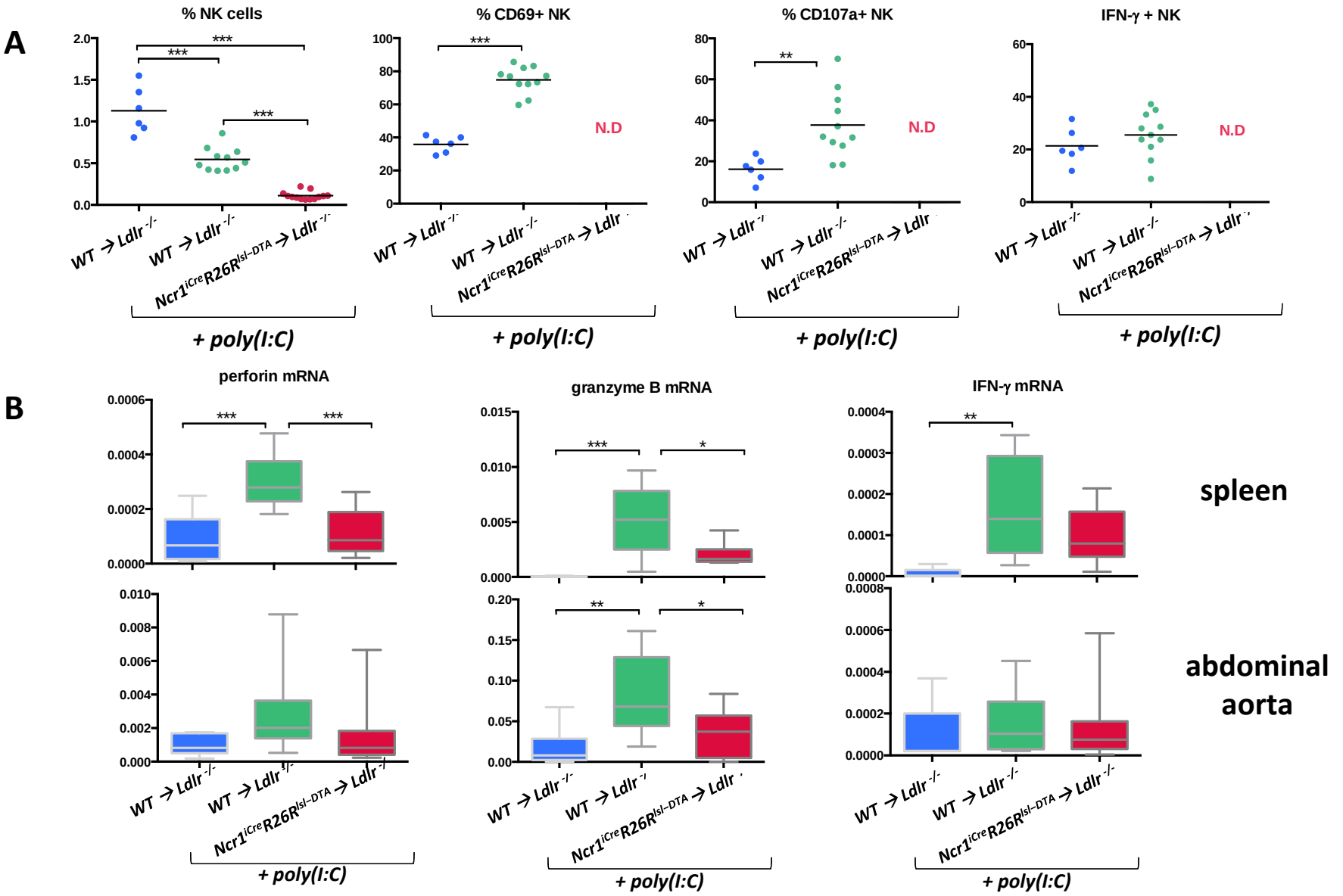
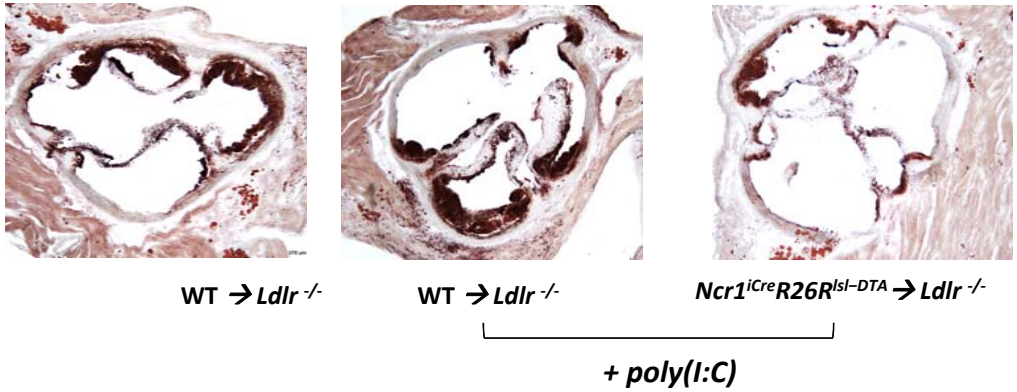


Figure 6 CIRCRES/2017/311743/R1

A



B

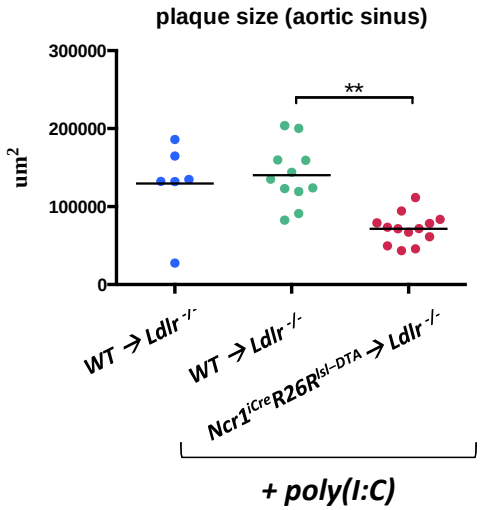


Figure 7 CIRCRES/2017/311743/R1