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Dendritic cell marker Clec4a4 deficiency limits atherosclerosis progression☆

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ABSTRACT

Background and aims: Atherogenesis results from altered lipid metabolism and impaired immune response. Emerging evidence has suggested that dendritic cells (DCs) participate to atherosclerosis-related immune response, but their impact is scarcely characterized. Clec4a4 or DCIR2 (Dendritic cell immunoreceptor 2) is a C-type lectin receptor, mainly expressed by CD8α^- DCs, able to modulate T cell immunity. However, whether this DC subset could play a role in the atherogenesis is still poorly understood. Thus, the aim of this study is to investigate whether the absence of Clec4a4 could affect atherosclerosis-related immune response and atherosclerosis itself.

Methods: *Dcir2*$^{-/-}$ *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were fed a standard diet or cholesterol-enriched diet for 12 weeks. Subsequently, the profile of circulating and lymph nodes-resident immune cells was investigated together with the analysis of plasma lipid levels and atherosclerotic plaque extension in the aorta.

Results: Here, we show that *Clec4a4* expression is downregulated under hypercholesterolemia and its deficiency in *Ldlr*$^{-/-}$ mice results in the reduction of atherosclerotic plaque formation, together with altered lipid metabolism and impaired myeloid immune cell distribution.

Conclusions: Our findings suggest a pro-atherosclerotic role of Clec4a4 in experimental atherosclerosis.

Clec4a4 is one of the prototypic dendritic cell (DC) immunoreceptor (DCIR) that belongs to the C-type lectin receptors (CTLRs), a superfamily of proteins able to bind carbohydrate moieties in a Ca^{2+}-dependent manner [1]. While in mouse DCIR family consists of four members, only one receptor has been identified in humans [2]. The peculiarity of DCIR family is the presence of an immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmatic tail that counterbalances the response of other activating receptors, such as Toll-like receptors, thus modulating the activation of immune cells [3,4]. It should be noted that, despite its name, DCIR family members are expressed not only by dendritic cells but also by other myeloid cells including macrophages, monocytes and granulocytes [5]. Nevertheless, DCIR2 (or Clec4a4) is considered the marker for CD8$^-$ DCs [6], a subset of type 2 conventional dendritic cells (cDC2); these, together with cDC1, are responsible of the activation of CD4$^+$ Th cells or CD8$^+$ cytotoxic T cells respectively [7].

Recently, DCIR2 has been proven to suppress autoimmunity by downregulating T cell priming [6] thus suggesting a strong contribution of the receptor in the modulation of T cell response exerted by DCs [8]. In addition to this, DCIR2$^+$ DCs ameliorate diseases with a strong immune inflammatory component, such as experimental autoimmune encephalomyelitis (EAE) [9], experimental melanoma [10] and diabetes [11], and more recently another DCIR member, DCIR1 (or Clec4a2), has been shown to protect against atherosclerosis by maintaining macrophage homeostasis [12]. Despite DCs play a role during atherogenesis development [13,14], the contribution of different DC subsets under hypercholesterolemic conditions is more controversial [15]. Therefore, as DCIR2 represents a specific DC subset, we investigated whether the absence of this receptor could affect atherosclerosis-related immunoinflammatory response and, accordingly, atherosclerosis itself.

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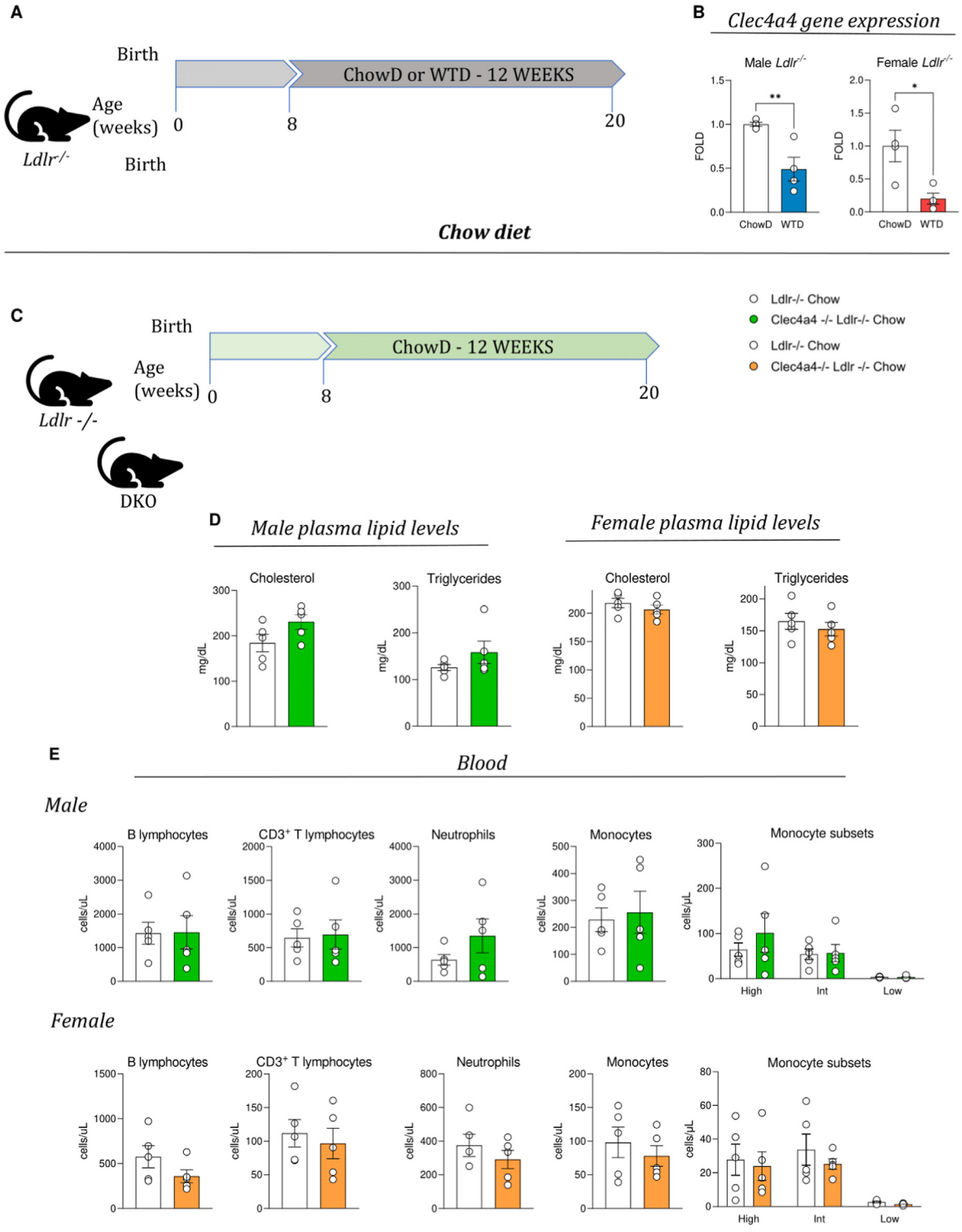

Fig. 1. Role of *Clec4a4* under dyslipidemic conditions.
(A) Experimental timeline: 8 weeks-old *Ldlr*$^{-/-}$ mice were fed for 12weeks with standard diet (ChowD) or Western-type diet (WTD). (B) Gene expression of *Clec4a4* in the spleen of *Ldlr*$^{-/-}$ male and female under ChowD or WTD. n = 4 mice per group. (C) Experimental timeline: 8 weeks-old male and female *Ldlr*$^{-/-}$ mice or *Clec4a4*$^{-/-}$ *Ldlr*$^{-/-}$ (DKO) mice were fed for 12weeks with ChowD. (D) Plasma cholesterol and triglycerides levels of male and female mice 12weeks-fed ChowD expressed as mg/dL. (E) Panels reporting immune populations detected by flow cytometry in *Ldlr*$^{-/-}$ and DKO after 12weeks of standard diet. Respectively, bar graphs referring to total number of circulating lymphoid (in order B and T lymphocytes) and myeloid leukocytes (in order neutrophils, monocytes and monocyte subsets discriminated based on the expression of Ly6C marker) of male and female mice, normalized on µL of blood. Data are presented as mean ± SEM. n = 5 mice per group. Statistical analyses were performed with *unpaired t-test*. *p < 0,05; **p < 0,01.

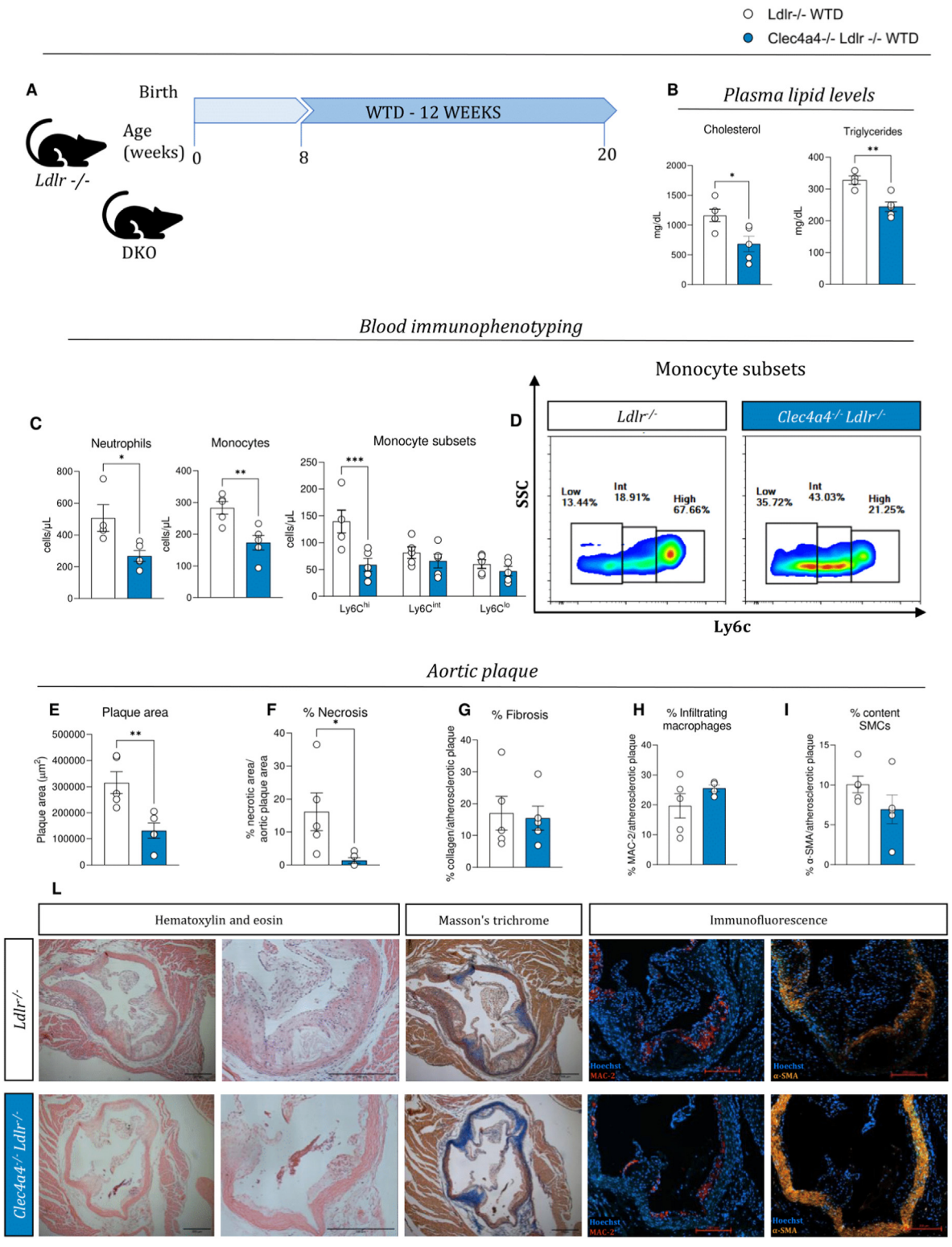

Fig. 2. Role of Clec4a4 deficiency in atherosclerotic *Lldr*$^{-/-}$ mice.
(A) Experimental timeline: 8 weeks-old male and female *Ldlr*$^{-/-}$ mice or *Clec4a4*$^{-/-}$ *Ldlr*$^{-/-}$ (DKO) mice were fed for 12 weeks with WTD. (B) Plasma cholesterol and triglycerides levels of male mice 12 week-fed WTD expressed as mg/dL. (C) Panels reporting total number of circulating myeloid leukocytes analysed by flow cytometry in *Ldlr*$^{-/-}$ and DKO after 12 weeks of WTD normalized on µL of blood. (D) Representative images of monocytes subsets discriminated based on the expression of Ly6C marker. (E–G) Quantification of total lesion area, percentage of necrotic core and of collagen content in the atherosclerotic plaque at the aortic sinus. (H–I) Percentage of macrophage and smooth muscle cells content within aortic plaque. (L) Representative images of aortic sections of *Ldlr*$^{-/-}$ (upper row) and DKO male mice (lower row) are presented: 5x and 10x images of hematoxylin and eosin-stained sections (first and second column), 5x images of Masson's trichrome stained sections (third column), 5x of Mac-2 and α-SMA immunofluorescence staining (last two columns). Results are expressed as mean ± SEM. n = 5 mice per group. Statistical analyses were performed with *unpaired t-test.**p < 0,05; **p < 0,01; ***p < 0,001.

To this aim, we first tested the effects of hyperlipidaemia on Clec4a4 expression in *Ldlr*$^{-/-}$ mice fed cholesterol-enriched diet (WTD) compared to fed standard (ChowD) diet. Notably, after 12 weeks of WTD feeding (Fig. 1A), a significant reduction of splenic *Clec4a4* expression compared to the standard-fed counterparts (Fig. 1B) was observed in both male and female *Ldlr*$^{-/-}$ mice. This prompted us to test whether Clec4a4 could be causally involved in atherogenesis. To this aim, we generated double knock-out (DKO) mice lacking *Clec4a4* on *Ldlr*$^{-/-}$ background.

After ChowD feeding for 12 weeks, *Ldlr*$^{-/-}$ and DKO mice presented a similar plasma lipid profile (Fig. 1C and D) and no differences in circulating immune profile in both male and female (Fig. 1E) were observed.

We next investigated the implication of *Clec4a4* deletion under hypercholesterolaemic conditions with a focus on male mice. *Ldlr*$^{-/-}$ and DKO mice were fed for 12 weeks with cholesterol enriched diet (WTD) (Fig. 2A) to induce hyperlipidemia and atherosclerosis. DKO mice presented decreased cholesterol and triglyceride plasma levels (Fig. 2B and Suppl.1) that was associated with a decreased circulating level of neutrophils and inflammatory monocytes (Ly6C^{high}) (Fig. 2C and D). The reduction of myeloid subsets was not the result of increased leukocyte extravasation. Indeed, the gene expression analysis in the aorta of *Ccr1 and Cx3cr1* - chemokine receptors critical for immune cell recruitment particularly within the aorta [16] - highlighted a decreased expression of these receptors in DKO mice compared to the control counterpart (Suppl.3A). Intriguingly, this effect appeared to be specific for these two subsets, as no differences were observed in the phenotype of DCs and T lymphocytes both analysed in blood (Suppl.3B) and T lymphocytes within mediastinal lymph nodes (i.e. those draining immune cells by aortic arch) (Suppl.3C) thus limiting the impact of Clec4a4 on adaptive immune response [6]. Of note, care must be taken regarding the activation state of these immune cells since diet-induced metabolic switch could result in their different activation and function [17–19]. For this reason, the possibility that the absence of Clec4a4 also affects the metabolic reprogramming of dendritic cells and, accordingly, T lymphocytes cannot be excluded.

Next, we investigated whether this phenotype could mirror significant changes in atherosclerosis progression and indeed we observed a significant reduction of the plaque area at the aortic sinus (Fig. 2E) and along the first 300 μm of the aorta (suppl.4A,B) in DKO compared to *Ldlr*$^{-/-}$ mice. These changes were paralleled by a decreased necrotic core content (Fig. 2F). Notably, no major differences in fibrosis, smooth muscle cells and infiltrated macrophages content were observed (Fig. 2G–I) in DKO compared to *Ldlr*$^{-/-}$ mice. DKO female mice presented a similar trend in atherosclerotic plaque reduction (Suppl.5B,C), in spite of no differences in plasma cholesterol levels (Suppl.5E) and in immune cell profile compared to *Ldlr*$^{-/-}$ mice (Suppl.6A-C). The discrepancies between female and male plasma lipid profile could result from sexual hormones-related differences of lipid metabolism. Indeed, although the gene expression analysis on DKO male liver did not show differences in genes related to lipid metabolism as compared to those of *Ldlr*$^{-/-}$, except for the decreased expression of the scavenger receptor CD36 (Suppl.7A), the same was not true for female one. The higher expression of the rate-limiting enzyme of the mevalonate pathway – *HMG-CoAr* – as well as of *SREBP1* and *FASN* (Suppl.7B)- which together sustain the synthesis of fatty acids [20] - in DKO female mice were in line with the increased trend of plasma lipids levels observed in female DKO compared the control counterpart (Suppl.5E).

While these data point to an atheroprotective effect of *Clec4a4* deficiency in atherosclerosis, the underlying molecular mechanism remains elusive. The observation of increased levels of Granulocyte-Monocyte Progenitors (GMP) - the precursors of both neutrophils and monocytes – within the bone marrow (Suppl.8E) – suggests that Clec4a4^{+} DCs might impact progenitors' mobilization from the bone marrow under hypercholesterolaemic conditions. This is in line with the observations regarding the role of DCs in the modulation of hematopoietic stem cell and progenitor cell trafficking [21].

In conclusion, these data strongly indicate that *Clec4a4* deficiency under hypercholesterolemic condition translates into decreased atherosclerosis development mainly impacting innate immune cells response.

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Authors' contribution

Conceived and designed the experiments: FB, GDN. Provided experimental models: YR, ON. Performed the experiments: RB, AM, JN, PU. Analysed the data: RB, FB. Data interpretation and discussion: RB, FB, GDN, AM. Wrote the paper: RB, FB, GDN. Revised the manuscript: FB, GDN, AM, YR.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2022.12.001.

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