Deficiency of HIF1α in Antigen-Presenting Cells Aggravates Atherosclerosis and Type 1 T-Helper Cell Responses in Mice

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Objective—Although immune responses drive the pathogenesis of atherosclerosis, mechanisms that control antigen-presenting cell (APC)–mediated immune activation in atherosclerosis remain elusive. We here investigated the function of hypoxia-inducible factor (HIF)-1α in APCs in atherosclerosis.

Approach and Results—We found upregulated HIF1α expression in CD11c⁺ APCs within atherosclerotic plaques of low-density lipoprotein receptor–deficient (Ldlr⁻/⁻) mice. Conditional deletion of Hif1a in CD11c⁺ APCs in high-fat diet–fed Ldlr⁻/⁻ mice accelerated atherosclerotic plaque formation and increased lesional T-cell infiltrates, revealing a protective role of this transcription factor. HIF1α directly controls Signal Transducers and Activators of Transcription 3 (Stat3), and a reduced Stat3 expression was found in HIF1α-deficient APCs and aortic tissue, together with an upregulated interleukin-12 expression and expansion of T-helper cells. Overexpression of Stat3 in Hif1a-deficient APCs in bone marrow reversed enhanced atherosclerotic lesion formation and reduced T-helper cell expansion in chimeric Ldlr⁻/⁻ mice. Notably, deletion of Hif1a in LysM⁺ bone marrow cells in Ldlr⁻/⁻ mice did not affect lesion formation or T-cell activation. In human atherosclerotic lesions, HIF1α, Stat3, and interleukin-12 protein were found to colocalize with APCs.

Conclusions—Our findings identify HIF1α to antagonize APC activation and T-helper polarization during atherogenesis in Ldlr⁻/⁻ mice and to attenuate the progression of atherosclerosis. These data substantiate the critical role of APCs in controlling immune mechanisms that drive atherosclerotic lesion development. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBHA.115.306171.)

Key Words: antigen-presenting cells ■ atherosclerosis ■ diet, high-fat ■ inflammation ■ leukocytes

Atherosclerosis is a chronic and systemic inflammatory disease characterized by the accumulation of immune cells in the vessel wall.¹,² Dendritic cells (DCs) localize to the intima and adventitia in healthy arteries in regions predisposed to atherosclerosis and accumulate in atherosclerotic lesions.³,⁴ DCs are increasingly regarded to play important roles in immune mechanisms governing atherogenesis.³,⁴ Both local and systemic adaptive immune responses control atherogenesis, and pro- and antiatherogenic CD4⁺ T-helper (Th) cell subsets and their cytokines have been defined.⁵ In particular, CD4⁺ type 1 Th1 and their cytokine interferon (IFN)-γ promote atherosclerosis, whereas regulatory T cells inhibit vascular inflammation.⁶,⁷ The function of the Th17 subtype is still unclear, as contradictory reports have been published.⁷ The hypoxia-inducible factor (HIF)-1α is among the primary transcription factors induced under hypoxic conditions, but can also be upregulated by inflammatory stimuli, such as oxidized low-density lipoprotein (LDL) and tumor necrosis factor-α in normoxia.⁸ In addition to regulating cell responses to hypoxia, fo example, glycolysis, and angiogenesis, HIF1α was identified to modulate adaptive and innate immune responses.⁹,¹⁰ Because of the high metabolic activity of inflammatory cells within lesions, and the reduced availability of oxygen...
Nonstandard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<td>BM</td>
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<td>BMDC</td>
<td>bone marrow–derived DC</td>
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<td>Ldlr</td>
<td>low-density lipoprotein receptor</td>
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<td>Stat</td>
<td>Signal Transducers and Activators of Transcription</td>
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<td>Th</td>
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confirmed by a marked deletion of *Hif1a* DNA (=80%) in isolated CD11c-cre *Hif1a* flox/flox *Ldlr*+ (Hif1a-conditional knockout [CKO] *Ldlr*−) when compared with CD11c-cre *Hif1a*+L *Ldlr*− (Hif1a-wild-type [WT] *Ldlr*−) APCs. In CD4+ T cells, only a marginal reduction was observed (≈5%; Figure 1A in the online-only Data Supplement). Likewise, *Hif1a* mRNA expression was reduced in isolated *Hif1a*-CKO *Ldlr*− APCs and *Hif1a*-CKO bone marrow–derived DCs (BMDCs)15 when compared with controls (Figure IB and IC in the online-only Data Supplement).

To study atherosclerotic lesion formation,15 *Hif1a*-WT *Ldlr*− and *Hif1a*-CKO *Ldlr*− mice were placed on a high-fat diet for 8 weeks. Body weight (28.9±0.7 versus 30.3±1.3 g), serum total cholesterol (12.7±1.0 versus 12.9±1.8×1000 μg/mL), and triglyceride levels (6.1±0.2 versus 5.6±0.1 mmol/L) did not differ between *Hif1a*-WT *Ldlr*− and *Hif1a*-CKO *Ldlr*− mice. We observed a 2.3-fold increase in atherosclerotic plaque growth in the aortic root and a 1.6-fold increase in the aorta of *Hif1a*-CKO *Ldlr*− when compared with that of *Hif1a*-WT *Ldlr*− mice (Figure 2A and 2B). Plaque cell density was unaltered between groups (4420±241.6 versus 3763±382.6 cells/mm² plaque area in *Hif1a*-CKO *Ldlr*− versus *Hif1a*-WT *Ldlr*− mice; n.s.), and no differences in plaque Mac-2+ macrophages, SMC numbers, or CD11c+ APCs were detected (Figure 2C–2E). A marked increase in relative necrotic core area was observed in plaques of *Hif1a*-CKO *Ldlr*− mice versus *Hif1a*-WT *Ldlr*− mice (33.0±2.9% versus 22.2±1.7% plaque area; P=0.0029), in line with a more advanced plaque phenotype. Notably, a 1.8-fold increase in numbers of CD3+ T cells was detected in lesions of *Hif1a*-CKO *Ldlr*− mice (Figure 2F), indicating that an enhanced accumulation of T cells within lesions was associated with an accelerated plaque growth because of deficiency of HIF1α in APCs.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**HIF1α Expression in Atherosclerosis**

Hypoxic areas, as well as *Hif1α* expression, have previously been demonstrated in human and murine atherosclerotic lesions.11,14 Consistent with this, we detected hypoxic regions in atherosclerotic plaques in aortic roots of *Ldlr*−/− mice fed a high-fat diet for 8 weeks (Figure 1A) but not in healthy 6-week-old *Ldlr*−/− mice on normal chow by hypoxyprobe staining (not shown); no staining was seen in *Ldlr*−/− mice without hypoxyprobe performed as a negative control (Figure 1A). Likewise, *Hif1α* mRNA and protein expressions were significantly upregulated in aortae of atherosclerotic *Ldlr*−/− mice than those of healthy *Ldlr*−/− controls (Figure 1B and 1C). Double-immunofluorescence staining revealed abundant expression of HIF1α protein within lesions (Figure 1D), and the majority of CD11c+ cells showed colocalization with HIF1α (Figure 1D), indicating HIF1α expression in lesional APCs. Moreover, increased expression of *Hif1α* mRNA was observed in splenic APCs from atherosclerotic *Ldlr*−/− mice (Figure 1E), indicating systemic upregulation of HIF1α in addition to localized effects in aortic lesions.

**Targeted Deletion of Hif1α in CD11c+ APCs Accelerates Atherosclerotic Lesion Formation**

To address the function of HIF1α in APCs, mice with a CD11c-specific deletion of *Hif1α* were generated, as

in deeper plaque areas, atherosclerotic lesions harbor areas of hypoxia,11 and HIF-1α can be detected in atherosclerotic lesions in both mice and humans.11-14 The direct cell-specific role of HIF1α in atherosclerosis in vivo, however, has not been addressed previously.

We here investigated the function of HIF1α in atherosclerosis in antigen-presenting cells (APCs). By deleting *Hif1α* specifically in CD11c+ cells, we here reveal a critical role of HIF1α in balancing APC-driven Th1 polarization during atherogenesis in LDL receptor–deficient (*Ldlr*−/−) mice and to attenuate the progression of atherosclerosis in *Ldlr*−/− mice.

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Hif1a-CKO versus Hif1a-WT mice on normal chow (Figure III in the online-only Data Supplement). These data suggest that HIF1α activation in APCs plays a crucial role in restraining T-cell activation and Th1 cell differentiation under inflammatory conditions in atherosclerosis, while being dispensable under homeostatic conditions. Notably, an increased percentage of IFN-γ+ CD4 T cells was also evidenced in LNs and spleens of Hif1a-CKO when compared with those of Hif1a-WT mice after immunization with OVA protein as an artificial model antigen (Figure IV in the online-only Data Supplement), corroborating an important role of HIF1α in controlling APC-driven Th1 T-cell polarization also under systemic inflammatory conditions unrelated to atherosclerosis.

Macrophages and DCs share phenotypic features. In particular, CD11c is expressed by both DCs and some macrophage subsets. To gain insight into the potential role of HIF1α in macrophages versus DCs among total APCs, atherosclerotic lesion formation was also assessed in Ldlr−/− mice reconstituted with BM of Hif1a-CKO or Ldlr−/− mice and Ldlr−/− mice fed a HFD for 8 weeks, normalized to Hprt and relative to Ldlr−/− healthy mice (n=8 each). *P<0.05. C, Analysis of HIF1α protein expression by Western blot in aortic tissue of Chow-fed Ldlr−/− mice and Ldlr−/− mice fed a HFD for 8 weeks. β-actin serves as a loading control. D, Double-immunofluorescence staining of HIF1α (green) and CD11c (red) in the atherosclerotic aortic root plaque of a Ldlr−/− mouse fed a HFD for 8 weeks. Cell nuclei were counterstained with DAPI (blue; scale bars, 50 μm). E, Analyses of Hif1a mRNA expression by qPCR in splenic antigen-presenting cells isolated from healthy Chow-fed Ldlr−/− mice and Ldlr−/− mice fed a HFD for 8 weeks, normalized to Hprt and relative to Ldlr−/− healthy mice (n=4 each). **P<0.01.

Furthermore, an alternative approach supports the importance of HIF1α in APCs, as untreated and tumor necrosis factor-α–stimulated BM-derived macrophages18 from Hif1a-CKO and Hif1a-WT mice did not display any consistent differences in proinflammatory Il12, Nfκb2, or anti-inflammatory Mrc1 and Tgfb mRNA expression (Figure VD in the online-only Data Supplement).

**HIF1α Controls Inflammatory IL-12 Expression in APCs by Regulating Signal Transducers and Activators of Transcription 3 Expression**

The migration of APCs is essential for efficient T-cell activation and controlled by CCR7.19 However, unchanged Ccr7 expression in Hif1a-KO BMDCs or APCs from atherosclerotic Hif1a-CKO Ldlr−/− mice and BMDC migration toward CCL19 (Figure VIA–VIC in the online-only Data Supplement) point toward effects of HIF1α unrelated to CCR7-driven APC migration.

T-cell activation and Th cell polarization are shaped by costimulatory molecule engagement and exposure to a specific cytokine milieu, with Th1 cells critically depending on IL-12 secretion from DCs.20,21 No significant changes in mRNA or surface protein expression of MHC-II, CD80, and CD86 were noted in tumor necrosis factor-α–matured Hif1a-CKO versus Hif1a-WT BMDCs, as assessed by quantitative polymerase chain reaction and flow cytometry (Figure VIIA and VIIC in the online-only Data Supplement). However, a significant increase in the mRNA expression of Il12 together with elevated IL-12 protein levels in supernatants of Hif1a-CKO BMDCs was observed, whereas Il4, Il6, Il10, Tgfb, or Tnfa were unaltered (Figure VIIIA and VIIIB in the online-only Data Supplement).
HIF1α has been shown to induce and to synergistically act with nuclear factor-kB. However, tmpkR/Grp105 and Relap65 transcript or protein expression (Figure IX in the online-only Data Supplement, and data not shown) were similar in mature Hif1α-CKO versus Hif1α-WT BMDCs, as assessed by quantitative polymerase chain reaction and flow cytometry (Figure VIIIC and VIIID in the online-only Data Supplement), suggesting that HIF1α-dependent changes in STAT3 expression may regulate IL-12 production. Indeed, overexpression of STAT3 in BMDCs decreased Il12α mRNA, whereas overexpression of a dominant negative form of STAT3 in BMDCs decreased Il12α mRNA and protein expression (Figure VIIE in the online-only Data Supplement, and data not shown) were similar in mature Hif1α-CKO versus Hif1α-WT BMDCs, as assessed by HIF1α has been shown to induce and to synergistically act with nuclear factor-kB. However, Nfkbia/p405 and Relap65 transcript or protein expression (Figure IX in the online-only Data Supplement, and data not shown) were similar in mature Hif1α-CKO versus Hif1α-WT BMDCs, as assessed by quantitative polymerase chain reaction and flow cytometry (Figure VIIIC and VIIID in the online-only Data Supplement), suggesting that HIF1α-dependent changes in STAT3 expression may regulate IL-12 production. Indeed, overexpression of STAT3 in BMDCs decreased Il12α mRNA, whereas overexpression of a dominant negative form of STAT3 in BMDCs decreased Il12α mRNA and protein expression (Figure VIIE in the online-only Data Supplement, and data not shown) were similar in mature Hif1α-CKO versus Hif1α-WT BMDCs, as assessed by flow cytometric analysis of T-cell distributions in spleens from atherosclerotic Hif1α-WT mice (Figure VIIIIE in the online-only Data Supplement).
HIF1α Controls Inflammatory T-Cell Responses in Atherosclerosis

Importantly, HIF1α also functions to promote STAT3 expression in APCs in atherosclerosis in vivo, as witnessed by a significant reduction in both STAT3 transcript and protein levels in splenic APCs from atherosclerotic Hif1a-CKO Ldlr−/− versus Hif1a-WT Ldlr−/− mice (Figure 4A and 4B). Further recapitulating findings in vitro, no significant changes in Cd74, Cld80, Cld86, and Nfkβ mRNA expression were noted (Figures VIIB and IXB in the online-only Data Supplement), but a significant increase in Il12 mRNA expression was observed in these APCs (Figure 4C and 4D).

We further assessed the propensity of splenic APCs isolated from atherosclerotic Hif1a-WT Ldlr−/− and Hif1a-CKO Ldlr−/− mice to antigen specifically activate OT-II T cells that express a T-cell receptor specific for the model antigen OVA. Although APCs of either genotype did not trigger noticeable activation of CFSE-labeled naïve CD4+ OT-II T cells in the absence of cognate antigen (not shown), significantly increased rates of T-cell proliferation and an expansion in IFN-γ-producing T cells were observed in cocultures with OVA-loaded Hif1a-CKO Ldlr−/− compared with Hif1a-WT Ldlr−/− APCs (Figure 4E and 4F). In contrast, no alterations in IL-17+ Th17 and Foxp3+CD25+ regulatory CD4+ T cells were detected (Figure 4F, and data not shown), congruent with the T-cell phenotype observed in atherosclerotic Hif1a-CKO Ldlr−/− mice. These data clearly indicate that APC-intrinsic deficiency in HIF1α promotes antigen-specific Th1 polarization. In line with known functions of IL-12 in T-cell activation, the presence of IL-12–blocking antibody significantly

**Figure 4.** Phenotype and functions of antigen-presenting cells (APCs) from atherosclerotic mice deficient in hypoxia-inducible factor (HIF)-1α. A, Signal Transducers and Activators of Transcription 3 (Stat3) mRNA (n=14 mice each) and (B) intracellular protein expression (n=5 mice each), and (C) mRNA expression of indicated cytokines (3 independent experiments, n=3–4 mice per experiment) in APCs isolated from spleens of Hif1a-wild-type (WT) low-density lipoprotein receptor-deficient (Ldlr−/−) and Hif1a-conditional knockout (CKO) Ldlr−/− mice fed a high-fat diet (HFD) for 8 weeks, analyzed by quantitative polymerase chain reaction and flow cytometry. mRNA expression was normalized to Hprt and presented relative to WT controls. D, Percent of interleukin (IL)-12+ cells among the APC population, analyzed by flow cytometry (n=6). Representative dot plots are shown (values indicate gated events among APCs). E, F, and G, APCs isolated from spleens of Hif1a-WT Ldlr−/− and Hif1a-CKO Ldlr−/− mice fed a HFD for 8 weeks and pulsed with OVA323-339 peptide were cocultured with naive CD4+ OT-II T cells for 3 days. T-cell proliferation was analyzed by CFSE dilution (E) and polarization by intracellular staining for interferon (IFN)-γ and IL-17a (F). Quantification and representative dot plots are shown (values indicate gated events among CD4+ T cells, 3 independent experiments, n=3–5 mice per experiment). G, mRNA expression of Stat3, Il12, and Ifng in whole aortae of Hif1a-WT Ldlr−/− and Hif1a-CKO Ldlr−/− mice fed a HFD for 8 weeks (normalized to Hprt and expressed relative to WT controls, n=3 mice). H, Intracellular STAT3 protein expression in CD11c+MHC-II+ APCs in the aorta of Hif1a-WT Ldlr−/− and Hif1a-CKO Ldlr−/− mice fed a HFD for 8 weeks (n=5 mice per group), analyzed by flow cytometry. Representative histograms for STAT3 fluorescence are shown (solid line, Hif1a-WT; dotted line, Hif1a-CKO; filled dark gray line, Hif1a-WT fluorescence minus 1 control (FMO), filled faint gray line Hif1a-CKO FMO). I, Frequencies of IFN-γ+CD4+ T cells in the aorta of Hif1a-WT Ldlr−/− and Hif1a-CKO Ldlr−/− mice fed a HFD for 8 weeks (n=3–4 per group). **P<0.01, ***P<0.001.
reduced IFN-γ-producing T-cell frequencies in cocultures with Hif1a-CKO Ldlr−/− APCs (not shown).

Notably, a significant decrease in Stat3 mRNA expression was observed in aortic tissue and in STAT3 protein levels in lesional APCs of atherosclerotic Hif1a-CKO Ldlr−/− mice, associated with a significant increase in Il12 and Ifng transcript expression (Figure 4G and 4H). Furthermore, an increased frequency of IFN-γ-producing T cells among CD4+ T cells was observed in the aorta of Hif1a-CKO Ldlr−/− mice (Figure 4I), suggesting that HIF1α may also control APC functions within lesions.

**HIF1α-Deficient APCs Promote Atherosclerosis Because Of Reduced STAT3 Expression**

To further confirm that Hif1a-CKO APCs promote atherosclerosis in a STAT3-dependent manner, we used a Cre-dependent system for STAT3 expression (pLB2-Ubi-FLIP). We generated a vector in which Stat3 cDNA was cloned in the reverse orientation and flanked by inverted loxP sequences, such that Cre-induced recombination irreversibly flipped Stat3 to a sense orientation, resulting in expression of STAT3 under the ubiquitin promoter in all Cre-expressing cells. The specificity of the Cre system was validated in vitro (Figure XA in the online-only Data Supplement). Transduction of BM cells from Cd11c-cre+ mice with lentivirus carrying the pLB2-Ubi-FLIP-STAT3 vector, but not an empty control vector, confirmed significantly elevated Stat3 mRNA expression in differentiated BMDCs after 7 days (Figure XB in the online-only Data Supplement).

Hif1a-WT and Hif1a-CKO BM cells were transduced with lentivirus containing control or pLB2-Ubi-FLIP-STAT3 vector and transplanted into lethally irradiated Ldlr−/− mice. Notably, increased lesion formation in Ldlr−/− mice carrying control virus-transduced Hif1a-CKO BM (CKO-BM+Ctrl-virus→Ldlr−/−) versus Hif1a-WT BM (WT-BM+Ctrl-virus→Ldlr−/−) in the aortic root and aorta was completely prevented by transduction with the pLB2-Ubi-FLIP-STAT3 vector (CKO-BM+STAT3-virus→Ldlr−/−) after 4 weeks of high-fat diet, and similar to levels seen in WT-BM+STAT3-virus→Ldlr−/− mice (Figure 5A–5C). However, this was paralleled by a reduction of the elevated total numbers of T cells in the aorta, and an abrogation of increased frequencies of IFN-γ+ CD4+ T cells in spleens of these mice (Figure 5D and 5E), clearly indicating that diminished STAT3 entails proatherogenic effects of Hif1α deficiency in APCs. WT-BM+Stat3-virus→Ldlr−/− mice displayed a reduction in atherosclerotic lesion formation in the aortic root but not in the aorta, no changes in aortic T-cell frequencies and a small trend toward decreased Th1 cell responses in the spleen when compared with WT-BM+Ctrl-virus→Ldlr−/− mice (Figure 5A and 5B), suggesting that prevailing actions of natural HIF1α on STAT3 expression in WT APCs dampen effects of an additional overexpression in atherosclerosis. Splenic APC isolated from WT-BM+Stat3-virus→Ldlr−/− and CKO-BM+Stat3-virus→Ldlr−/− mice displayed an enhanced expression of Stat3 when compared with WT-BM+Ctrl-virus→Ldlr−/− or CKO-BM+Ctrl-virus→Ldlr−/− mice (Figure 5C in the online-only Data Supplement), confirming overexpression of Stat3 in APCs in vivo.

![Figure 5](https://example.com/five.png)

**Expression of HIF1α, STAT3, and IL-12 in APCs in Human Atherosclerotic Lesions**

To finally assess whether these mechanisms may also be relevant to human disease, immunostaining of human atherosclerotic carotid artery plaques was performed. Similar to findings in mice and previous reports,11–14 we detected hypoxia and abundant HIF1α protein expression in atherosclerotic carotid artery plaque tissue (Figure 6A). Costaining for the APC markers S100 and CD11c revealed that the majority of APCs were hypoxic and expressed HIF1α, respectively (Figure 6A–6C). Furthermore, we could detect both STAT3 and IL-12 protein in colocalization with S100+ APCs (Figure 6A). These data indicate that APCs express HIF1α, STAT3, and IL-12 in human atherosclerotic lesions. In addition, real-time-polymerase chain reaction analyses of atherosclerotic plaques obtained from patients with high-grade carotid artery stenosis, histologically classified as early and advanced stages...
lesion formation was observed in mice deficient in HIF-1α in APCs, together with an expansion of proinflammatory Th1 cells both locally within lesions and systemically, indicating that APC-expressed HIF-1α is of paramount importance in balancing uncontrolled Th1-cell responses and atherosclerosis in Ldlr−/− mice. Mechanistically, we could demonstrate that HIF1α directly binds the Stat3 promoter to control its transcription. Overexpression of Stat3 in Hif1a-deficient APCs in BM reversed enhanced atherosclerotic lesion formation and reduced Th1-cell expansion in chimeric Ldlr−/− mice. These findings offer unique insight into the regulatory function of HIF1α in APCs (Figure XI in the online-only Data Supplement), and substantiate the critical role of APCs in controlling immune mechanisms that drive atherogenesis.

In humans and apolipoprotein E–deficient mice, HIF1α expression was detected in atherosclerotic lesions and to increase from early to stable lesions. In line, we were able to detect an upregulation of HIF1α expression in atherosclerotic aortae and aortic roots of Ldlr−/− mice when compared with healthy controls, and in advanced versus early human atherosclerotic lesions. Expressed in many cell types, HIF1α was also found to colocalize with hypoxic CD11c+ cells within lesions in both mice and humans.

Limited evidence on the role of HIF1α in atherosclerotic lesion formation exists. Mice lacking HIF1α in CD4+ T cells were previously shown to display increased T-cell activation, associated with an augmented neointimal femoral artery hyperplasia after cuff placement. Systemic hydrodynamic injection of plasmids encoding constitutively active Hif1a into Apoe−/− mice, resulting in HIF1α overexpression predominantly in CD4+ T cells, lead to a reduction in lesion formation, associated with a shift toward an anti-inflammatory cytokine expression profile in CD4+ T cells. However, in contrast to these studies, which would be consistent with an induction of Foxp3 and regulatory T cells by HIF1α, and the demonstration of a protective function of regulatory T cells in atherosclerosis, deficiency in HIF1α was more recently shown to diminish Th17 but to enhance regulatory T-cell development in CD4+ T cells.

In macrophages, HIF1α has been described to be critical for maintaining intracellular energy homeostasis, and Hif1a-deficient LysM-cre+ macrophages were shown to display normal cytokine production but an abrogated migratory capacity, preventing skin infiltration and inflammation. However, a reduced production of proinflammatory cytokines was demonstrated in Hif1a-deficient LysM-cre+ macrophages in response to lipopolysaccharide, together with a protection from lipopolysaccharide-induced sepsis. In the context of atherosclerosis, HIF1α was suggested to exert proatherogenic functions in cultured macrophages by promoting cholesterol accumulation. Variable effects of hypoxia-induced HIF-1α expression have also been shown in DCs. For instance, a reduction in costimulatory molecule expression and of the stimulatory capacity for T-cell functions was observed in Ldlr−/− mice, whereas increased expression of costimulatory molecules and an induction of allogeneic lymphocyte proliferation in response to lipopolysaccharide was noted in another report in vitro, whereas both studies described an upregulated production of
proinflammatory cytokines. Before our study, the direct in vivo role of HIF1α in APCs in atherosclerosis had not been addressed.

We here deleted Hif1a specifically in CD11c+ APCs, allowing a definite assessment of its role under physiological conditions and in atherosclerosis in vivo. APCs differentiated normally with no differences in their numbers or maturation in Hif1a-deficient mice. Moreover, no differences in APC phenotype and T-cell activation were noted in young, healthy mice, indicating that HIF1α plays a subordinate role in maintaining homeostatic APC functions. In atherosclerotic Ldlr−/− mice, however, a significant increase in IL-12 was observed in Hif1a-deficient APCs, whereas other cytokines and the expression of MHC-II and costimulatory molecules were unaltered. Moreover, an enhanced activation of CD4+ T cells and increased frequencies of Th1 cells were observed in Hif1a-CKO Ldlr−/− versus Hif1a-WT Ldlr−/− mice in vivo in the aorta and spleen, and in cocultures with Hif1a-deficient APCs isolated from atherosclerotic Ldlr−/− mice and loaded with OVA as a model antigen in vitro. These data suggest that Hif1a deficiency in APCs drives T-cell activation and Th1 differentiation, and that the effects of HIF1α deficiency are systemic.

Hypoxia can frequently be detected in atherosclerotic plaques. Hypoxyprobe (pimonidazole) is metabolized in living cells experiencing oxygen levels below 10 mm Hg (=1% O2). Cells positive for pimonidazole are thus viable and hypoxic, but do not experience a total lack of oxygen (anoxia). Both the thickness of the plaque exceeding the maximum oxygen diffusion distance, and more importantly, the high metabolic demand of cells within chronically inflamed tissue contribute to plaque hypoxia also within the oxygen diffusion limit in symptomatic patients, rabbits, and mice. In line, we detected hypoxic regions in atherosclerotic lesions of Ldlr−/− mice in luminal and intramural plaque cells. In addition to hypoxia, however, HIF1α expression can also be triggered and potentiated by oxidized LDL, lipopolysaccharides, and proinflammatory cytokines. Hence, HIF1α expression in hypoxic vascular APCs, known to ingest lipids and to be exposed to cytokines, may arise from a combination of these factors. Likewise, increased HIF1α in splenic APCs may have been activated by systemically increased lipid mediators or atherogenic cytokines, possibly in combination with relative hypoxia because of higher oxygen consumption under conditions of splenic inflammation. In this regard, it is interesting that similar changes in Th1 polarization were observed on systemic immunization with OVA in otherwise healthy CKO mice, providing further evidence that HIF1α controls APC-driven T-cell responses in inflammation also unrelated to atherosclerosis.

Increased atherosclerotic lesion size in Hif1a-CKO Ldlr−/− mice was accompanied by an increased necrotic core area. It was recently shown that silencing of HIF1α provokes a loss in viability with increased rates of apoptosis and necrosis in cultured human macrophages, potentiated in the presence of oxidized LDL or under hypoxic conditions. Although the potential impact of reduced monocyte/macrophage viability in atherosclerotic plaques is unclear and may depend on plaque stage, an increased apoptosis/necrosis of HIF1α-deficient APCs may have contributed to the expansion of the necrotic core in our model, warranting further investigations of this mechanism and its impact on atherogenesis in the future.

Notably, Ldlr−/− mice carrying LysMCre+ Hif1aflx/fox BM did not display any differences in plaque size or T-cell activation. This may appear counterintuitive as a substantial proportion of CD11c+ APCs, for example, monocyte-derived DCs and CD11c+ macrophages, would also lose expression of HIF1α in this model. However, HIF1α may have proatherogenic functions in CD11c+ myeloid cell subsets that promote atherosclerosis, such as Ly6C<sup>hi</sup> monocytes, macrophages, and neutrophils that contrast with its protective role in CD11c+ APCs. For instance, several reports have described a proinflammatory function of HIF1α in neutrophils or macrophages. Hence, the loss of protective HIF1α signaling in some CD11c+ cells may have been counterbalanced by the loss of its proatherogenic functions in other cell types in LysMCre+ Hif1aflx/fox mice. Alternatively, the phenotype observed in CD11c+cre+ Hif1aflx/fox mice may be preferentially related to atheroprotective functions of HIF1α in classical DCs. In the future, lineage-specific deletion of HIF1α in novel models may provide a clearer picture of its role in these various cell populations.

We did not detect any alterations in nuclear factor-κB, IKKα, or Ikβα expression in APCs deficient in HIF1α, similar to Hif1α-deficient LysM-Cre<sup>+</sup> macrophages, indicating that the deletion of Hif1a does not directly affect the nuclear factor-κB pathway per se. In agreement with the identification of binding sites in silico, ChIP analyses demonstrated direct binding of HIF1α to the Stat3 promoter, and Hif1a-deficient APCs to display a reduction in Stat3 mRNA and protein expression. These findings reveal HIF1α as an important regulator of STAT3 expression. Interestingly, STAT3 is known to exert immune-suppressive and anti-inflammatory functions in myeloid cells, and mice with Stat3-deficient APCs were previously shown to produce significantly more IL-12 in response to lipopolysaccharide, associated with an increased capacity to stimulate T-cell proliferation and IFN-γ secretion. Accordingly, overexpression of STAT3 reduced Il12 transcript levels in BMDCs, whereas a dominant negative mutant of STAT3 elevated Il12 expression, corroborating evidence that STAT3 interferes with Il12 transcription. Importantly, these effects occurred downstream of HIF1α, as also evidenced in Hif1α-deficient APCs. Lentiviral transduction of Hif1a-CKO BM with overexpression of STAT3 in APCs reversed the enhanced atherosclerotic lesion formation, decreased T-cell infiltrates, and reduced Th1-cell polarization in chimeric Ldlr<sup>−/−</sup> mice, confirming that increased levels of HIF1α and STAT3 in APCs are pivotal in controlling atherosclerotic plaque formation. In line with a clear but nonsignificant trend toward increased STAT3 expression in splenic APCs, marginal effects on plaque size and unaffected aortic T-cell accumulation and Th1 cell responses were observed in WT-BM+Ctrl-virus→Ldlr−/− versus WT-BM+STAT3-virus→Ldlr−/− mice. This may indicate that reduced STAT3 availability in CKO APCs rather than its additional supplementation in WT APCs plays a subordinate role in healthy mice, indicating that HIF1α deficiency in APCs drives T-cell activation and Th1 differentiation.
APCs that already inherently display increased HIF1α and STAT3 levels in atherosclerosis determines disease development in this setting.

Interestingly, human APCs exposed to hypoxia that display increased levels of HIF1α showed a reduced secretion of IL-12 and induced lower T-cell IFN-γ production, suggesting an HIF1α-triggered pathway restraining Th1 responses in human APCs. Notably, extending previous findings describing the presence of hypoxia and HIF1α in human atherosclerotic lesions, we here demonstrate that APCs express HIF1α, STAT3, and IL-12 in human atherosclerotic lesions. Furthermore, an increased HIF1α and STAT3 but a decreased IL-12 mRNA expression was observed in advanced versus early carotid artery plaques. These data suggest that the regulatory signaling axis revealed in our study in APCs in mice may also be operative in human disease. Interestingly, IL-12 expression in plasma and plaque tissue was previously shown to correlate with IFN-γ expression, with T cells being the principal source of IFN-γ in the arterial wall in humans, in line with the notion that IL-12-controlled Th1 T-cell responses are of primary importance during plaque development.

The bidirectional effects of HIF1α-deficiency in APCs on other lesional cell types and their contribution to lesion formation remain to be addressed. For instance, mast cells are present in atherosclerotic plaques and are considered to promote lesion growth and plaque destabilization. Mast cell-derived cytokines, via an induction of HIF1α in APCs, may lead to an attenuated proatherogenic APC phenotype balancing overshooting inflammation in atherosclerosis, and be in line with mast cells often ensuing Th2-type inflammatory responses. However, proinflammatory mediators released by mast cells, as induced by the contact with T cells, which showed an increased activation in CKO Ldlr−/− mice, may have also contributed to enhanced inflammation and plaque progression in our study.

Although it is widely acknowledged that Th1-mediated immune responses drive atherosclerotic lesion formation, still little is known about the pathways and transcription factors in APCs that drive T-cell polarization in atherosclerosis. Moreover, although HIF-1α can be detected in atherosclerotic lesions in both mice and humans and can modulate immune responses, its cell-specific role in atherosclerosis had not been addressed previously. Although dispensable under homeostatic conditions, our findings demonstrate that HIF1α balances APC activation and Th1 polarization during atherogenesis in Ldlr−/− mice and attenuates disease progression.

Acknowledgments

We thank Melanie Schott for excellent technical assistance, Dr Peilin Zheng for help with lentivirus production, and Dr Eliana Ribechini for help with the immunization assay.

Sources of Funding

This work was supported by the VENI fellowship of the Netherlands Organization of Scientific research (016.116.017) to J.C. Sluimer, and the Deutsche Forschungsgemeinschaft (ZE827/1-2, SFB688 TPA22) to A. Zernecke. S. Kessler is the recipient of a Career Development Award from Juvenile Diabetes Research Foundation (2-2010-383).

Disclosures

None.

References

Atherosclerosis remains the number one cause of death in the Western world. Insights into the mechanisms of disease development are still limited. The transcription factor hypoxia-inducible factor (HIF)-1α is induced under hypoxic conditions, but can also be upregulated by inflammatory stimuli. We here show that atherosclerotic lesion formation is associated with an upregulated expression of HIF1α in atherosclerotic lesions and antigen-presenting cells in atherosclerosis-prone mice. By conditionally deleting HIF1α in CD11c+ cells, we reveal that HIF1α balances excessive antigen-presenting cell-mediated proatherogenic T-cell proliferation and Th1 polarization. In contrast, deletion of HIF1α in LysM+ BM cells in Ldr−/− mice did not affect lesion formation or T-cell activation. These findings offer unprecedented insights into the function of HIF1α in antigen-presenting cells in atherosclerosis, and provide the first evidence that this transcription factor restrains DC-driven T-cell responses in atherosclerosis.

Significance
Deficiency of HIF1α in Antigen-Presenting Cells Aggravates Atherosclerosis and Type 1 T-Helper Cell Responses in Mice
Sweena M. Chaudhari, Judith C. Sluimer, Miriam Koch, Thomas L. Theelen, Helga D. Manthey, Martin Busch, Celina Caballero-Franco, Frederick Vogel, Clement Cochain, Jaroslav Pelisek, Mat J. Daemen, Manfred B. Lutz, Agnes Görlach, Stephan Kessler, Heike M. Hermanns and Alma Zernecke

Arterioscler Thromb Vasc Biol. published online September 24, 2015;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Deficiency of HIF1alpha in dendritic cells aggravates atherosclerosis and type 1 T helper cell responses in mice

Sweena M. Chaudhari, Judith C. Sluimer, Miriam Koch, Thomas L. Theelen, Helga D. Manthey, Martin Busch, Celia Caballero-Franco, Frederick Vogel, Clement Cochain, Jaroslav Pelisek, Mat J. Daemen, Manfred Lutz, Agnes Görlich, Stephan Kissler, Heike M. Hermanns, Alma Zernecke
Figure SI. Hif1a deletion efficiency
(a,b) CD11c+ APCs and CD4+ T cells were isolated from spleens of atherosclerotic Hif1a-WT Ldlr-/- and Hif1a-CKO Ldlr-/- mice, and Hif1a deletion from genomic DNA (a) and Hif1a mRNA expression (b) was analyzed by qPCR. mRNA expression was normalized to Hprt reference gene and relative to WT controls (n=3 mice). (c) Hif1a mRNA expression was also analyzed in Hif1a-WT and Hif1a-CKO BMDCs by qPCR (n=3 mice). mRNA expression was normalized to Hprt and presented relative to WT controls. Data are presented as mean ± SEM. *p<0.05, ***p<0.001.
Figure SII. Ldlr<sup>−/−</sup> mice deficient in Hif1α in APCs display enhanced T-cell activation.

(a-l) Flow cytometric analyses of T cell distributions in blood (a-f) and lymph nodes (g-l) of atherosclerotic Hif1α-WT Ldlr<sup>−/−</sup> (n=10) and Hif1α-CKO Ldlr<sup>−/−</sup> (n=7-12) mice fed a high fat diet for 8 weeks. Numbers of CD3<sup>+</sup> T cells (a,g), frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells among CD3<sup>+</sup> T cells (b,h), frequencies of activated CD44<sup>high</sup> CD62L<sup>low</sup> (c,i) and naïve CD62L<sup>high</sup> CD4<sup>+</sup> T cells (d,j), IFNγ<sup>+</sup> CD4<sup>+</sup> T cells and IL-17<sup>a</sup> CD4<sup>+</sup> T cells (e,k) and FoxP3<sup>+</sup> CD25<sup>−</sup> CD4<sup>+</sup> Tregs (f,l) were quantified. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
Figure SIII. Deficiency of HIF1α in APCs does not alter T-cell activation under homeostatic conditions.

(a-j) Flow cytometric analyses of T-cell distributions in spleen (a-e) and lymph nodes (f-j) of 8 week old Hif1α-WT (n=4) and Hif1α-CKO (n=4 mice). Numbers of CD3⁺ T cells (a,f), frequencies of CD4⁺ and CD8⁺ T cells among CD3⁺ T cells (b,g), frequencies of activated CD44⁺CD62L⁻ T cells (c,h) and naïve CD62L⁺CD44⁻ T cells (d,i), IFNγ⁺ CD4⁺ T cells and IL-17a⁺ CD4⁺ T cells (e,j) were quantitated. Representative dot plots showing intracellular IFNγ versus IL-17a expression are shown; values indicate gated events among CD4⁺ T cells. Data are presented as mean ± SEM.
Figure SIV. Mice with HIF1α deficient APCs show enhanced Th1 polarization in response to systemic immunization

WT and CKO mice were immunized with OVA protein and CFA, and Th1 polarization was analyzed by intracellular staining for IFNy by flow cytometry in lymph nodes (a) and spleens (b) (n=4 per group). Data are presented as mean ± SEM. ***p<0.001.
Figure SV. Deficiency of HIF1α in LysM+ myeloid cells does not affect plaque growth

(a) Hif1a mRNA expression in bone-marrow derived macrophages from LysM-cre*Hif1a+/+Ldlr-/- and LysM-cre*Hif1aflox/flox→Ldlr-/- mice in normoxia (0 hour) and under hypoxic conditions (0.2% O2) for 1 and 4 hours; mRNA expression was presented relative to WT controls. (n=3 per group). (c) Quantification of plaque area and (d) plaque Mac3+ macrophage content in aortic roots of atherosclerotic LysM-cre*Hif1a+/+Ldlr-/- and LysM-cre*Hif1aflox/flox→Ldlr-/- mice (n=16 each) fed a high fat diet for 6 weeks; representative H&E stained sections are shown (scale bars: 100μm). (e) mRNA expression of M1 markers Il12 and Nos2 and M2 markers Mrc1 and Igf1 in untreated (UT) or TNFα (TNF) treated BMMs from Hif1a-CKO mice and Hif1a-WT mice, as assessed by qPCR. mRNA expression was normalized to Hprt and presented relative to Hif1α-WT controls (n=5). Data are presented as mean ± SEM. *p<0.05.
Hif1a-deficient APCs show no alterations in CCR7 expression or migration capacity.

(a) Ccr7 mRNA expression in BMDCs from Hif1a-WT and Hif1a-CKO mice, analyzed by qPCR (n=5 mice per group). mRNA expression was normalized to Hprt and presented relative to controls. (b) Migration of BMDCs from Hif1a-WT and Hif1a-CKO mice towards CCL19, as assessed in in vitro migration assays. Values are expressed relative to the random migration of WT BMDCs without chemotactic stimulation (blank, n=3 independent experiments, performed in triplicates). (c) CCR7 protein expression on splenic APCs from Hif1a-WT Ldlr<sup>-/-</sup> and Hif1a-CKO Ldlr<sup>-/-</sup> mice fed a high fat diet for 8 weeks (n=6 mice per group). Representative histograms depicting CCR7 expression in Hif1a-WT Ldlr<sup>-/-</sup> and Hif1a-CKO Ldlr<sup>-/-</sup> splenic APCs (solid line - Hif1a-WT Ldlr<sup>-/-</sup>, dotted line - Hif1a-CKO Ldlr<sup>-/-</sup>, filled dark grey line - Hif1a-WT Ldlr<sup>-/-</sup> FMO, filled faint grey line - Hif1a-CKO Ldlr<sup>-/-</sup> FMO). Data are presented as mean ± SEM.
Figure SVII.

(a) mRNA expression of indicated co-stimulatory molecules in mature BMDCs (n=3 mice) generated from Hif1a-WT and Hif1a-CKO mice and in (b) APCs isolated from spleens of Hif1a-WT Ldlr^-/- and Hif1a-CKO Ldlr^-/- mice fed a high fat diet for 8 weeks (n=12 mice), as analyzed by qPCR. mRNA expression was normalized to Hprt and presented relative to Hif1a-WT controls. (c) Representative histogram depicting co-stimulatory molecule expression in Hif1a-WT and Hif1a-CKO BMDCs (solid line - Hif1a-WT, dotted line - Hif1a-CKO, filled dark grey line - Hif1a-WT FMO, filled faint grey line - Hif1a-CKO-FMO). Data are presented as mean ± SEM.
Figure SVIII

(a) mRNA expression of indicated cytokines, assessed by qPCR (3 independent experiments, n=3-4 mice per experiment), (b) IL-12 protein levels in culture supernatants determined by ELISA (n=3 mice each), (c) Stat3 mRNA expression assessed by qPCR (n=5 mice) and (d) STAT3 intracellular protein expression (n=4 mice) analyzed by flow cytometry in TNFα-matured BMDCs generated from Hif1a-WT and Hif1a-CKO mice. mRNA expression was normalized to Hprt and presented relative to WT controls. Representative histograms for STAT3 fluorescence are shown (solid line - Hif1a-WT, dotted line - Hif1a-CKO, filled dark grey line - Hif1a-WT fluorescence minus one control (FMO), filled faint grey line Hif1a- CKO FMO). (e) Il12 mRNA expression in Hif1a-WT and Hif1a-CKO BMDCs transfected with pCAGGS-STAT3 wild type vector, pCAGGS-STAT3D dominant negative mutant vector, or empty vector, analyzed by qPCR (normalized to Hprt and expressed relative to Hif1a-WT empty vector, 3 independent experiments, n=3 mice per experiment). (f) ChIP assay was performed using anti-HIF1α antibody or respective IgG control antibody for HIF1α precipitation from C57BL/6J BMDCs. Immunoprecipitated chromatin was analyzed by qPCR using primers to HIF1α binding-sites on the Stat3 promoter. Results were normalized to input chromatin (representative with n=3 mice from 2 independent experiments); a representative agarose gel of PCR-amplified samples is shown. *p<0.05, **p<0.01, ***p<0.001.

Figure SVIII. HIF1α-deficient BMDCs display increased IL-12 and decreased STAT3 expression.
Figure SIX. *Hif1a*-deficient APCs show no changes in *Nfkb* expression.

qPCR was performed to analyze mRNA expression of *Nfkb1/p105* and *Rela/p65* in (a) mature BMDCs from *Hif1a*-WT and *Hif1a*-CKO mice (n=3 mice) and (b) splenic APCs isolated from *Hif1a*-WT *Ldlr*⁻/⁻ and *Hif1a*-CKO *Ldlr*⁻/⁻ mice fed a high fat diet for 8 weeks (n=12 mice). mRNA expression was normalized to *Hprt* and presented relative to controls. Data are presented as mean ± SEM.
Figure SX. CD11c-specific Stat3 mRNA expression
(a-c) qPCR analysis of Stat3 mRNA expression in (a) HEK293F cells co-transfected with the STAT3 overexpressing vector pLB2-Ubi-FLIP-STAT3 and with or without a Cre producing pLC-cre vector (n=6 each), (b) BMDCs differentiated from BM of Cd11c-cre+ mice transduced with control or pLB2-Ubi-FLIP-STAT3 lentivirus (n=3 each) and (c) splenic APCs isolated from WT-BM+Ctrl-virus→Ldlr−/− mice, WT-BM+STAT3-virus→Ldlr−/− mice, CKO-BM+Ctrl-virus→Ldlr−/− mice and CKO-BM+STAT3-virus→Ldlr−/− mice (n=5-8 each). mRNA expression was normalized to Hprt and presented relative to controls. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
**Figure SXI.**

**Summary of main findings.**
The transcription factor HIF1α is upregulated in atherosclerotic lesions in response to hypoxia or inflammatory stimuli. HIF1α directly binds the Stat3 promoter to control its transcription. STAT3 in turn dampens pro-inflammatory IL-12 secretion, which triggers Th1 T cell activation. In CKO mice with a deletion of Hif1a in CD11c⁺ APCs in Ldlr⁻/⁻ mice, STAT3 expression is decreased, whereas IL-12 expression is increased, promoting an expansion of pro-inflammatory Th1 T cells and an accelerated atherosclerotic lesion formation. HIF1α thus functions to balance APC-activation and Th1-polarization during atherogenesis in Ldlr⁻/⁻ mice, and to attenuate the progression of atherosclerosis.
Supplementary Table SI

Cholesterol levels and T cell distributions in atherosclerotic \( Ldlr^{-/-}(\text{LysM-cre}^{+}\text{Hif1}^{\alpha\text{flox/flox}}\text{BM}) \) mice.

<table>
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<tr>
<th></th>
<th>( \text{LysM-cre}^{+}\text{Hif1}^{\alpha^+/+} )</th>
<th>( \text{LysM-cre}^{+}\text{Hif1}^{\alpha\text{flox/flox}} )</th>
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<td>Serum cholesterol (μg/ml)</td>
<td>9,384 ± 498</td>
<td>8,964 ± 353</td>
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<tr>
<td>Lymph nodes</td>
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<td>CD4⁺:CD8⁺</td>
<td>0.62 ± 0.06</td>
<td>0.60 ± 0.05</td>
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<td>%CD4⁺CD25⁺</td>
<td>2.72 ± 0.13</td>
<td>2.74 ± 0.22</td>
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<tr>
<td>%CD3⁺CD69⁺</td>
<td>33.16 ± 0.83</td>
<td>36.18 ± 0.80</td>
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<td>Spleen</td>
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<td></td>
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<tr>
<td>%CD4⁺/CD8⁺</td>
<td>1.00 ± 0.09</td>
<td>1.27 ± 0.11</td>
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<tr>
<td>%CD4⁺CD25⁺</td>
<td>1.44 ± 0.05</td>
<td>1.55 ± 0.10</td>
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<tr>
<td>%CD3⁺CD69⁺</td>
<td>24.72 ± 0.42</td>
<td>26.66 ± 1.52</td>
</tr>
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</table>
Materials and Methods

Animals
C57BL/6J mice, Ldlr<sup>-/-</sup> mice, Hif1α<sup>fl ox/ fl ox</sup> mice, Cd11c-cre mice, and OT-II mice (all C57BL/6J background) were obtained from the Jackson laboratory or Charles River. Cd11c-cre mice, Hif1α<sup>fl ox/ fl ox</sup> mice and Ldlr<sup>-/-</sup> mice were crossed to generate littermate Cd11c<sup>-cre+</sup> Hif1α<sup>fl ox/ fl ox</sup> (Hif1α-CKO) and Cd11c<sup>-cre+</sup> Hif1α<sup>+/+</sup> (Hif1α-WT) mice, as well as Cd11c<sup>-cre+</sup> Hif1α<sup>fl ox/ fl ox</sup> Ldlr<sup>-/-</sup> (Hif1α-CKO Ldlr<sup>-/-</sup>) and Cd11c<sup>-cre+</sup> Hif1α<sup>+/-</sup> Ldlr<sup>-/-</sup> (Hif1α-WT Ldlr<sup>-/-</sup>) mice. 8 week old male Hif1α-WT Ldlr<sup>-/-</sup> and Hif1α-CKO Ldlr<sup>-/-</sup> mice or Ldlr<sup>-/-</sup> mice were placed on a high fat diet (15% fat, 1.25% cholesterol, Altromin) for 8 weeks. LysM-cre<sup>+</sup>Hif1α<sup>fl ox/ fl ox</sup> mice (C57BL/6J background)<sup>1</sup> were kindly provided by Randall Johnson. All experiments were approved by local authorities (Regierung von Unterfranken, or Maastricht University) and performed in compliance with directive 2010/63/EU of the European Parliament.

Immunohistochemistry and atherosclerotic lesion quantification
Hypoxia was detected using the Hypoxyprobe Plus kit (Hypoxyprobe Inc) according to the manufacturer’s instructions. Briefly, mice were injected with 60 mg/kg Pimonidazole hydrochloride and the hearts were harvested after 90 minutes to generate fixed frozen cryosections (5 μm). Hypoxia was detected using the provided FITC-MAb1 primary antibody and anti-FITC-HRP secondary reagent and visualized by the DAB peroxidase substrate kit (Vector laboratories). CD11c<sup>+</sup> cells and HIF1α were stained in cryosections of the aortic root using biotin-labeled anti-CD11c (Biolegend) and anti-HIF1α (Novus Biologics) antibodies, detected by Alexa-Fluor-555-Streptavidin (Molecular probes, Life Technologies) and Alexa-Fluor-488 anti-rabbit IgG (Molecular probes, Life Technologies), respectively. Nuclei were counter-stained by 4’,6-Diamidino-2-phenylindole (DAPI, Vector laboratories). The extent of atherosclerosis was assessed in serial sections of paraffin-embedded aortic roots stained with aldehyde fuchsin (basic fuchsin and acid aldehyde, Sigma Aldrich) or hematoxylin-eosin (Sigma) and in en face prepared aortas by staining for lipid depositions with Oil-Red-O<sup>2</sup>. Macrophages, T cells and SMCs were immunostained using specific antibodies to Mac-2 (Cedarlane labs) or Mac-3 (Becton Dickinson), CD3 (AbD Serotec) and α-actin (Sigma Aldrich), respectively, using standard immunohistochemistry techniques<sup>2</sup>. The co-localization of different DC markers with markers for hypoxia in human plaques was measured by multispectral imaging of immunohistochemical staining. Paraffin embedded and frozen human plaque sections were stained for the DC marker S100 (Dako) or CD11c (BD Biosciences) and the hypoxia marker pimonidazole (Hypoxyprobe store) or HIF-1α (Novus Biologicals), respectively. In addition, APCs were visualized by staining for the DC marker S100 in combination with STAT3 (Cell Signaling Technology) or IL12 (abcam), on paraffin embedded human plaque sections. From double staining, spectral imaging data sets from maximal three random regions of interest were taken between 420-720 nm (10 nm interval) at a 20x (plan apo.) magnification using a Nuance spectral imaging system (Perkin Elmer/Caliper Life Sciences, Hopkinton, MA, USA) mounted on a Zeiss Axiophot microscope. Slides stained for a single chromogen only (Vector Red and Vector Blue, both Vector Laboratories) were used to create a spectral library. The spectral library was used for computational segregation of the individual image components using the Nuance™ 3.0.2 software as described<sup>3</sup>. After spectral unmixing, pseudo-colors were assigned to unmixed images, and composite images showing co-localization were generated with the Nuance 3.0.2 software. Co-localization was quantified on a pixel-based measurement of the individual markers per microscopic field using the same software.

Quantitative real-time RT-PCR
Total RNA was isolated from APCs and BMMs using the RNeasy Mini kit (Qiagen), and from aortas using the RNeasy Micro kit (Qiagen) according to the manufacturer’s instructions. cDNA was reverse transcribed from total RNA using random primers and first strand cDNA synthesis kit (Fermentas). Genomic DNA (gDNA) was prepared from isolated CD11c<sup>+</sup> and CD4<sup>+</sup> T cells. qPCR analyses were performed from 20 ng cDNA or gDNA using SYBR green mix (Fermentas) and gene specific primer sets (Hprt: 5’-TCCTCCTCAGACCCGCTT-3’, 5’-
CCTGGTTCATCATCGCTAATC-3';  Hif1a: 5'-CAAGATCTCGGCGAAGCAA-3', 5'-GGTGAGCCTCATAACAGAAGCTTT-3'; Cd80: 5'-TCGTCTTTCACAAGTGTCTTCAG-3', 5'-TTGCCAGTAGATTCGGTCTTC-3'; Cd86: 5'-GAAGCCGAATCAGCCTAGC-3', 5'-CAGCGTTACTATCCCGCTCT-3'; Cd74: 5'-CACCGAGGCTCCACCTAA-3', 5'-GCAGGGATGTGGCTGACT-3'; Il4: 5'-CAACGAAGAACACCACAAGAG-3', 5'-ATGAATCCAGGCATCGAAAAGC-3'; Il6: 5'-GTGGCTAAGGACCAAGACCA-3', 5'-ACCACAGTGAGGAATGTCCA-3'; Il10: 5'-TGCACTACCAAAGCCACAAGG-3', 5'-TGGGAAGTGGGTGCAGTTATTG-3'; Il12a: Quantitect primer assay QT01048334 (Qiagen); Tgfb: 5'-GACGTCACTGGAGTTGTACGG-3', 5'-GGTTCATGTCATGGATGGTGC-3'; Tnfa: 5'-CTGTAGCCCACGTCGTAGC-3', 5'-GGTTGTCTTTGAGATCCATGC-3'; Nfkb1/p105: 5'-GAACCTCTCGGACAGCTTCG-3', 5'-TGTTGATCCATACCAAAGG-3'; Rela/p65: 5'-CATGCGATTCCGCTATAAATG-3', 5'-TCTGTGTAGCCATTGATCTTG-3'; Stat3: 5'-CTACCTCTACCGACATTCCC-3', 5'-GATGACCTTGCTGCTTCTTGCAG-3'; Nos2: 5'-GTGTTCTCGACCAACTACAAGA-3', 5'-GTGGAGGGTGGTGCAGTTCAC-3'; Igf1: 5'-CTGGCTCTCAGTACCACACT-3', 5'-TCCTGTGATCCATTGCTTGG-3'; Mrc1: 5'-CACCTCATACCTACAAACCAAG-3', 5'-CAGGAGGACCAGGTGAC-3'; Ccr7: 5'-CTCCTTGTCATTTCAGCGATTTGTAAT-3') in triplicates (7900HT Thermal cycler, Applied Biosystems). For analysis of the expression of genes in human atherosclerotic lesions, RNA was isolated from paraffin-embedded tissue sections adjacent to slides used for histological characterisation by the High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. In all cases 2 sections of 10 µm thickness were used, yielding 10-20 ng/µl of RNA per tissue sample. Isolated RNA was reverse transcribed using the cDNA Synthesis Kit RevertAid and oligo-dT primers supplied by the manufacturer (Fermentas, St. Leon-Rot, Germany). Quantitative Real-Time PCR was performed with cDNA using SYBRgreen fluorescence dye (peqLab, Erlangen, Germany) and specific primer pairs from Qiagen, QuantiTect Primer Assays using the ABI Prism 7700 Sequence Detector (Applied Biosystems).

Western blotting
Whole cellular lysates were separated by 8% or 10% SDS-PAGE, followed by semi-dry Western blotting onto a Nitrocellulose or PVDF-membrane (Whatman, GE Healthcare). Protein detection was conducted using antibodies from Cell Signaling Technology to NFκB p65 (C22B4), phosphor-NFκB p65-Ser536 (93H1), IkBα (polyclonal), GAPDH (14C10), from Sigma Aldrich to tubulin (B-5-1-2), Santa Cruz biotechnology to β-Actin (J1509) and from Abcam to HIF1α (GR30496-1), NFκB p105/p50 (E381), and enhanced chemiluminescence kit (Thermo Fisher Scientific Inc.) according to the manufacturers’ instructions. Quantification of the chemiluminescence signal was carried out on the FluorChemQ using the AlphaView® software (ProteinSimple). Equal loading of the gel was verified by stripping the membrane in 62.5 mM Tris HCl (pH 6.7) containing 2% SDS and 100 mM β-mercaptoethanol at 70°C for 20 minutes and redetection with antibodies recognizing the protein irrespective of its phosphorylation status or by detection of β-Actin, tubulin or GAPDH.

Serum cholesterol and triglyceride assays
Serum cholesterol and triglyceride levels were quantified using the Amplex Red cholesterol assay kit (Life technologies) or standard enzymatic techniques automated on the Cobas Fara centrifugal analyzer (Roche) and Triglyceride assay kit (Biotrend), according to the manufacturer’s instructions.

Flow cytometric analysis
Spleens and lymph nodes from mice were dissociated into single-cell suspensions. Blood and spleen were subjected to erythrocyte lysis. Aortic tissue was enzymatically dissociated using Liberase Blendzyme TL solution (Roche). Staining was performed using combinations of specific antibodies from BD Biosciences to CD19 (1D3), CD3 (500A2 or 17A2), CD45 (30-F11), CD8a (53-6.7), Gr1 (RB6-8C5), IFNγ (XMG1.2), MHC II (2G9); from eBioscience to CD115 (AF588), CD11b (M1/70), CD11c (N418), CD4 (RM4-5 or GK1.5), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL1), F4/80
BM8, Foxp3 (FJK-16s), IL-12 (C15.6), IL-17a (eBio17B7), CCR7 (4B12); from Cell Signaling Technology to STAT3 (124H6); R&D Systems to STAT3 (232209). Intracellular staining was performed using the Cytofix/Cytoperm solution (BD Biosciences) on cells treated with 2.5 µg/ml brefeldin, 50 ng/ml PMA and 750 ng/ml ionomycin for IFNγ and IL-12, and 2.5 µg/ml brefeldin and 1 µg/ml LPS for IL-12. Intracellular staining for Foxp3 and STAT3 was performed using the Transcription factor staining buffer set (eBioscience). Data were acquired using a FACSCanto II (BD Bioscience) or a FACS Calibur (BD Biosciences), and results analyzed by FlowJo 7.6 software (Tree Star).

**Immunization assay**
Mice were subcutaneously injected with 100 µg OVA protein (Sigma Aldrich) and complete Freund’s adjuvant (Sigma Aldrich). After 10 days, spleens and inguinal lymph nodes were harvested, and single cell suspensions re-stimulated with 5 µg/ml brefeldin, 10 ng/ml PMA and 1 µg/ml ionomycin overnight. Thereafter, cells were stained for IFNγ as described above and analyzed by flow cytometry.

**Bone marrow transplantation (BMT)**
Recipient 12 week old female Ldlr-/- mice were given acidified water (pH=2) containing neomycin (100 mg/L, Gibco) and polymyxin B sulphate (6x10^4 U/L, Gibco) from 1 week before until 4 weeks after BMT. One day after lethal irradiation (10 Gy), Ldlr-/- mice were injected intravenously with bone marrow cells (10^7 cells) isolated from femurs and tibias of male donor LysM-cre*Hif1a*floxflox and LysM-cre*Hif1a*+/+ mice. Mice were placed on a high fat diet (20% fat, 1.25% cholesterol, Research Diets) starting 4 weeks after BMT for a total of 6 weeks.

**APC isolation, bone marrow derived dendritic cells (BMDCs) and macrophages (BMMs)**
Splenic APCs were isolated using CD11c-microbeads (Miltenyi Biotec). For bone marrow-derived cell cultures, femurs were excised and cells flushed to prepare single cell suspension. For the generation of BMDCs, cells were cultured in RPMI-1640 (with 2 mM L-Glutamine, Gibco) supplemented with 10% FCS (PAA), 100 U/ml penicillin-streptomycin (Gibco), 50 µM β-mercaptoethanol (Gibco) and 50 ng/ml murine GM-CSF (Peprotech) for 7 days and matured with 100 ng/ml murine TNFα (Peprotech) for 24 h to induce Hif1a mRNA expression (2.4±0.3 fold over unstimulated controls, n=5, p<0.001). Alternatively, cells were cultured in RPMI-1640 (with 2 mM L-Glutamine) supplemented with 10% FCS, 100 U/ml Penicillin-Streptomycin, 50 µM β-mercaptoethanol and 15% L929-conditioned medium for 7 days to generate BMMs. Some BMMs were treated with 100 ng/ml murine TNFα (Peprotech) for 24 h.

**BMDC transmigration assay**
Serum starved BMDCs were allowed to migrate towards 100 ng/ml CCL19 (Peprotech) through 8 µm transmigration inserts (ThinCert, Greiner Bio-one) for 2 hours at 37°C. Transmigrated cells collected from the bottom chamber were enumerated using a Neubauer chamber.

**ELISA**
IL-12 protein levels were quantified in cell culture supernatants using the murine IL-12 mini ELISA development kit (Peprotech) according to the manufacturer’s instructions.

**Transfection of STAT3 vectors in BMDCs**
BMDCs were transfected with pCAGGS-STAT3 wild type vector, pCAGGS-STAT3D dominant negative mutant vector (kindly provided by K. Nakajima and T. Hirano), or empty vector using the Amaxa P4 Primary Cell 4D-Nucleofector kit (Lonza) following manufacturer’s instructions. Transfected cells were cultured in RPMI-1640 (with 2 mM L-Glutamine, Gibco) supplemented with 10% FCS (PAA), 100 U/ml penicillin-streptomycin (Gibco), 50 µM β-mercaptoethanol (Gibco) and 100 ng/ml murine TNFα (Peprotech) for 16 hours and then harvested for RNA isolation. Vector overexpression was confirmed by observing dramatically increased Stat3 mRNA expression in Hif1α-WT and Hif1α-CKO BMDCs transfected with wild
type (2,982 ± 756, and 1,186 ± 490 fold increase, respectively, n=3 each) and mutant vector (3,221 ± 1,045, and 3,530 ± 2,425 fold increase, respectively, n=3 each).

**Chromatin Immunoprecipitation (ChIP) assay**

Predicted HIF1α binding sites were determined within the Stat3 promoter using the MatInspector software (Genomatix) (GATTCCACGTGGTAAG at anchor position 117bp and CGCCCCCACGTGGGTGCC at anchor position 147bp from start of Stat3 gene; bases in bold show core HRE sequence). ChIP assays were performed using the ExactaChIP Human/Mouse HIF1α chromatin immunoprecipitation kit (R&D) according to the manufacturer’s instructions. In brief, BMDCs generated from C57BL/6J mice and treated with CoCl₂ (Sigma Aldrich) in vitro for 16 hours were cross-linked using 1% formaldehyde (Sigma Aldrich). After formaldehyde quenching with 125 mM glycine (Sigma Aldrich), cells were lysed, the lysates sonicated to shear chromatin and lysate supernatants prepared (serving as input DNA sample). The remaining supernatants were incubated with biotin anti-HIF1α or biotin normal goat IgG antibody overnight at 4°C. Antibodies were precipitated using Streptavidin agarose beads (Sigma Aldrich) and washed using kit buffers. Reverse cross-linking was done by boiling the samples with a Chelating Resin solution. DNA fragments were purified using the QIAquick DNA purification kit (Qiagen) and equal volumes from the elute were added to a PCR reaction with primers against HIF1α binding site in the Stat3 gene promoter region (5'-GCCCTGATACGGCTCGCTTCTGC-3', 5'-TGGGGACCGCCTAAGTGGCTG-3'). Quantitative PCR was carried out at an annealing temperature of 60°C and results were analyzed and presented as percent input. Amplified products were also separated by gel electrophoresis (1% agarose).

**Antigen-specific T cell proliferation and polarization**

Naïve CD4⁺ T cells were isolated from spleens and lymph nodes of OT-II mice using the CD4⁺CD62L⁺ T cell isolation kit (Miltenyi Biotec) and labeled with 5µM CFSE (Sigma Aldrich) for 10 minutes at 37°C. APCs were loaded with 1 µg/ml OVA 323-339 peptide (AnaSpec Inc.) for 45 minutes at 37°C. Naïve CD4⁺ T cells (1.5 x 10⁵ cells) were co-cultured with OVA peptide-loaded CD11c⁺ APCs (0.5 x 10⁵ cells) in RPMI-1640 medium (supplemented with 2mM L-glutamine, 10% FCS, 100 U/ml Penicillin/Streptomycin and 50 µM β-mercaptoethanol) for 3 days. For IL-12 blockade, neutralizing αIL-12 p35 antibody (eBioscience) was added. Flow cytometric analysis was performed to quantify T cell proliferation and polarization by CFSE dye-dilution and intracellular cytokine staining respectively.

**Transplantation of lentivirus transduced bone marrow cells**

A pLB2-Ubi-FLIP-STAT3 vector was prepared by cloning Stat3 cDNA (Open Biosystems library, clone ID 4923137) in the reverse orientation and flanked by inverted repeats of loxP sequences behind the Ubiquitin promoter. To test the specificity of the vector, HEK293F cells were co-transfected with the pLB2-Ubi-FLIP-STAT3 vector and a Cre recombine producing pIC-cre vector (kindly provided by Dr. Bernhard Nieswandt, Würzburg, Germany). Lentivirus particles incorporating the pLB2-Ubi-FLIP-STAT3 or empty control vector were generated as described. BM cells from Cd11c-cre⁺ mice or lineage-depleted BM cells from Hif1a-WT and Hif1a-CKO mice (Lineage Cell Depletion Kit, Miltenyi Biotec) were transduced with lentivirus particles (50 MOI) in the presence of DEAE-dextran (10 µg/ml, Sigma Aldrich). After 24 hours, cells were washed to remove free virus, and differentiated into BMDCs, or injected retro-orbital into lethally irradiated Ldlr⁻⁻ recipients (1x10⁶ cells) treated with neomycin sulphate (2g/L, Bela pharm) for 2 weeks before switching to a high fat diet for 4 weeks.

**Human atherosclerotic plaques**

For immunohistochemistry, a total of 40 atherosclerotic carotid arteries with advanced atherosclerotic lesions were obtained from patients undergoing vascular surgery or at autopsy to analyze expression in cryosections or paraffin-embedded sections, respectively. In addition, in 7 symptomatic patients the hypoxia marker pimonidazole (Hypoxygenprobe-1, 0.5 g/m² hypoxyprobe store) was used to detect hypoxia in human carotid atherosclerosis. The
investigation was approved by an external ethical committee and written informed consent was obtained. All patient data has previously been described. For real-time PCR analyses, atherosclerotic plaques were obtained from patients with high-grade carotid artery stenosis (>70%) after carotid endarterectomy. The study was performed according to the Guidelines of the World Medical Association Declaration of Helsinki. The local ethics committee (Klinikum rechts der Isar der Technischen Universität München) approved the study and written informed consent for being included in the Biobank was given by all patients. Carotid plaques were segmented in blocks of 3 to 4 mm, fixed in formalin and embedded in paraffin. Haematoxylin-eosin and Elastica van Gieson staining were performed in order to assess the stage/type of atherosclerosis. Histological classification of carotid atherosclerotic lesions was performed as described by Stary et al.12, 13 by two independent investigators blinded for the study in close collaboration with an experienced pathologist. Study specimens were divided into groups of early (stage I-III, n=10) and advanced atherosclerosis (stage V-VII, n=10).

Statistics
Data are presented as mean ± SEM. Data were analyzed by unpaired Student t tests, and if more than 2 groups were compared by ANOVA 1-way analysis of variance followed by Tukey’s or Dunnett’s multiple comparison post-hoc tests. Differences with p<0.05 were considered to be statistically significant.

References