NR4A1 deletion in marginal zone B cells exacerbates atherosclerosis in mice

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**Short title:** B cells Nr4a1 in atherosclerosis

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Supplementary Figure I. Characterization of the partial irradiation model.
(A) Graph showing percentage of CD45.1\(^+\) MZB, FOB and Non-B cells (including FSC\(^{low}\); other lymphocytes and FSC\(^{hi}\): myeloid) cells in CD45.2 Ldlr\(^{-/-}\); Cd79a\(^{Cre/+}\); Rbpj\(^{flox/flox}\) mice injected with CD45.1 BM donor cells after partial irradiation with a single dose of 4 Gy. There were 3 experiments with 3 mice per group. (B) Total numbers of B2 cell subsets from males and females Ldlr\(^{-/-}\); Cd79a\(^{Cre/+}\); Rbpj\(^{flox/flox}\) mice partially irradiated and injected with WT (for reconstitution with WT MZB cells) or Nr4a1\(^{-/-}\) (for reconstitution with Nr4a1\(^{-/-}\) MZB cells) BM and fed a HF/HC diet for 8 weeks. Two-way ANOVA.
Supplementary Figure II. Representative immunofluorescence images and flow cytometry plots for immune cell subsets. Representative images of aortic root sections from mice used in this paper showing: (A) Necrotic core (n); (B) Mac3 staining (red) for macrophages in atherosclerotic plaques, and (C) CD3⁺ (green) and (D) Ly6G⁺ cells (red) in atherosclerotic plaques and in adventitia. Original magnification 10x (A) and 20x (B-D). Scale bar 50 μm. Representative plots and gating strategy for (E) B cell subsets: B cells (IgM⁺ B220⁺); FOB (IgM⁺ B220⁺ CD23⁺ CD21low); and MZB cells (IgM⁺ B220⁺ CD23® CD21hi); (F) GC B cells (CD19⁺ B220⁺ CD95hi SL7hi); (G) CD4 T cells (CD3⁺ CD4⁺); TEM (CD3⁺ CD4⁺ CD44hi CD62L); Tfh (CD3⁺ CD4⁺ CD44hi CXCR5⁺ PD1⁺); Tregs (CD3⁺ CD4⁺ CD44⁺ CD25⁺ Foxp3⁺ CXCR5⁻ PD1⁻); TfrCD25⁺ (CD3⁺ CD4⁺ CD44⁺ CD25⁺ Foxp3⁺ CXCR5⁻ PD1⁺); TfrCD25⁺ (CD3⁺ CD4⁺ CD44⁺ CD25⁺ Foxp3⁺ CXCR5⁻ PD1⁻); TfrCD25⁺ (CD3⁺ CD4⁺ CD44⁺ CD25⁺ Foxp3⁺ CXCR5⁻ PD1⁺); H) DC (CD11c⁺); neutrophils (Ly6G⁺); Monocytes (CD11c⁺ Ly6G⁻ Ly6C⁺/low).
Supplementary Figure III. Further characterization of atherosclerotic plaques and blood from Ldlr<sup>−/−</sup> reconstituted with WT or Nr4a1<sup>−/−</sup> B cells. Ldlr<sup>−/−</sup> male mice were transplanted with a mixed chimera containing 80% µMT + 20% WT (for reconstitution with WT B cells) or 20% Nr4a1<sup>−/−</sup> (for reconstitution with Nr4a1<sup>−/−</sup> B cells) BM and fed a HF/HC diet for 8 weeks (A-K). (A) Numbers of splenic MZB and FOB cells. (B) Quantification of Oil red O en face staining of aortic arches. Quantification of (C) necrotic core area, (D) Mac3 and (E) CD3 positive staining in atherosclerotic plaques of aortic roots. Quantification of (F) CD3 positive cells and (G) neutrophils (Ly6G positive cells) in adventitia of aortic root sections. For (C-G) 3 representative sections were analyzed for each mouse with n=6-8 per group. Percentage of blood (H) neutrophils, (I) B cells, (J) T cells and (K) monocytes. For (B-K) each symbol represents one mouse and horizontal bars are group mean ± s.e.m. Two-tailed unpaired Student t-test *p<0.05.
Supplementary Figure IV. Further splenic immunophenotyping of Ldlr<sup>–/–</sup> reconstituted with WT or Nr4a1<sup>–/–</sup> B cells. Ldlr<sup>–/–</sup> male mice were transplanted with a mixed chimera containing 80% μMT + 20% WT (for reconstitution with WT B cells) or 20% Nr4a1<sup>–/–</sup> (for reconstitution with Nr4a1<sup>–/–</sup> B cells) BM and fed a HF/HC diet for 8 weeks (A-H). Total numbers of splenic (A) CD11c positive (dendritic cells); (B) Ly6G<sup>+</sup> (neutrophils); (C-D) Ly6Ch<sub>high</sub> (monocytes); (E) T effector memory (TEM) cells; (F) T regulatory Foxp3<sup>+</sup> cells; (G) T follicular regulatory CD25<sup>+</sup> cells and (H) T follicular regulatory CD25<sup>+</sup> cells. Each symbol represents one mouse; horizontal bars are group mean ± s.e.m.. Two-tailed unpaired Student t-test.
Supplementary Figure V. Further characterization of atherosclerotic plaques of \( \text{LDLR}^{+/+} \), \( \text{Cd79a}^{\text{Cre}+/+} \), \( \text{Rbpjk}^{\text{floxt/floxt}} \) mice reconstituted with WT or \( \text{Nr4a1}^{-/-} \) MZB cells. \( \text{LDLR}^{+/+} \), \( \text{Cd79a}^{\text{Cre}+/+} \), \( \text{Rbpjk}^{\text{floxt/floxt}} \) male and female mice were partially irradiated and injected with WT (for reconstitution with WT MZB cells) or \( \text{Nr4a1}^{-/-} \) (for reconstitution with \( \text{Nr4a1}^{-/-} \) MZB cells) BM and fed a HF/HC diet for 8 weeks. (A) Quantification of Oil red O en face staining of aortic arches. Quantification of (B) Mac3 and (C) CD3 positive staining and (D) necrotic core area, in atherosclerotic plaques of aortic roots. Quantification of (E) CD3 positive cells and (F) neutrophils (Ly6G\(^{+}\) cells) in adventitia of aortic root sections. For (B-F) 3 representative sections were analyzed for each mouse with \( n=10-15 \) per group. Total plasma (G) IgM, (H) CuOxLDL specific IgM and (I) CuOxLDL specific IgG1 antibody titres. Each symbol represents one mouse and horizontal bars are group mean ± s.e.m. Two-tailed unpaired Student t-test *\( p<0.05 \).
Supplementary Figure VI. Further splenic immunophenotyping of Ldlr<sup>cre<sup>-<sup>/; Cd79a<sup>cre<sup>+/; Rbp<sub>flox/flox<sup> mice reconstituted with WT or Nr4a1<sup>−/−<sup> MZB cells. Ldlr<sup>−/−<sup>; Cd79a<sup>cre<sup>+/; Rbp<sub>flox/flox<sup> male and female mice were partially irradiated and injected with WT (for reconstitution with WT MZB cells) or Nr4a1<sup>−/−<sup> (for reconstitution with Nr4a1<sup>−/−<sup> MZB cells) BM and fed a consumption of a HF/HC diet for 8 weeks. Total numbers of splenic (A) T effector memory (TEM) cells; (B) T regulatory Foxp<sub>3<sup>+<sup> cells; (C) T follicular regulatory CD25<sup>+<sup> cells and (D) T follicular regulatory CD25<sup>+<sup> cells; (E) CD11c<sup>+<sup> (dendritic cells); (F) Ly6G<sup>+<sup> (neutrophils); (G-H) Ly6C<sub>hi</sub> (monocytes). Each symbol represents one mouse; horizontal bars are group mean ± s.e.m. Two-tailed unpaired Student t-test. *p<0.05.
Supplementary Figure VII. NR4A1 modulates PDL1 expression in MZB cells in a BCR-independent manner. MZB cells were sorted from WT or Nr4a1^{-/-} mice and were treated in vitro with anti-IgM for (A) 6 hours prior to assessment of PDL1 protein expression by flow cytometry, or for 3 hours prior to assessment of (B) Nr4a1 and (C) Cd274 expression by qRT-PCR, or (D) for 30 min prior to assessment of Btk phosphorylation (pBtk) by flow cytometry. (n=4-6 biological replicates per group). Horizontal bars are mean ± s.e.m. Two-tailed unpaired Student t-test and two-way ANOVA followed by Bonferroni’s post-hoc analysis. * p<0.05; **p<0.01 and ***p<0.001.
Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

| Species      | Vendor or Source | Background Strain | Sex | Persistent ID / URL                                                                 |
|--------------|------------------|-------------------|-----|-------------------------------------------------------------------------------------|
| Mice WT      | Charles River    | C57Bl/6J          | M-F | https://www.criver.com/products-services/find-model/c57bl6-mouse?region=3671        |

Genetically Modified Animals

| Parent - Male | Species | Vendor or Source | Background Strain | Other Information | Persistent ID / URL |
|---------------|---------|------------------|-------------------|-------------------|---------------------|
| LDLr/-/       | Jackson Labs | C57Bl/6J | MGI: 1857212      |
| LDLr/-/       | Jackson Labs | C57Bl/6J | MGI: 1857212      |
| RBPf/f        | T. Honjo  | C57Bl/6J         | MGI:3583755       |
| RBPf/f        | T. Honjo  | C57Bl/6J         | MGI:3583755       |
| CD79aCre/+    | Jackson Labs | C57Bl/6J | MGI:3687451       |
| CD79a+/+      | Jackson Labs | C57Bl/6J | MGI:3687451       |
| Nr4a1/-/      | Jackson Labs | C57Bl/6J | MGI:1352454       |
| Nr4a1/-/      | Jackson Labs | C57Bl/6J | MGI:1352454       |

Antibodies

| Target antigen | Vendor or Source | Catalog # | Working concentration | Lot # (prefered but not required) | Persistent ID / URL                                                                 |
|----------------|------------------|-----------|------------------------|-----------------------------------|-------------------------------------------------------------------------------------|
| B220           | eBioscience     | 10324-3   | 1ug/mL                 |                                   | https://www.biolegend.com/en-us/products.brilliant-violet-605-anti-mouse-human-b220-antibody-7870 |
| IgM            | eBio            | 48-5890-82| 1ug/mL                 |                                   | https://www.thermofisher.com/antibody/product/IgM-Antibody-clone-eB121-15F9-Monoclonal/48-5890-82 |
| MHC-II         | Biolegend       | 10762-0   | 1ug/mL                 |                                   | https://www.biolegend.com/en-us/products/pacific-blue-anti-mouse-i-a-i-e-antibody-3136 |
| CD23           | eBioscience     | 12-0232-82| 1ug/mL                 |                                   | https://www.thermofisher.com/antibody/product/CD23-Antibody-clone-B3B4-Monoclonal/12-0232-82 |
| CD21           | eBioscience     | 12341-8   | 1ug/mL                 |                                   | https://www.thermofisher.com/antibody/product/CD21-Antibody-clone-HB5-Monoclonal/11-0219-42 |
| Antibody | Vendor | Catalog Number | Concentration | Reference |
|----------|--------|----------------|---------------|-----------|
| CD69     | Biolegend | 104508 | 2ug/mL | https://www.biolegend.com/en-us/products/pe-anti-mouse-cd69-antibody-265 |
| CD44     | Biolegend | 103047 | 1ug/mL | https://www.biolegend.com/en-us/products/anti-human-cd44-antibody-8807 |
| CD4      | Biolegend | 100536 | 1ug/mL | https://www.biolegend.com/en-us/products/alexa-fluor-700-anti-mouse-cd4-antibody-3386 |
| CD3      | Biolegend | 100321 | 1ug/mL | https://www.biolegend.com/en-us/products/alexa-fluor-488-anti-mouse-cd3-antibody-2676 |
| CD62L    | Biolegend | 104424 | 1ug/mL | https://www.biolegend.com/en-us/products/pacific-blue-anti-mouse-cd62l-antibody-3117 |
| CD95     | BD     | 557653 | 2ug/mL | https://www.bdbiosciences.com/eu/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/pe-cy7-hamster-anti-mouse-cd95-jo2/p/557653 |
| GL7      | Biolegend | 144607 | 2ug/mL | https://www.biolegend.com/en-us/products/pe-anti-human-gl7-antigen-t-and-b-cell-activation-marker-antibody-9122 |
| CD19     | BD     | 553785 | 2ug/mL | https://www.bdbiosciences.com/eu/applications/research/stem-cell-research/hematopoietic-stem-cell-markers/mouse/negative-markers/fitc-anti-mouse-cd19-1d3/p/553785 |
| CD13     | Biolegend | 142505 | 2ug/mL | https://www.biolegend.com/en-us/products/apc-anti-mouse-cd138-syndecan-1-antibody-7572 |
| CXCR5    | Biolegend | 145503 | 2ug/mL | https://www.biolegend.com/en-us/products/pe-anti-mouse-cd185-cxcr5-antibody-8455 |
| ICOS     | eBioscience | 17-9949-82 | 2ug/mL | https://www.thermofisher.com/antibody/product/CD278-ICOS-Antibody-clone-C398-4A-Monoclonal/17-9949-82 |
| PD1      | Biolegend | 135241 | 2ug/mL | https://www.biolegend.com/en-us/products/brilliant-violet-510-anti-mouse-cd279-pd-1-antibody-14923 |
| pBtk     | eBioscience | 17-9015-42 | 8.3 ug/mL | https://www.thermofisher.com/antibody/product/Phospho-BTK-ITK-Tyr551-Tyr511-Antibody-clone-M4G3LN-Monoclonal/17-9015-42 |
| PDL1     | eBioscience | 46-5982-82 | 2ug/mL | https://www.thermofisher.com/antibody/product/CD274-PD-L1-B7-H1-Antibody-clone-MIH5-Monoclonal/46-5982-82 |

**DNA/cDNA Clones**

N/A

**Cultured Cells**

N/A

**Data & Code Availability**

N/A

**Other**

N/A