The Dendritic Cell Receptor DNGR-1 Promotes the Development of Atherosclerosis in Mice

Yacine Haddad, Charlotte Lahoute, Marc Clément, Ludivine Laurans, Sarvenaz Metghalchi, Lynda Zeboudj, Andreas Giraud, Xavier Loyer, Marie Vandestienne, Julien Wain-Hobson, Bruno Esposito, Stéphane Potteaux, Hafid Ait-Oufella, Alain Tedgui, Ziad Mallat, Soraya Taleb

**Rationale:** Necrotic core formation during the development of atherosclerosis is associated with a chronic inflammatory response and promotes accelerated plaque development and instability. However, the molecular links between necrosis and the development of atherosclerosis are not completely understood. Clec9a (C-type lectin receptor) or DNGR-1 (dendritic cell NK lectin group receptor-1) is preferentially expressed by the CD8α subset of dendritic cells (CD8α+ DCs) and is involved in sensing necrotic cells. We hypothesized that sensing of necrotic cells by DNGR-1 plays a determinant role in the inflammatory response of atherosclerosis.

**Objective:** We sought to address the impact of total, bone marrow–restricted, or CD8α+ DC–restricted deletion of DNGR-1 on atherosclerosis development.

**Methods and Results:** We show that total absence of DNGR-1 in Apoe (apolipoprotein e)–deficient mice (Apoe<sup>−/−</sup>) and bone marrow–restricted deletion of DNGR-1 in Ldlr (low-density lipoprotein receptor)–deficient mice (Ldlr<sup>−/−</sup>) significantly reduce inflammatory cell content within arterial plaques and limit atherosclerosis development in a context of moderate hypercholesterolemia. This is associated with a significant increase of the expression of interleukin-10 (IL-10). The atheroprotective effect of DNGR-1 deletion is completely abrogated in the absence of bone marrow–derived IL-10. Furthermore, a specific deletion of DNGR-1 in CD8α+ DCs significantly increases IL-10 expression, reduces macrophage and T-cell contents within the lesions, and limits the development of atherosclerosis.

**Conclusions:** Our results unravel a new role of DNGR-1 in regulating vascular inflammation and atherosclerosis and potentially identify a new target for disease modulation. (Circ Res. 2017;121:234-243. DOI: 10.1161/CIRCRESAHA.117.310960.)

**Key words:** atherosclerosis ■ dendritic cell ■ DNGR-1 ■ inflammation ■ interleukin-10 ■ necrosis

Accumulation of apoptotic cells and necrotic debris within the lipid core is a major feature of advanced atherosclerotic lesions and has been associated with increased susceptibility to thrombotic complications after plaque rupture. We and others have shown that accumulation of apoptotic and secondary necrotic debris in atherosclerotic lesions results from defective efferocytosis or clearance of apoptotic cells. This in turn leads to activation of immune-inflammatory responses and accelerates the progression of atherosclerosis. Mouse models of decreased efferocytosis, such as mice lacking the receptor MERTK (myeloid-epithelial-reproductive tyrosine kinase) or the bridging molecule MFGE8 (milk fat globule EGF-like factor 8) showed an alteration of the protective immune response, associated with increased vascular inflammation and enhanced necrotic core formation. However, the mechanisms involved in necrosis-induced inflammation during atherosclerosis development are not fully understood.

In This Issue, see p 197

Dying cells release endogenous molecules known as damage-associated molecular patterns that are recognized by pattern recognition receptors in immune cells. TLRs (toll-like receptors) and CLRs (C-type lectin receptors) are 2 main pattern recognition receptor families expressed on immune cells, including macrophages and dendritic cells (DCs). DNGR-1 (dendritic cell NK lectin group receptor-1), also known as CLEC9a, is a type II transmembrane CLR containing a single extracellular C-type lectin-like domain and a cytoplasmic tail with a semi-immunoreceptor tyrosine-based activation motif that allows recruitment/activation of the SYK (spleen tyrosine

© 2017 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.117.310960
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLEC4e</td>
<td>C-type lectin receptor 4e</td>
</tr>
<tr>
<td>CLRs</td>
<td>C-type lectin receptors</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNGR-1</td>
<td>dendritic cell NK lectin group receptor-1</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HFD</td>
<td>high-fat diet</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin-10</td>
</tr>
<tr>
<td>MERTK</td>
<td>myeloid-epithelial-reproductive tyrosine kinase</td>
</tr>
<tr>
<td>MFGE8</td>
<td>milk fat globule EGF-like factor 8</td>
</tr>
<tr>
<td>SYK</td>
<td>spleen tyrosine kinase</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
</tbody>
</table>

Novelty and Significance

What Is Known?

- Advanced atherosclerotic plaques are characterized by an increase in necrotic cells.
- A necrotic core increases plaque instability.
- The DNGR-1 (dendritic cell NK lectin group receptor-1) is involved in sensing necrotic cells.

What New Information Does This Article Contribute?

- The DNGR-1, specifically expressed by a subtype of dendritic cells (CD8α+ DCs), has proinflammatory and proatherogenic effects that depend on interleukin-10.
- A new role of DNGR-1 in regulating vascular inflammation and atherosclerosis, making it a potential target that could be blocked to promote plaque stabilization.

Accumulation of necrotic debris increases inflammation in atherosclerotic lesions and has been associated with higher susceptibility to plaque rupture. However, the mechanisms that contribute to necrosis-induced inflammation during atherosclerosis remain unclear. DNGR-1 is a sensor of necrotic cells that is preferentially expressed in a subtype of dendritic cells (CD8α+ DCs). We have shown that in a context of moderate hypercholesterolemia the total, bone marrow and CD8α+ DC-specific DNGR-1 deletion decreased the infiltration of T lymphocytes and macrophages within lesions and plaque size. Mechanistically, the absence of DNGR-1 led to increased expression of IL-10 in CD8α+ DCs, which also appeared to impact the production of interleukin-10 by CD4+ cells. Moreover, deletion of IL-10 in CD8α+ DCs abolished the atheroprotective effects of DNGR-1 deficiency. Further studies are required to determine whether increased DNGR-1 is associated with the formation of thin-cap fibroatheromatous lesions in humans and whether blockage of this protein would promote plaque stabilization.

kinase) pathway. DNGR-1 is mostly expressed by the CD8α+ subset of DCs (CD8α+ DCs) as a receptor for necrotic cells, which favors cross-priming of cytotoxic T lymphocytes to dead cell–associated antigens in mice. Although this receptor is not essential for particle uptake via phagocytosis, DNGR-1 is not essential for particle uptake via phagocytosis,11 DNGR-1, leading to disease progression. Here, we show that DNGR-1 expression is abrogated by the insertion of a gene encoding a membrane-anchored form of GFP (green fluorescent protein) into the Clec9a open-reading frame.11 CD8α+ DC-deficient (Cd11cCre+/Irf8flox/flox) mice were obtained by crossing Irf8flox/flox (Jax) with Cd11cCre+ (Jax). Mfge8−/− mice were previously described. Apoe−/−Clec9a−/−, Clec9a−/−Il10−/−, and Clec9a−/−Mfge8−/− mice were generated by crossing, respectively, Apoe−/− to Clec9a−/−, Il10−/− to Clec9a−/−, and Mfge8−/− to Clec9a−/− mice. Apoe−/− and Apoe−/−Cl ec9a−/− male mice on either chow diet (20 weeks) or high-fat diet (HFD; 6 weeks) were used in some experiments. We subjected 7- to 10-week-old Ldlr−/− male mice to medullar aplasia by 9.5 gray lethal total body irradiation. We repopulated the mice with an intravenous injection of bone marrow cells isolated from femurs and tibias of age- and sex-matched C57Bl6d mice: Clec9a−/−, Clec9a−/−Il10−/− or Clec9a−/−Mfge8−/− or Clec9a−/−Mfge8−/− mice. After 4 weeks of recovery, mice were fed with a proatherogenic diet containing 15% fat, 1.25% cholesterol, and 0% cholate for 5, 7, or 13 weeks.

Finally, we irradiated Ldlr−/− mice and transplanted them with a 2:8 mixture of either control (Cd45.1) bone marrow or Clec9a−/− bone marrow mixed with bone marrow from CD8α+ DC-deficient (Cd11cCre+/Irf8flox/flox) mice. After 4 weeks of reconstitution, the mice were put on HFD diet for 7 weeks.

All mice used in these experiments were bred and housed in a specific pathogen-free barrier facility. Experiments were conducted according to the French veterinary guidelines and those formulated by the European community for experimental animal use (L358-86/609ECC).

Extent and Plaque Composition of Atherosclerotic Lesions

Quantification of lesion size and composition was performed as previously described.15

Flow Cytometry

Flow cytometric analysis was performed on single cell suspensions of spleen and peritoneal cells as mentioned before.16

Cell Recovery and Stimulation

Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mmol/L 2β-mercaptoethanol, and antibiotics. Splenocytes were stimulated with lipopolysaccharide (1 µg/mL; Sigma) for 48 hours, and then, supernatants were subjected to cytokine measurements. Spleen DCs, CD8α+ DCs, CD8α+ DCs, CD3+, and CD4+ T cells were purified using Miltenyi kits. To analyze for any new information, we focused on the following:

- Advanced atherosclerotic plaques are characterized by an increase in necrotic cells.
- A necrotic core increases plaque instability.
- The DNGR-1 (dendritic cell NK lectin group receptor-1) is involved in sensing necrotic cells.

Methods

Detailed methods can be found in the Online Data Supplement.

Animals

Il10−/−, Apoe−/−, and Ldlr−/− mice were from the Jackson Laboratory. C57BL/6 Clec9a−/− mice are from Reis e Sousa laboratory.11 Clec9a−/− mice were backcrossed on C57Bl6 for >10 generations. Littermates C57BL/6 Clec9a−/− and C57BL/6 Clec9α−/+ were used in all the experiments. Briefly, these mice were Clec9a−/+ and in which DNGR-1 expression is abrogated by the insertion of a gene encoding a membrane-anchored form of GFP (green fluorescent protein) into the Clec9a open-reading frame.11 CD8α+ DC-deficient (Cd11cCre+/Irf8flox/flox) mice were obtained by crossing Irf8flox/flox (Jax) with Cd11cCre+ (Jax). Mfge8−/− mice were previously described.14 Apoe−/−Clec9a−/−, Clec9a−/−Il10−/−, and Clec9a−/−Mfge8−/− mice were generated by crossing, respectively, Apoe−/− to Clec9a−/−, Il10−/− to Clec9a−/−, and Mfge8−/− to Clec9a−/− mice. Apoe−/− and Apoe−/−Cl ec9a−/− male mice on either chow diet (20 weeks) or high-fat diet (HFD; 6 weeks) were used in some experiments. We subjected 7- to 10-week-old Ldlr−/− male mice to medullar aplasia by 9.5 gray lethal total body irradiation. We repopulated the mice with an intravenous injection of bone marrow cells isolated from femurs and tibias of age- and sex-matched C57Bl6d mice: Clec9a−/−, Clec9a−/−Il10−/− or Clec9a−/−Mfge8−/− or Clec9a−/−Mfge8−/− mice. After 4 weeks of recovery, mice were fed with a proatherogenic diet containing 15% fat, 1.25% cholesterol, and 0% cholate for 5, 7, or 13 weeks.

Finally, we irradiated Ldlr−/− mice and transplanted them with a 2:8 mixture of either control (Cd45.1) bone marrow or Clec9a−/− bone marrow mixed with bone marrow from CD8α+ DC-deficient (Cd11cCre+/Irf8flox/flox) mice. After 4 weeks of reconstitution, the mice were put on HFD diet for 7 weeks.

All mice used in these experiments were bred and housed in a specific pathogen-free barrier facility. Experiments were conducted according to the French veterinary guidelines and those formulated by the European community for experimental animal use (L358-86/609ECC).
cytokine production by T cell, 10^5 CD3^+ or CD4^+ T cells were cultured alone or cocultured with 10^5 CD8^+ DCs in 96-well microwells precoated with anti-CD3 antibody (5 μg/mL; Pharmingen) and anti-CD28 antibody (1 μg/mL; Pharmingen) during 48 hours in the presence of lipopolysaccharide (1 μg/mL). Neutralizing anti–IL-10 receptor antibody or control IgG (R&D Systems) and water-soluble cyclodextrin-cholesterol (Sigma) (1 μL/well) were performed to measure IL-10, IL-17, tumor necrosis factor-α, IL-6, and interferon-γ (BD) of the different conditions.

**Statistical Analysis**

Values are expressed as means±SEM. Differences between values were examined using Student t test. Mann-Whitney test is applied in the animal studies. Values were considered significant at P≤0.05.

**Results**

**DNGR-1 Deficiency Decreases the Development of Atherosclerosis**

To study the role of DNGR-1 in atherosclerosis, we crossed Clec9a−/− mice with atherosclerosis-susceptible Apoe−/− mice and generated Apoe−/−Clec9a−/− mice and Apoe−/−Clec9a+/+ littermate controls. Both groups were put on chow diet until euthanasia at 20 weeks of age. As shown in Figure 1, validation of DNGR-1 led to a marked 60% decrease of plaque size (P=0.002), despite no significant change in plasma cholesterol levels (Figure 1A through 1C). We also reconstituted lethally irradiated Ldlr−/− mice with Clec9a+/+ or Clec9a−/− bone marrow. After 4 weeks of recovery, mice were put on HFD for 5 or 7 weeks to study early disease stages and for 13 weeks to study advanced stages of atherosclerosis. As expected, plasma cholesterol levels significantly increased over time during HFD, without any significant differences between mice transplanted with Clec9a+/− or Clec9a+/+ bone marrow cells (Online Figure 1A). Mice reconstituted with Clec9a+/− bone marrow showed significant reduction in atherosclerosis at the aortic sinus (Figure 1D and 1E) compared with mice reconstituted with Clec9a+/+ bone marrow after 5 or 7 weeks of HFD, confirming the atheroprotective effect of DNGR-1 deletion. Plaque area was also significantly decreased in the thoracic aorta after 7 weeks of HFD in mice reconstituted with Clec9a−/− compared with Clec9a−/− bone marrow (Figure 1F).

This was a surprising finding given that necrotic cores, which may activate signaling by DNGR-1, show increased accumulation in lesions at the advanced stages of atherosclerosis (Online Figure ID). To account for that finding, we hypothesized that the absence of DNGR-1 proatherogenic effects after 13 weeks of HFD in Ldlr−/− mice or after HFD in Apoe−/− mice was because of the high plasma cholesterol levels observed in these mice, rather than of the advanced stage of the plaques.

We then examined Clec9a mRNA in the spleen, a tissue known to readily express Clec9a17 and to play a major role in atherosclerosis.18,19 As shown in Online Figure IIA, Clec9a chimerism was high at all time points (7 or 13 weeks), as assessed by the almost total absence of Clec9a expression in

![Figure 1. DNGR-1 (dendritic cell NK lectin group receptor-1) deletion in bone marrow protects against atherosclerosis.](a.png)

A. Plasma cholesterol levels in 20-wk-old male Apoe−/− (n=10) and male Apoe−/−Clec9a+/− (n=13) put on chow diet during 20 wk. B and C. Representative photomicrographs and quantification of lesion size in aortic sinus of male Apoe−/− (n=10) and male Apoe−/−Clec9a−/− mice (n=13). D and E. Representative photomicrographs and quantification of lesion size in Ldr−/− mice reconstituted with either control (Clec9a−/−→Ldr−/−) or Clec9a−/− (Clec9a−/−→Ldr−/−) bone marrow after 5 wk (control [n=7] and Clec9a−/− [n=8]) or 7 wk (control [n=12] and Clec9a−/− [n=16]) or 13 wk (control [n=8] and Clec9a−/− [n=9]) of high-fat diet (HFD). F. Representative photomicrographs and quantification of lesion size in the thoracic aorta reconstituted with either control (Clec9a−/−→Ldr−/− [n=12]) or Clec9a−/− (Clec9a−/−→Ldr−/− [n=16]) bone marrow after 7 wk of HFD. Mean values±SEM are shown. *P<0.05, **P<0.001, ***P<0.0001.
spleens of mice reconstituted with DNGR-1–deficient bone marrow. However, Cleca9 expression in spleens of Ldlr−/− mice reconstituted with Cleca9+/+ bone marrow markedly decreased after 13 weeks of HFD, compared with that of Ldlr−/− mice on chow diet or after 7 weeks of HFD (Online Figure IIA). This may suggest that Cleca9 expression was downregulated by the prolonged and severe hypercholesterolemia after 13 weeks of HFD. Consistent with this hypothesis, in vitro incubation of splenocytes from Cleca9Gfp/Gfp mice, in which the Cleca9 gene was replaced by a GFP reporter gene (see Materials and Methods) in the presence of cholesterol markedly decreased GFP expression (Online Figure IIB).

To investigate the role of Cleca9 in advanced atherosclerosis while avoiding severe and prolonged hypercholesterolemia, we generated Cleca9−/−Mfge8−/− mice by crossing Mfge8−/− mice, in which the accumulation of late apoptotic/necrotic cells is increased because of defective efferocytosis,20,21 with Cleca9−/− mice. Then, lethally irradiated male Ldlr−/− mice were reconstituted with either Mfge8−/− or Cleca9−/−Mfge8−/− bone marrow cells and put on HFD for 7 weeks. As expected, a small apoptotic area as assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining was detected in plaques of Ldlr−/− mice reconstituted with Cleca9+/+ or Cleca9−/− bone marrow at this early stage of disease development (Online Figure IIIA and IIIB). As previously published,9 Mfge8 deficiency markedly increased apoptotic TUNEL+ area (Online Figure IIIA and IIIB) and accelerated lesion development (Online Figure IIIC and IIID) under moderate hypercholesterolemia (5.01±0.66 versus 5.86±0.45 g/L; *P=0.33, in wild-type and Mfge8−/− mice, respectively). No significant changes of necrotic core area was observed between Cleca9−/−Mfge8−/− and Mfge8−/− groups (data not shown). Interestingly, atherosclerotic lesion size in Ldlr−/− mice reconstituted with Mfge8−/− bone marrow at 7 weeks of HFD was similar to that of Ldlr−/− mice fed a HFD for 13 weeks. Remarkably, Ldlr−/− mice transplanted with Cleca9−/−Mfge8−/− bone marrow showed a marked reduction of atherosclerosis (Online Figure IIC and IIID) as compared with mice transplanted with Mfge8−/− bone marrow, despite no change in plasma cholesterol levels (5.86±0.45 versus 6.19±0.33 g/L; **P=0.56, in Mfge8−/− and Cleca9−/−Mfge8−/− mice, respectively). This indicates that DNGR-1 promotes atherosclerosis even at advanced stages of lesion development but only in a context of moderate hypercholesterolemia.

**DNGR-1 Mediates Proatherogenic Effects Through Downregulation of IL-10**

We next assessed lesion composition in Cleca9−/−→Ldlr−/− and Cleca9+/+→Ldlr−/− mice after 5, 7, and 13 weeks of HFD. The percentage of macrophages (MOMA-2 staining/plaque surface) showed a significant decrease in mice reconstituted with DNGR-1–deficient bone marrow after 5, 7, or 13 weeks of HFD, compared with chimeric Cleca9+/+ mice (Online Figure IVA). T-lymphocyte accumulation within the lesions (CD3 staining) significantly decreased in mice transplanted with Cleca9−/−, compared with Cleca9+/+ at 7 weeks of HFD (P=0.047; Figure 2A), but this effect was lost at 13 weeks of HFD (P=0.77; Figure 2B) when plasma cholesterol levels were excessively high.

![Figure 2. DNGR-1 (dendritic cell NK lectin group receptor-1) deletion protects against vascular inflammation. A and B, Representative photomicrographs and quantitative analysis of lesional T-cell infiltration in Ldlr−/− mice reconstituted with either control (n=12) and Cleca9−/− (n=16) bone marrow after 7 wk or control (n=8) and Cleca9−/− (n=9) after 13 wk of high-fat diet (HFD). Plaque (P) and media (M) are indicated. The results showed a significant decrease of T cells % (CD3 area: plaque area) in the absence of DNGR-1 after 7 wk but not after 13 wk of HFD. C, Cleca9a, Tgf-β, and Il-10 mRNA in spleens of Ldlr−/− mice transplanted with Cont (n=7) or Cleca9a−/− (n=9) bone marrow after 7 wk of HFD. The results show a significant increase of Tgf-β and Il-10 expression in the absence of DNGR-1. Mean values±SEM are shown. **P<0.05, ***P<0.001, ****P<0.0001.](http://circres.ahajournals.org/DownloadedFrom/6a86-ahajournals.org)
We then examined the inflammatory profile in spleens of Ldlr−/− mice reconstituted with either Clec9a+/+ or Clec9a−/− bone marrow. The expression of major inflammatory cytokines involved in atherosclerosis, including Ifnγ, Il1β, and Il6 was not different between the 2 groups of mice after 7 weeks of HFD (Online Figure IVB). However, as shown in Online Figure VA and VB and Figure 2C, DNGR-1 deficiency after 5 and 7 weeks, but not after 13 weeks of HFD, markedly increased Tgfβ and Il10 expression, suggesting an anti-inflammatory and antiatherogenic phenotype.23–26 We also found a significant increase of IL-10 production by splenocytes in the absence of DNGR-1 after lipopolysaccharide-TLR4 stimulation (Online Figure VC), without significant changes in tumor necrosis factor-α or IL-6 (data not shown), suggesting that DNGR-1 acts as a regulator of IL-10.

To directly address the physiological relevance of enhanced expression of IL-10 by DNGR-1-deficient bone marrow cells, we generated Clec9a−/−Il10−/− mice. We recovered bone marrow from Il10−/− or Clec9a−/−Il10−/− mice to reconstitute lethally irradiated Ldlr−/− mice. After 4 weeks of recovery, mice were put on HFD for 7 weeks. As previously reported,27 IL-10 deficiency in the bone marrow accelerated lesion development (Figure 3A and 3B), despite no change in plasma cholesterol levels (5.00±0.78 versus 4.57±0.20 g/L; P=0.93). Strikingly, the observed atheroprotective effect of Clec9a deficiency (Figure 1D) was completely abolished in Clec9a−/−Il10−/−→Ldlr−/− mice (Figure 3A and 3B), despite no impact on plasma cholesterol levels (4.57±0.20 versus 4.81±0.20 g/L; P=0.53). Clec9a expression in Il10−/−→Ldlr−/− spleens is comparable to Il10−/−→Ldlr−/−, indicating that inflammation is not involved in the modulation of Clec9a expression (data not shown). Moreover, no significant differences in necrotic core area, macrophage, or T-cell infiltration were observed between Ldlr−/− mice reconstituted with bone marrow cells from either Il10−/− or Clec9a−/− Il10−/− (Figure 3C through 3E). These data further demonstrate that the modulation of IL-10 expression is an important mechanism through which DNGR-1 mediates its proatherogenic effects.

DNGR-1 Expressed in CD8α+ DCs Regulates IL-10 Production and Atherosclerosis Development

DNGR-1 is highly expressed in CD8α+ DCs.11 However, the role of CD8α+ DCs in atherosclerosis is still poorly understood.28,29 To address the direct role of CD8α+ DC–specific DNGR-1 in atherosclerosis, we used irradiated Ldlr−/− mice transplanted with a 8:2 mixture of CD8α+ DC–deficient (Cd11cCre+/Irf8flox/flox; Online Figure VIA) CD45.2 bone marrow (80%), mixed with either Clec9a+/+ (CD45.1) or Clec9a−/− bone marrow (20%). In these chimeric mice, at least 80% of spleen cells other than CD8α+ DCs, including T cells (CD4+ and CD8+), B cells (B220+), CD8α+ DCs, macrophages (F4/80+), and neutrophils (GR1+), derived from Clec9a−/−Il10−/−→Ldlr−/− mice (Figure 3A and 3B), despite no impact on plasma cholesterol levels (4.57±0.20 versus 4.81±0.20 g/L; P=0.53). Clec9a expression in Il10−/−→Ldlr−/− spleens is comparable to Il10−/−→Ldlr−/−, indicating that inflammation is not involved in the modulation of Clec9a expression (data not shown). Moreover, no significant differences in necrotic core area, macrophage, or T-cell infiltration were observed between Ldlr−/− mice reconstituted with bone marrow cells from either Il10−/− or Clec9a−/− Il10−/− (Figure 3C through 3E). These data further demonstrate that the modulation of IL-10 expression is an important mechanism through which DNGR-1 mediates its proatherogenic effects.

**Figure 3.** IL-10 (interleukin-10) deficiency abrogates atheroprotection in DNGR-1 (dendritic cell NK lectin group receptor-1)–deficient mice. A and B, Representative photomicrographs and quantitative measurement of lesion size in the aortic sinus of lethally irradiated male Ldlr−/− mice reconstituted with bone marrow from Clec9a+/+ (n=4) or Clec9a−/− (n=5) or Il10−/− (n=6) or Clec9a−/−Il10−/− (n=7) mice after 7 wk of high-fat diet (HFD). C–E, Representative photomicrographs and quantitative analysis of necrotic cores, lesional macrophage, and T-cell infiltration in Ldlr−/− mice reconstituted with either Il10−/− (n=6) or Clec9a−/−Il10−/− (n=7) bone marrow after 7 weeks of HFD. The results show the importance of IL-10 in DNGR-1–mediated proatherogenic effects. Results were obtained after 4 weeks of recovery from bone marrow transplantation followed by 7 wk on a HFD. Mean values±SEM are shown. **P<0.05, ***P<0.001, ****P<0.0001.
the 80% Cd11c<sup>Cre+/Irf8flox/flox</sup> (CD45.2) bone marrow (Online Figure VIIB; Figure 4A). On the contrary, we expected all CD8α<sup>+</sup> DCs to reconstitute almost exclusively from the 20% bone marrow (Clec9a<sup>+/+</sup> or Clec9a<sup>−/−</sup>). In agreement with this, Clec9a mRNA was highly expressed in purified spleen CD8α<sup>+</sup> DCs of mice reconstituted with 80% Cd11c<sup>Cre+/Irf8flox/flox</sup>+20% Clec9a<sup>−/−</sup> bone marrow (CD8α<sup>+</sup> DC control) but markedly decreased in purified spleen CD8α<sup>+</sup> DCs of mice reconstituted with 80% Cd11c<sup>Cre+/Irf8flox/flox</sup>+20% Clec9a<sup>−/−</sup> bone marrow (CD8α<sup>+</sup> DC-Clec9a<sup>−/−</sup>; Figure 4B). As expected, DNGR-1 was barely expressed in purified CD8α<sup>+</sup> DCs from spleens of both groups of mice (Figure 4B). Reconstitution of spleen DCs, and more particularly CD8α<sup>+</sup> DCs, was similar between the 2 groups of mice (Online Figure VIC).

Remarkably, the selective absence of DNGR-1 in CD8α<sup>+</sup> DCs significantly decreased plaque size (P=0.04; Figure 4C and 4D), despite no change in plasma cholesterol levels (6.69±0.25 versus 6.44±0.31 g/L; P=0.46). Moreover, macrophage and T-cell infiltration within the plaques were significantly decreased in the absence of DNGR-1 in CD8α<sup>+</sup> DCs, indicating that the expression of DNGR-1 in CD8α<sup>+</sup> DCs is required to mediate its proatherogenic effects (Online Figure VIIA; Figure 5A and 5B).

DNGR-1 has previously been shown to induce CD8<sup>+</sup> T-cell responses against dying cells. However, we found no differences in spleen CD8<sup>+</sup> T-cell percentage or activation between the 2 groups of mice (Online Figure VIIIB), suggesting the involvement of other mechanisms.

Interestingly, as in the total DNGR-1–deficient model, the specific deletion of DNGR-1 in CD8α<sup>+</sup> DCs led to a marked increase of IL-10 expression in those cells (Online Figure VIIIC) but also more generally in the spleen (Figure 5C), showing that DNGR-1 specifically expressed by CD8α<sup>+</sup> DCs plays a major role in the regulation of IL-10. DNGR-1 was also detected in CD45<sup>+</sup> cells purified from atherosclerotic plaque aorta (Online Figure VIIIDA), suggesting potential local effects. We also found higher Il10 mRNA levels in aortas of mice with DNGR-1 deletion in CD8α<sup>+</sup> DCs compared with controls, without significant changes of Tnfα, Il6, and Tgfβ expression (Online Figure VIIIB).

The expression of Il10 in CD8α<sup>+</sup> DCs and DCs in general was relatively weak, which suggests that DNGR-1 deficiency in CD8α<sup>+</sup> DCs likely increased IL-10 expression in other cell types. Accordingly, it was previously shown that IL-10, and particularly DC-derived IL-10, promoted IL-10–producing T regulatory (Tr1) cells. In this regard, we found that cocculture of splenic CD8α<sup>+</sup> DCs purified from Il10<sup>−/−</sup> mice with
Il10+/+ CD3+ T cells resulted in a significantly lower IL-10 production as compared with the coculture of Il10+/+ CD8α+ DCs with Il10+/+ CD3+ T cells, further highlighting the importance of CD8α+ DC-derived IL-10 to stimulate IL-10 production by T cells (Online Figure IXA). Furthermore, coculture of DNGR-1–deficient CD8α+ DCs with CD3+ T cells significantly increased IL-10 production by T cells without significant changes of interferon-γ or IL-17 (Online Figure IXB through IXD). In line with these results, the selective absence of DNGR-1 in CD8α+ DCs significantly increased IL-10 expression in T-CD4+ cells in vivo (Figure 5D), suggesting a specific bystander effect of DNGR-1 expressed in CD8α+ DCs on the regulation of IL-10 production by CD4+ cells. Of note, DNGR-1 in CD8α+ DCs did not affect the levels of T regulatory cells (CD4+CD25+FOX3+; Online Figure XA).

To directly test the specific role of IL-10 expression in CD8α+ DCs in the presence or absence of DNGR-1, we irradiated and transplanted Ldr−/− mice with 8:2 bone marrow from Cd11cCre+/Irf8flox/flox (80%) mice, mixed with 20% of bone marrow from control (n=8), Clec9a−/− (n=9), Il10−/− (n=8), or Clec9a−/−Il10−/− (n=9) mice. Then, the mice were put on HFD during 7 weeks. As shown in Figure 6A through 6D, specific IL-10 deletion in CD8α+ DC abrogated the atheroprotection observed in absence of DNGR-1 in this cell type, without any significant change of plasma cholesterol levels. This was associated with the inability of Clec9a deficiency to increase IL-10 production by splenocytes (Figure 6E). Furthermore, coculture of CD8α+ DCs from each group with wild-type CD4+ T cells showed that CD8α+ DCs promoted increased IL-10 production by T cells in the absence of DNGR-1, which was dependent on IL-10 expression in CD8α+ DC (Online Figure XB) and most likely IL-10 receptor signaling in CD4+ T cells (Online Figure XC).

Discussion

Accumulation of apoptotic cells or debris during the development of atherosclerosis promotes inflammation and contributes to plaque growth and thrombosis on plaque rupture,3,33 We recently showed that CLEC4e (C-type lectin receptor 4e), known to be involved in the sensing of necrotic cores, promoted atherosclerosis through induction of endoplasmic reticulum stress–dependent macrophage proliferation, inflammation, and foam cell formation.34 However, other C-type lectin receptors could recognize necrotic cores and stimulate inflammation and plaque progression.

Our findings provide in vivo and in vitro evidence that DNGR-1, known to be involved in the sensing and presentation of antigens derived from necrotic cells,12 is proatherogenic,
and its deletion in both Ldlr−/− and Apoe−/− mouse models promotes an anti-inflammatory response and deactivates important proatherogenic mechanisms. We have identified a new DNGR-1-regulated IL-10 pathway that controls vascular inflammation and substantially impacts lesion development. DNGR-1 deficiency inhibited macrophage and T-cell infiltration within the plaques and upregulated Tgfb and Il10, 2 major anti-inflammatory and antiatherogenic genes.2,25 Intriguingly, the atheroprotective effect of DNGR-1 deletion was already observed at the early stages of atherosclerosis (5 or 7 weeks of HFD) when plaques were still relatively small and apoptotic cell death was barely detectable. Even though we cannot rule out the possibility that DNGR-1 was activated by some necrotic debris, it is likely that other pattern recognition receptors, including TLRs, were activated during atherosclerosis35 and interacted with DNGR-1 signaling to activate inflammatory pathways. In agreement with this, our results showed that the absence of IL-10 abrogates the protective effects of DNGR-1 deletion in CD8α DCs. Mean values±SEM are shown. P<0.05, **P<0.001, ***P<0.0001.

Figure 6. Interleukin-10 (IL-10) deletion abrogates protective effects of DNGR-1 (dendritic cell NK lectin group receptor-1) absence in CD8α+ subset of dendritic cells (CD8α+ DCs). A and B, Representative photomicrographs and quantification of lesion size in aortic sinus. C, Plasma cholesterol in chimeric Ldlr−/− mice transplanted with 2:8 mix of bone marrow from control (n=8) or Clec9a−/− (n=9) or IL-10−/− (n=9) bone marrow cells mixed with Cd11cCre+/Irf8flox/flox bone marrow after 7 wk of high-fat diet (HFD). D, Clec9a and Il-10 mRNA in pooled purified CD8α+ DCs isolated from either control group (CD8α+ DC-Cont, n=8) or DNGR-1–deficient CD8α+ DC group (CD8α+ DC-Clec9a−/−, n=9) or IL-10–deficient CD8α+ DC group (CD8α+ DC-Il-10−/−, n=8) or DNGR-1/IL-10–deficient CD8α+ DC group (CD8α+ DC-Clec9a−/−Il-10−/−, n=9). E, IL-10 production by anti-CD3 (5 µg/mL) and anti-CD28 (1 µg/mL) stimulated splenocytes from either CD8α+ DC-Cont (n=6) or CD8α+ DC-Clec9a−/− (n=9) or CD8α+ DC-Il-10−/− (n=8) or CD8α+ DC-Clec9a−/−Il-10−/− (n=9), in presence of lipopolysaccharide (1 µg/mL). The results show that the deletion of IL-10 abrogates the protective effects of DNGR-1 deletion in CD8α+ DCs, as indicated by our in vitro experiments showing reduced CLEC9a expression by CD8α+ DCs in the presence of high cholesterol. In agreement with this, we found that DNGR-1 deletion was still atheroprotective in mice deficient for Mfge8, which exhibited after only 7 weeks of HFD (moderate hypercholesterolemia) increased accumulation of apoptotic/necrotic debris, and plaques as large as those found in Mfge8+/+ mice after 13 weeks of HFD (severe hypercholesterolemia). In addition, previous studies showed that T cells become dispensable for atherosclerosis development after prolonged and severe hypercholesterolemia.36,37 More recently, Tsaousi et al38 reported that when Apoe−/− mice were fed a chow diet for 35 weeks, lesions were approximately the same size as after 12 weeks of HFD. Yet, T-bet–deficient Apoe−/− mice, in which T cells are unable to differentiate into a Th1 phenotype, exhibited fewer plaques compared with immunocompetent Apoe−/− mice after 35 weeks of chow diet, whereas T-bet deficiency had no atheroprotective effects in Apoe−/− mice fed a HFD for 12 weeks. As our data suggest that the protective effects of DNGR-1 deletion are dependent on T cells, this may explain why the atheroprotective effect is lost at high plasma cholesterol levels.

In agreement with our hypothesis of a role for DNGR-1 in adaptive immune responses, earlier studies showed a restricted DNGR-1 expression in CD8α+ DCs,12,29 However,
existing reports about the role of CD8α+ DCs in atherosclerosis are contradictory. One study used FMS-like tyrosine kinase (flt3−/−) Ldlr−/− double deficient mice, which displayed marked depletion of CD8α+ DCs, suggested a proatherogenic role of CD8α+ DCs.28 However, flt3−/− mice are also deficient for many other conventional DC subtypes. Moreover, in contrast to those results, atherosclerotic plaque formation and stability were not altered in chimeric Ldlr−/− mice transplanted with bone marrow from Batf3−/− mice transplanted with control CD8α+ DCs showing that CD8α+ DC subset, compared with CD8α−/− mice, were not altered in chimeric Ldlr−/− mice transplanted with bone marrow from Batf3−/− mice transplanted with control CD8α+ DCs. However, atherosclerotic plaque formation and stability were not altered in chimeric mice transplanted with bone marrow from Batf3−/− mice transplanted with control CD8α+ DCs.29 Interestingly, we found that the absence of DNGR-1 in CD8α+ DCs promotes atherosclerosis, identifying a previously unrecognized proatherogenic function of CD8α+ DCs in the development of atherosclerosis.

DNGR-1 stimulation in CD8α+ DCs has been previously shown to mediate cross-presentation of dead cell–associated antigens leading to CD8+ T-cell activation.11,40,41 Thus, we expected that DNGR-1 expressed in CD8α+ DCs under conditions of hyperlipidemia would lead to CD8+ T-cell activation. However, our results showed no significant changes in spleen CD8+ T-cell count or activation between control and DNGR-1–deficient mice, suggesting that cross-presentation may not be the main mechanism accounting for DNGR-1–mediated proatherogenic effects. In addition, the role of CD8+ T cells and cross-presentation in atherosclerosis is still debated.29,41–43 Interestingly, we found that DNGR-1 deletion in CD8α+ DCs increased the expression of the anti-inflammatory and antiatherogenic IL-10 in spleens, suggesting the involvement of this cytokine in the observed atheroprotective effects. Noteworthy, spleen CD8α+ DCs purified from mice reconstituted with DNGR-1–deficient CD8α+ DCs showed a significantly higher IL-10 expression compared with control CD8α+ DCs. Yet, IL-10 expression by CD8α+ DCs was weak, suggesting an additional impact of DNGR-1 deficiency in CD8α+ DCs on IL-10 expression in other cell types. In agreement with this, we found that the absence of DNGR-1 in CD8α+ DCs led to increased IL-10 (but not IL-17 nor interferon-γ) production by CD4+ T cells, suggesting the induction of an anti-inflammatory and antiatherogenic T-cell response.

Our findings point to an important role of IL-10 as the main atheroprotective factor in the absence of DNGR-1. First, IL-10 expression was upregulated at 5 and 7 weeks of HFD in the absence of DNGR-1. However, after 13 weeks of high diet, when no difference of plaque size was observed, IL-10 expression remained unchanged in the absence of DNGR-1. Moreover, double deficiency in DNGR-1 and IL-10, specifically in CD8α+ DCs, completely abrogated the protective effects of DNGR-1 deletion. The mechanisms leading to an increase of IL-10 in the absence of DNGR-1 are still unknown and will require further investigation. One likely mechanism is that interaction between TLRs and DNGR-1 signaling modulates IL-10 production through the SYK pathway.10,11,44,45

In conclusion, our results identify a proatherogenic role for the necrotic cell sensor DNGR-1 through the regulation of IL-10 production and suggest a new potential therapeutic target to combat atherosclerosis.

Acknowledgments

The DNGR-1–deficient mice were provided by Caetano Reis e Sousa (Immunobiology Laboratory, London, UK). We thank members of our animal facility for their assistance in mouse care. We also thank Jose Vilar for his technical help. We gratefully thank David Sancho and Caetano Reis e Sousa for their critical reading of the article.

Sources of Funding

This work was supported by Inserm, European Research Council grant (Z. Mallat), the British Heart Foundation (Z. Mallat), United Kingdom, and the Fondation pour la Recherche Medicale, France. Y. Haddad is a recipient of fellowship from COOQIM, Ile-de-France, Paris. C. Lahoute was a recipient of fellowship from Fondation pour la Recherche Medicale.

Disclosures

None.

References


References


The Dendritic Cell Receptor DNGR-1 Promotes the Development of Atherosclerosis in Mice

Yacine Haddad, Charlotte Lahoute, Marc Clément, Ludivine Laurans, Sarvenaz Metghalchi, Lynda Zeboudj, Andreas Giraud, Xavier Loyer, Marie Vandestienne, Julien Wain-Hobson, Bruno Esposito, Stephane Potteaux, Hafid Ait-Oufella, Alain Tedgui, Ziad Mallat and Soraya Taleb

Circ Res. 2017;121:234-243; originally published online June 12, 2017;
doi: 10.1161/CIRCRESAHA.117.310960

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/121/3/234

Data Supplement (unedited) at:
http://circres.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Extent and plaque composition of atherosclerotic lesions

Mice were anesthetized with isoflurane before sacrifice. Plasma cholesterol was measured using a commercial cholesterol kit (Sigma). The heart and aorta, including the brachiocephalic artery, were taken off, fixed in 4% paraformaldehyde for 2 hours. Lesion extent in the thoracic aorta represents the percentage of Oil red O staining. Necrotic cores were visualized using Masson’s Trichrome. The presence of macrophages and T cells within plaques were studied using respectively an anti-macrophages/macrophages antibody, MOMA-2 (EMD Millipore) and anti-CD3 (DAKO, Trappes, France). At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used. Terminal dUTP nick end-labeling (TUNEL; ApopDETEK kit, ENZO Diagnostics, DAKO) was considered as positive when at least 2 of 6 sections per mouse showed TUNEL staining. We performed morphometric studies using Histolab software (Microvisions).

Quantitative Real time PCR

Cells were lysed in detergent buffer RLT and spleens were lysed in Trizol and then subjected to RNA extraction and reverse transcription (Qiagen). Then, quantitative real-time PCR was performed on an ABI PRISM 7700 (Applied Biosystems) in triplicates. Cycle threshold for Gapdh (primers: Gapdh-R, 5'-CGTCCCGTAGACAAATGTTGAA-3'; Gapdh-L, 5'-GCCGTGAGTGAGTCTAGTGGAACA-3') was used to normalize gene expression. Primers for Il10, Clec9α, Tgfβ, Il6, Ifnγ and Il1β are respectively: Il10 (primers: Il10-R, 5'-AAGTGATGCCCAAGGCA-3'; Il10-L 5'-TCTCACCCAGGAATCTCAA-3'), Clec9α (primers: Clec9α-R, 5'-TTTGCACCAATACACGACAG-3'; Clec9α-L, 5'-TGTTGACTGCTCCACACTGG-3'), Tgfβ (primers: Tgfβ-R: 5'CGGAGAGCCCTGGATACCAACTA-3'; Tgfβ-L, 5'-CCGCACACAGCAGTCTTCTCT-3'), Il6 (primers Il6-R: 5'-GGTCTTTTGCTTCTTTCTGT-3'; Il6-L, 5'-AAAGACAAAGCCAGTAGCTCTTTAGAGAT-3'), Ifnγ (primers Ifnγ-R, 5'-AGCACAAGCAAGGCGAAA-3'; Ifnγ-L, 5'-CTGGACCTGTGGGTGTGTTGA-3'), Il1b (primers Il1β-R: 5'-GGGTGTGCCGTCTTCTCATTA-3'; Il1β-L: 5'-GAAGAGCCCCATCTTCTGTG-3').

PCR conditions were 10 min at 95°C; 35 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s; and a final extension at 72°C for 20s.

Flow cytometry

Cell surface staining are the following: APC-conjugated CD45.1 (A20), FITC-conjugated CD45.2 (104), PE-Cy7 conjugated CD11c (HL3), Alexa fluor 700-conjugated CD8 (53-6.7), PE-conjugated F4/80 (BM8) (BD Biosciences), and eFluor 450-conjugated CD62L (clone MEL-14), APC-conjugated CD44 (IM7) (eBioscience), PercC-Cy5.5-conjugated GR-1 (RB6-8C5), APC-conjugated CD25 (PC61.5) (eBioscience), and V-500-conjugated B220 (RA3-6B2). Intracellular staining of forkhead box P3 (PE-Cy7-conjugated FOXP3) (FJK-16s) (eBioscience) was performed. Splenocytes were stimulated in vitro for 4 h using leukocyte activation cocktail (BD) for intracellular staining. Briefly, cells were stained for surface markers followed by fixation and permeabilization using a kit (eBioscience) for intracellular staining. Then, cells were stained with APC-conjugated Il10 (clone JESS-16E3) (eBioscience). For CD8α+DCs staining, we used anti-CD11c-PE-Cy7 (clone: N418, Biolegend), MHC II Pacific blue (clone: M5/114.15.2, Biolegend), CD8α BV786 (clone: 53-6.7, Biolegend) and CD11b A700 (clone: M1/70, BDBioscience).

Samples were acquired using a flow cytometer (LSRFortessa, Becton Dickinson) and data was analyzed using FlowJo software (TreeStar, OR, USA). Cell doublets were excluded using forward (FSC) and side (SSC) light scattering area (A) vs. width (W).
Supplementary Figure I DNGR1 deletion effects in atherosclerosis. A plasma cholesterol in Ldlr−/− mice reconstituted with either control (Clec9a+/+ → Ldlr−/−) or Clec9a−/− (Clec9a−/− → Ldlr−/−) bone marrow after 5 weeks (control (n=7) and Clec9a−/− (n=8)) or 7 weeks (control (n=12) and Clec9a−/− (n=16)) or 13 weeks (control (n=8) and Clec9a−/− (n=9)) of high fat diet (HFD). B plasma cholesterol C representative pictures and quantification of plaque size in aortic sinus of Apoe−/− (n=9) and Apoe−/−Clec9a−/− (n=9) mice fed with HFD during 6 weeks D representative pictures and quantification of necrotic cores in plaques of Ldlr−/− mice reconstituted with either (control (n=8) and Clec9a−/− (n=9)) after 13 weeks of HFD. Mean values ± SEM are shown. *P<0.05, **P<0.001, ***P<0.0001.
Supplementary Figure II High levels of cholesterol decreases DNGR1 expression. A mRNA expression of Clec9a in spleens of male Ldlr<sup>−/−</sup> mice on chow diet (CD) (n=3) or male Ldlr<sup>−/−</sup> mice reconstituted with either control ([Clec9a<sup>+/+</sup> → Ldlr<sup>−/−</sup>]) or Clec9a<sup>−/−</sup> ([Clec9a<sup>−/−</sup> → Ldlr<sup>−/−</sup>]) bone marrow after 7 weeks (control (n=6) or Clec9a<sup>−/−</sup> (n=8)) or 13 weeks (control (n=7) or Clec9a<sup>−/−</sup> (n=7)) of high fat diet (HFD). B Representative examples and quantitative analysis of flow cytometry-based GFP expression in splenocytes from either control (Clec9a<sup>+/+</sup>, GFP<sup>−</sup>) or Clec9a<sup>−/−</sup> (GFP<sup>+</sup>) (see materials and methods). Splenocytes were incubated in absence (NS) or in presence of cyclodextrin-cholesterol at different concentrations (5, 10, 25, 50, 100 ug/ml) during 16 h. One representative experiment out of two is shown. Mean values ± SEM are shown. * P<0.05, **P<0.001, ***P<0.0001.
Supplementary Figure III. DNGR1 deletion in bone marrow protects against atherosclerosis in mice bearing Mfge8 deletion with accumulation of apoptotic/necrotic cells. A-B Representative photomicrographs and quantifications of TUNEL staining in lethally-irradiated male Ldlr−/− mice reconstituted with bone marrow from wild type control (n=4) or Clec9a−/− (n=5) or Mfge8−/− (n=7) or Mfge8−/−Clec9a−/− (n=15) mice. Percent lesion area occupied by acellular (apoptotic/necrotic) material are shown. Results were obtained after 4 weeks of recovery from bone marrow transplantation followed by 7 weeks on a high fat diet (HFD). C-D Representative photomicrographs and quantification of lesion size in Ldlr−/− mice reconstituted with either Control (n=4) and Clec9a−/− (n=5) or Mfge8−/− (n=7) or Mfge8−/−Clec9a−/− (n=15) bone marrow after 7 weeks of HFD. Results were obtained after 4 weeks of recovery from bone marrow transplantation followed by 7 weeks on a HFD. Mean values ± SEM are shown. *P<0.05, **P<0.001, ***P<0.0001.
Supplementary Figure IV DNGR1 effects on macrophage infiltration within plaques and on gene expression of inflammatory factors. A Representative photomicrographs and quantitative analysis of lesional macrophage infiltration in Ldlr⁻/⁻ mice reconstituted with either Control (n=7) and Clec9a⁻/⁻ (n=8) after 5 weeks of high fat diet (HFD), or either Control (n=12) and Clec9a⁻/⁻ (n=16) after 7 weeks of HFD, or either Control (n=8) and Clec9a⁻/⁻ (n=9) after 13 weeks of HFD. B DNGR1 deletion has no effect on mRNA of Il-6, Ifn-γ, Il-1β in spleens of Ldlr⁻/⁻ reconstituted with either control or Clec9a⁻/⁻ bone marrow after 7 weeks of HFD. Mean values ± SEM are shown. *p<0.05,**P<0.001, ***P<0.0001.
**Supplementary Figure V** DNGR1 deletion has anti-inflammatory effects at 5 weeks but not at 13 weeks of HFD. A-B DNGR1 deletion effects on *il-10* and *tgf-β* mRNA in spleens of *Ldlr*⁻/⁻ mice reconstituted with either control (*Clec9a⁺⁺ → Ldlr⁻/⁻*) or *Clec9a⁻/⁻* (*Clec9a⁻/⁻ → Ldlr⁻/⁻*) bone marrow after 5 weeks (control (n=7) and *Clec9a⁻/⁻* (n=8)) or 13 weeks (control (n=8) and *Clec9a⁻/⁻* (n=9)) of high fat diet (HFD). C DNGR1 deletion increases IL-10 production in supernatants of splenocytes from Apoe⁻/⁻ (n=5) and Apoe⁻/⁻*Clec9a⁻/⁻* (n=5) at 20 weeks-old after stimulation with LPS (1μg/ml) during 48 h. * P<0.05, **P<0.001, ***P<0.0001.
Supplementary Figure VI Constitution of control and Clec9a<sup>−/−</sup>-CD8α<sup>+</sup>DC chimeric mice. A Frequency of DCs (CD11c<sup>high</sup>MHC II<sup>high</sup>) and CD8α<sup>+</sup>DCs in the spleen of Cd11c<sup>Cre−/IRF8<sup>fl</sup>ox/</sup>flox<sup>−/−</sup> and Cd11c<sup>Cre+</sup>/IRF8<sup>fl</sup>ox/</sup>flox<sup>−/−</sup> analysed by flow cytometry (n=3/group). B Donor origin as defined by flow cytometry analysis of CD45.1 % and CD45.2 % of different cell types in spleens of chimeric Ldlr<sup>−/−</sup> mice transplanted with bone marrow mix (8:2) from Cd11c<sup>Cre+</sup>/IRF8<sup>fl</sup>ox/</sup>flox<sup>−/−</sup> (CD45.2) and control (CD45.1) mice (n=7). C Representation and quantification of CD8α<sup>+</sup>DC by flow cytometry in control group (CD8α<sup>+</sup>DC-Cont, n=7) or DNGR1-deficient CD8α<sup>+</sup>DC group (CD8α<sup>+</sup>DC-Clec9a<sup>−/−</sup>, n=8) after 7 weeks of HFD. Mean values ± SEM are shown. ***P<0.0001, *P≤0.05.
Supplementary Figure VII effects of DNGR1 deletion in CD8α+DCs. A photomicrographs and quantitative analysis of lesional macrophages in Ldlr−/− mice reconstituted with either control group (CD8α+DC-Cont, n=7) or Clec9a-deficient CD8α+DC group (CD8α+DC-Clec9a−/-, n=8) after 7 weeks of high fat diet (HFD) B No change in activated (CD44+) and naive (CD62L+) spleen CD8+ T cells in the Clec9a-deficient CD8α+DCs (n=8) compared to control-CD8α+DCs (n=7) groups of mice. C Il-10 mRNA in pooled Clec9a-deficient CD8α+DCs (n=3) or CD8α+DCs (n=3) compared to control-CD8α+DCs (n=3) or CD8α+DCs (n=3) isolated from chimeric Ldlr−/− mice transplanted with bone marrow mix (2:8) from control (CD45.1) (n=7) or Clec9a−/− (n=8) bone marrow cells mixed with Cd11cCre+/Irf8flox/flox (CD45.2) bone marrow. Mean values ± SEM are shown. *p<0.05, ***<0.0001.
Supplementary Figure VIII DNGR1 deletion in CD8α⁺DCs increases Il-10 expression in aorta. A Representative examples of flow cytometry-based GFP expression in purified CD45 cells from aorta of either control (Apoe⁺/⁻Clec9a⁺/-, GFP⁻) or Apoe⁻/⁻Clec9a⁻/- (GFP⁺) (see materials and methods). B mRNA of il10, tnfa, il6 and tgfβ in aorta from the DNGR1-deficient CD8α⁺DCs (n=8) compared to control-CD8α⁺DCs (n=7) groups of mice. Mean values ± SEM are shown. **P<0.001.
Supplementary Figure IX DNGR1 deletion in CD8α*DCs increases IL-10 production by T cells. A IL-10 production by anti-CD3 (5ug/ml) and anti-CD28 (1 ug/ml) stimulated spleen TCD3+ cells from Il-10+/+ mice, co-cultured in presence of LPS (1ug/ml)-treated spleen CD8α*DCs from Il-10+/+ or Il-10−/− mice, measured after 48 h of stimulation. B-D IL-10, Il-17 and IFN-γ production by anti-CD3 (5ug/ml) and anti-CD28 (1 ug/ml) stimulated spleen CD3+ cells from Apoe−/− mice (n=3) or Apoe−/−Clec9a−/− mice (n=3), co-cultured in presence of LPS (1ug/ml)-treated spleen CD8α*DCs from Apoe−/− mice (n=3) or Apoe−/−Clec9a−/− mice (n=3), measured after 48 h of stimulation. Both CD8α*DCs and T cells were isolated from Apoe−/− mice (n=3) or Apoe−/−Clec9a−/− mice (n=3) after 20 weeks of chow diet. Mean values ± SEM are shown. *p<0.05, **P<0.001, ***P<0.0001.
Supplementary Figure X DNGR1 deletion in CD8α+DCs increases IL-10 production by T cells. A Representative and quantification by flow cytometry of regulatory T cell (CD4+CD25+FOXP3+) B IL-10 production by anti-CD3 (5ug/ml) and anti-CD28 (1 ug/ml) stimulated spleen CD4+ cells from wild-type (WT) mice, co-cultured in presence of LPS (1ug/ml)-treated spleen CD8α+DCs from chimeric Ldlr−/− mice transplanted with 2:8 mix of bone marrow from control (n=8) or Clec9a−/− (n=9) or Il−10−/− (n=8) or Clec9a−/−Il−10−/− (n=9) bone marrow cells mixed with Cd11cCre+/Irf8flox/flox bone marrow, after 7 wks of HFD and IL-10 was measured after 48 h of stimulation. C IL-10 production by anti-CD3 (5ug/ml) and anti-CD28 (1 ug/ml) stimulated spleen CD4+ cells from WT mice, co-cultured in presence of LPS (1ug/ml)-treated spleen CD8α+DCs from chimeric Ldlr−/− mice transplanted with 2.8 mix of bone marrow from control (n=8) or Clec9a−/− (n=9) in presence of control IgG or anti-IL-10 receptor neutralizing antibody (anti-IL-10r Ab, 10µg/ml). Mean values ± SEM are shown. *p<0.05, **P<0.001, ***P<0.0001.