Indoleamine 2,3-dioxygenase-1 is protective in atherosclerosis and its metabolites provide new opportunities for drug development

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Atherosclerosis is the major cause of cardiovascular disease (CVD), the leading cause of death worldwide. Despite much focus on lipid abnormalities in atherosclerosis, it is clear that the immune system also has important pro- and antiatherogenic functions. The enzyme indoleamine-2,3-dioxygenase (IDO) catalyses degradation of the essential amino acid tryptophan into immunomodulatory metabolites. How IDO deficiency affects immune responses during atherogenesis is unknown and we explored potential mechanisms in models of murine and human atherosclerosis. IDO deficiency in hypercholesterolemic ApoE$^{-/-}$ mice caused a significant increase in lesion size and surrogate markers of plaque vulnerability. No significant changes in cholesterol levels were observed but decreases in IL-10 production were found in the peripheral blood, spleen and lymph node B cells of IDO-deficient compared with IDO-competent ApoE$^{-/-}$ mice. 3,4,-Dimethoxycinnamoyl anthranilic acid (3,4-DAA), an orally active synthetic derivative of the tryptophan metabolite anthranilic acid, but not L-kynurenine, enhanced production of IL-10 in cultured splenic B cells. Finally, 3,4-DAA treatment reduced lesion formation and inflammation after collar-induced arterial injury in ApoE$^{-/-}$ mice, and reduced cytokine and chemokine production in ex vivo human atheroma cell cultures. Our data demonstrate that endogenous production of tryptophan metabolites via IDO is an essential feedback loop that controls atherogenesis and athero-inflammation. We show that the IDO pathway induces production of IL-10 in B cells in vivo and in vitro, suggesting that IDO may induce immunoregulatory functions of B cells in atherosclerosis. The favorable effects of anthranilic acid derivatives in atherosclerosis indicate a novel approach toward therapy of CVD.

atherosclerosis | indoleamine 2,3-dioxygenase | inflammation | cardiovascular disease | B cell

Cardiovascular disease (CVD) remains the biggest killer worldwide (1) despite a reduction in the CVD mortality rate over recent decades. Considering our aging and increasingly obese and diabetic populations, mortality seems likely to rebound (2). The immune response represents an important component of the pathogenesis of CVD that has yet to be successfully targeted therapeutically. Currently we lack insight into which immune-regulatory pathways in vessels might be successfully exploited to generate medicines to use in conjunction with lipid lowering agents.

Indoleamine-2,3,-dioxygenase (IDO) is the rate-limiting enzyme in the formation of a spectrum of immune-regulatory tryptophan (Trp) metabolites, including kynurenine (Kyn) and anthranilic acid. IDO expression is induced by inflammatory mediators, such as IFN-γ (3) and Toll-like receptor ligands (4), and its expression by myeloid and endothelial cells mediates immune regulation and endothelium-dependent vasodilation, respectively (5, 6). The Kyn:Trp ratio reflects IDO activity and changes in this ratio have been described in vascular diseases, suggesting that this pathway is activated in humans. The Kyn:Trp ratio is augmented in patients with coronary artery disease (7) and positively correlates with levels of the inflammation marker C-reactive protein and negatively with high-density lipoprotein cholesterol levels (8, 9). Plasma Kyn levels are associated with increased risk of acute myocardial infarction in patients with stable angina pectoris (10). In the Tampere Vascular Study, increased IDO expression was observed in the macrophage-rich cores of human atherosclerotic plaques (11).

An orally active synthetic derivative of the Trp metabolite anthranilic acid [3,4,-dimethoxycinnamoyl anthranilic acid (3,4-DAA)], was tested in the Prevention of REStenosis with Tranilast and its Outcomes (PRESTO) trial. Although no benefit was shown on restenosis, a significant reduction in myocardial infarction was observed in the treated group (12). With no restenosis prevention, we speculate that 3,4-DAA's mechanism of action is not related to blockade of smooth muscle cell (SMC) proliferation, but because of its anti-inflammatory effects (13, 14). 3,4-DAA is an antiallergic drug approved for use for asthma treatment in Japan that was originally discovered for its inhibitory effect on mast cell degranulation (15). 3,4-DAA reversed paralysis in mice with experimental autoimmune encephalomyelitis, a model of multiple sclerosis and ameliorated experimental arthritis (13, 14).

In this study, the effect of IDO-deficiency on murine atherosclerosis was dramatic, causing a significant increase in lesion size and surrogate markers of plaque vulnerability, demonstrating that IDO is a key endogenous regulator of plaque stability and inflammation in atherosclerosis. We also show that IDO boosts IL-10 production in

Significance

Inflammation is an important component of the pathogenesis of cardiovascular disease, the world's biggest killer. No antiinflammatory treatments have yet been developed to treat cardiovascular disease. Indoleamine 2,3-dioxygenase (IDO) is a critical enzyme in the metabolism of tryptophan that has been shown to be immune-regulatory in many diseases. ApoE$^{-/-}$ mice deficient in IDO (ApoE$^{-/-}$Indo$^{-/-}$) developed larger atherosclerotic lesions and an unfavorable lesion phenotype that may predispose to cardiovascular complications. Furthermore, administration of an orally active synthetic tryptophan metabolite (3,4-DAA) reduced disease development in mice and cytokine production in human atheroma. Our data demonstrate that endogenous production of tryptophan metabolites via IDO is an essential feedback loop that controls atherogenesis and athero-inflammation, defining a path toward the development of new therapeutics.

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The authors declare no conflict of interest.

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B cells. Administration of 3,4-DAA was also effective in reducing lesion size and inflammation in an arterial injury model and a human ex vivo atheroma cell culture model, defining a path for the development of new therapeutics.

Results

INDO Deficiency Increases Atherogenesis. To determine the role of IDO and the endogenous metabolites generated during IDO-mediated Trp metabolism in the development of atherosclerosis, Indo$^{-/-}$ mice were crossed with hyperlipidemic apolipoprotein E deficient (ApoE$^{-/-}$) mice to generate IDO-deficient ApoE$^{-/-}$Indo$^{-/-}$ mice. IDO-competent ApoE$^{-/-}$Indo$^{+/+}$ (ApoE$^{-/-}$) and ApoE$^{-/-}$Indo$^{-/-}$ mice were fed a normal chow diet and were killed at 15, 20, or 30 wk of age. No statistically significant differences in either body weight or serum cholesterol levels were observed between the two groups of mice at any time point examined (Table S1). ApoE$^{-/-}$Indo$^{-/-}$ mice displayed an almost twofold increase in atherosclerotic lesion size in the aortic root compared with ApoE$^{-/-}$ mice ($P < 0.001$ at 15 wk; $P < 0.05$ at 20 wk) (Fig. 1). Percentage lesion area was also significantly increased in ApoE$^{-/-}$Indo$^{-/-}$ mice ($P < 0.01$ at 15 wk; $P < 0.05$ at 20 wk) (Fig. 1). At 30 wk of age, no difference in either absolute aortic root lesion area or aortic root lesion area fraction was observed between the two groups of mice (Fig. 1), suggesting that at very late time points IDO can no longer protect from the atherogenic drive toward lesion formation.

IDO Deficiency Affects Atherosclerotic Plaque Vulnerability. The phenotype of human atherosclerotic lesions is related to the risk of thrombosis (16). To examine the effect of IDO deficiency on atherosclerotic lesion composition, plaque sections were stained with antibodies against CD68 (monocytes/macrophages), CD4 (T cells) and α-SMC actin (SMCs). In addition, slides were stained with H&E to quantify necrosis. A striking difference in plaque composition was observed between lesions of IDO-deficient and IDO-competent ApoE$^{-/-}$ mice. At 15 wk of age, lesions in ApoE$^{-/-}$Indo$^{-/-}$ mice contained significantly more CD68^{+} macrophages and CD4^{+} T cells compared with lesions in ApoE$^{-/-}$ mice (Fig. 2 *A–D*). A significant increase in lesional macrophage content was also observed in 20-wk ApoE$^{-/-}$Indo$^{-/-}$ compared with ApoE$^{-/-}$ mice (Fig. S1). No difference in CD4^{+} cells was observed between the two groups of mice at 20 and 30 wk (Figs. S1 and S2).

Despite similarities in size, aortic root lesions in 30-wk ApoE$^{-/-}$Indo$^{-/-}$ mice had less SMC content and an increased necrotic core size compared with lesions in ApoE$^{-/-}$ mice (Fig. 2 *E–H*). No difference in SMC or necrotic core content was observed at earlier time points (Fig. S1). The increase in inflammatory cells in the atherosclerotic lesions of ApoE$^{-/-}$Indo$^{-/-}$ mice at early time points in disease development, together with the increase in necrotic core area and reduction in SMC content in ApoE$^{-/-}$Indo$^{-/-}$ mice at later time points, reveals an important role for IDO in maintaining plaque stability.

Quantitative PCR Data Reveal Changes in Inflammatory Gene Expression. Quantitative RT-PCR (qRT-PCR) was used to assess myeloid and T-cell phenotype markers in the spleen, lymph nodes (LN), and aortas of ApoE$^{-/-}$ vs. ApoE$^{-/-}$Indo$^{-/-}$ mice. An increase in GATA3 (transacting T-cell–specific transcription factor 3) expression was observed in the aorta of ApoE$^{-/-}$Indo$^{-/-}$ mice (Fig. 3), suggesting facilitation of a Th2 response. However, changes in aortic IL-4 expression were not significant, nor were there any changes in expression of T-cell–associated genes in either the spleens or LN of ApoE$^{-/-}$Indo$^{-/-}$ compared with ApoE$^{-/-}$ mice (Fig. 3 and Fig. S3). Aortas from ApoE$^{-/-}$Indo$^{-/-}$ mice also displayed significantly increased expression of CD11c, CD64, and costimulatory molecules CD80 and CD86 compared with aortas from ApoE$^{-/-}$ mice (Fig. 3), supporting the immunohistochemistry data showing increased macrophage accumulation in ApoE$^{-/-}$Indo$^{-/-}$ lesions.

Reduced Serum Kyn in IDO-Deficient ApoE$^{-/-}$ Mice. To investigate whether the athero-protective effects of IDO deficiency were because of Trp accumulation or endogenous Kyn depletion, serum Trp and Kyn levels were assessed. At all time points examined, ApoE$^{-/-}$Indo$^{-/-}$ mice had reduced serum Kyn levels compared

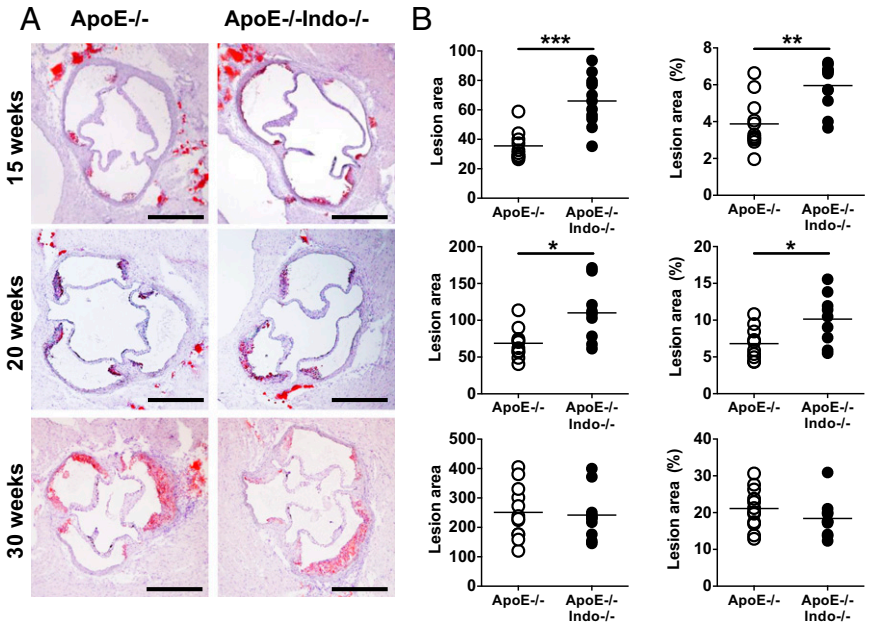

Fig. 1. IDO deficiency accelerates early atherosclerotic lesion formation in the aortic root. ApoE$^{-/-}$ and ApoE$^{-/-}$Indo$^{-/-}$ mice fed a normal chow diet were killed at 15, 20, or 30 wk of age. (*A*) Representative photomicrographs of aortic roots from 15-, 20-, or 30-wk-old mice stained with Oil red O and Hematoxylin. (Scale bars, 500 μm.) (*B*) Cross-sectional aortic root lesion size (×10^{3} μm^{2}, *Left*) and the percentage aortic root lesion area (percent, *Right*). Data show the mean lesional area per individual mouse. Line represents the group mean (n = 9–12; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

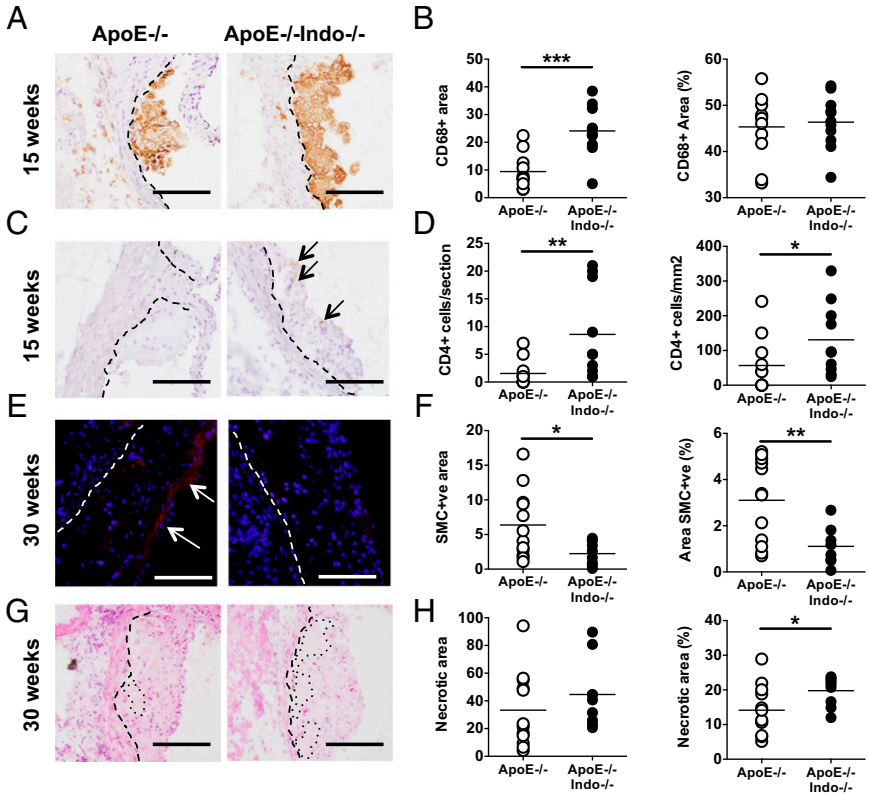

Fig. 2. IDO deficiency promotes a vulnerable plaque phenotype. (*A*) Representative photomicrographs of aortic root sections from 15-wk-old ApoE$^{-/-}$ and ApoE$^{-/-}$ Indo$^{-/-}$ mice stained with an antibody against CD68 (brown staining) and Hematoxylin. (*B*) Lesion area staining positive (×10^3 μm^2, *Left*, and percentage, *Right*) for CD68. (*C*) Representative photomicrographs of aortic root sections from 15-wk-old mice stained with an antibody against CD4 (brown staining) and Hematoxylin. Arrows denote positive cells. (*D*) Lesion area staining positive (cells per section, *Left*, and cells per square millimeter, *Right*) for CD4. (*E*) Representative photomicrographs of aortic root sections from 30-wk-old mice stained with an antibody against α-smooth muscle actin (Cy3-red) and DAPI (blue). (*F*) Lesion area staining positive (×10^3 μm^2, *Left* and percentage, *Right*) for SMC. (*G*) Representative photomicrographs of aortic root sections from 30-wk-old mice stained with H&E. Areas of necrosis denoted by dotted lines. (*H*) Necrotic lesion area (×10^3 μm^2, *Left*, and percentage, *Right*). (*A*, *C*, *E*, and *G*) Dashed lines denote internal elastic lamina. (Scale bars, 100 μm.) (*B*, *D*, *F*, and *H*) Line represents the group mean (n = 8–12; *P < 0.05, **P < 0.01, ***P < 0.001).

with ApoE$^{-/-}$ mice, whereas Trp levels remained similar (Fig. S4 *A* and *B*). This finding resulted in a highly significant reduction in the Kyn:Trp ratio in the ApoE$^{-/-}$Indo$^{-/-}$ mice (Fig. S4*C*), confirming the efficacy of the genetic deletion of IDO in depleting Trp metabolite formation.

Reduced IL-10 and IL-10–Expressing B Cells in ApoE$^{-/-}$Indo$^{-/-}$ Mice. Circulating IL-10 levels were significantly reduced in 15- and 20-wk ApoE$^{-/-}$Indo$^{-/-}$ compared with ApoE$^{-/-}$ mice (Fig. 4*A* and Fig. S5*A*). At 30 wk of age, IL-10 did not differ significantly (Fig. S5*A*). IL-12p40 levels were not significantly different in ApoE$^{-/-}$Indo$^{-/-}$ compared with ApoE$^{-/-}$ mice (Fig. S5*B*). Next, we attempted to identify whether the loss of IL-10 was associated with abnormalities in the levels of regulatory cell subsets via flow cytometry. A role for IDO in regulating Treg numbers has been described (17). No difference in Treg or in Tr1 cell content was observed in either the spleen or LN of ApoE$^{-/-}$ vs. ApoE$^{-/-}$Indo$^{-/-}$ mice (Fig. S6). In contrast, a significant decrease in IL-10–expressing B cells was observed in the spleens and LN of ApoE$^{-/-}$Indo$^{-/-}$ compared with ApoE$^{-/-}$ mice (Fig. 4 *B* and *C*), matching the IL-10 decrease in serum (Fig. 4*A*). Next we assessed whether the increase in IL-10 in IDO-competent mice was secondary to Trp-metabolites. To this aim, we cultured splenocytes from ApoE$^{-/-}$ mice in the presence of L-Kyn and 3,4-DAA, a synthetic derivative of anthranilic acid, downstream of Kyn in the Trp degradation pathway (18). Surprisingly, 3,4-DAA but not L-Kyn resulted in a dose-dependent increase in CD19$^+$IL-10$^+$ cells (Fig. 4 *D* and *E*). No significant changes were observed in other cell subsets.

Treatment with 3,4-DAA Inhibits Inflammatory Cytokine Production in Human Atheroma Cells. We have previously demonstrated that mixed human atheroma cell cultures spontaneously generate cytokines (19) and we used this model to assess the direct effect of Trp metabolites on cytokine production. Atheroma cells from five donors undergoing carotid endarterectomy were cultured for 48 h in the presence or absence of 3,4-DAA at various concentrations. An MTT assay confirmed that the drug was not cytotoxic at any concentration used (Fig. 5*F*). Multianalyte profiling was performed by Luminex. Of the 26 analytes studied, 10 were not reproducibly detectable in the culture. Out of the analytes detected, 3,4-DAA treatment reduced interleukin (IL) 6, granulocyte macrophage colony-stimulating factor (GMCSF), tumor necrosis factor alpha (TNFα), C-X-C motif ligand 1 (CXCL1), and IL-10 levels (Fig. 5 *A*–*E*) at the highest dose. Strikingly, no effect was observed with L-Kyn. These data suggest that 3,4-DAA is capable of inhibiting cytokine production in human atherosclerosis.

Effect of 3,4-DAA on Accelerated Atherosclerotic Lesion Formation and Macrophage Accumulation in Arterial Injury. To evaluate 3,4-DAA therapy in vivo, we used a perivascular collar induced injury-model of accelerated atherosclerosis (20). A perivascular collar was placed onto the carotid artery of ApoE$^{-/-}$ mice and collared mice were treated with either 400 mg/kg 3,4-DAA or vehicle (1% carboxymethylcellulose sodium salt) alone, daily for 3 wk. Body weight and serum cholesterol levels were unaffected by 3,4-DAA treatment (Table S1). The dose of 3,4-DAA used in this study was based upon the dose we previously used in arthritis studies and this dose gives comparable plasma concentrations to

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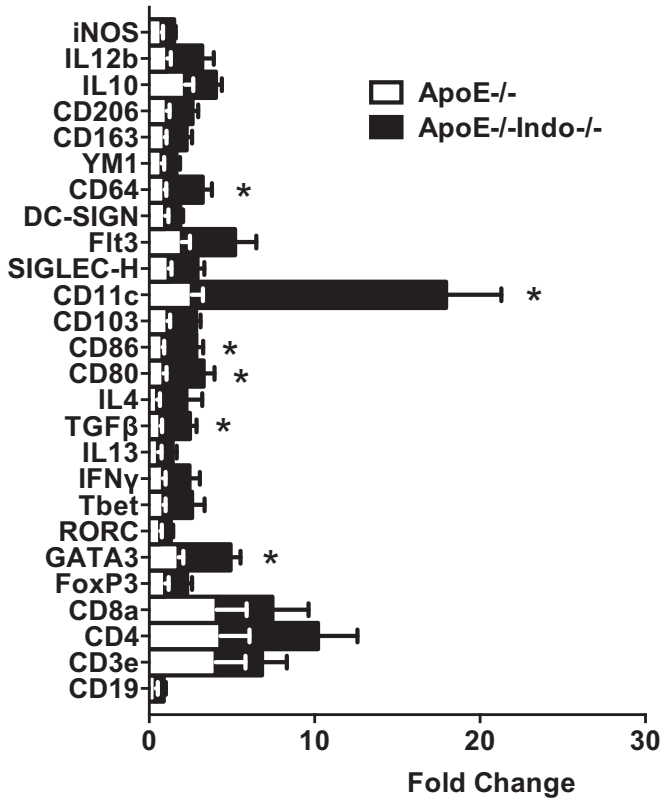

Fig. 3. Aortas of ApoE$^{-/-}$Indo$^{-/-}$ mice display increased myeloid-marker gene expression. Aortas from 15-wk-old ApoE$^{-/-}$ and ApoE$^{-/-}$Indo$^{-/-}$ mice were collected at killing and RNA extracted and gene expression of myeloid and lymphoid markers examined by RT-PCR. Stacked bars show mean + SEM. (n = 5–6; *P < 0.05).

those seen in human 3,4-DAA–treated patients (13). Injury-induced lesion formation, assessed by the intima/media ratio, was significantly reduced in 3,4-DAA- compared with vehicle-treated ApoE$^{-/-}$ mice (Fig. 6). Staining for CD68 revealed attenuated macrophage recruitment in the lesions of ApoE$^{-/-}$ mice treated with 3,4-DAA (Fig. S7). No difference in serum Trp or Kyn levels was observed between 3,4-DAA and vehicle-treated mice (Fig. S8).

Discussion

Since their adoption over 25 y ago, statins have remained the "gold standard" in terms of CVD treatment and prevention. Interestingly, statins are moderately anti-inflammatory in both animal models of inflammatory diseases (21–23) and in humans in rheumatoid arthritis trials (24), suggesting that the prognostic advantage of statins is also linked to their anti-inflammatory capacity. However, targeted therapies to further reduce inflammation and clinical events in atherosclerosis remain to be identified.

The endogenous mechanisms of immune regulation in vessels remain unclear. Trp, an essential amino acid, is precursor to several metabolic pathways in different cell types (e.g., synthesis of kynurenine, serotonin, and melatonin) (18). Trp metabolism by IDO is important to the pathogenesis of neuroinflammation, infection, and cancer (5). Using a combination of mouse models of atherosclerosis and functional studies in human atheroma, we demonstrate that the IDO pathway exerts a powerful athero-protective function via the control of inflammation within the lesions and the systemic production of IL-10, particularly by B cells. Our data suggest therapy with the orally active Trp metabolite analog 3,4-DAA reduces lesion formation in mice and inflammation in human atheroma, and it may be beneficial in CVD.

We show that IDO deficiency in normal chow-fed ApoE$^{-/-}$ mice increases atherosclerotic lesion size and the development of a vulnerable plaque phenotype with a reduced SMC content and an increased necrotic area. In previous studies, treating Western (i.e., high fat) diet-fed low-density lipoprotein receptor deficient (LDLR$^{-/-}$) mice with 3-hydroxyanthranilic acid (3HAA) reduced levels of proatherogenic lipid particles and lesion size (25), whereas administering 1-methyl-DL-tryptophan, a pharmacological IDO inhibitor to Western diet-fed ApoE$^{-/-}$ mice achieved the reverse (26), suggesting a lipid-lowering mechanism of atheroprotection for Trp metabolites. No clear changes were observed in surrogate markers of plaque vulnerability to rupture with either treatment (25, 26). We examined ApoE$^{-/-}$ and ApoE$^{-/-}$Indo$^{-/-}$ mice on normal fat chow diet and we did not observe changes in lipid metabolism, indicating that the immunoregulatory effects of the IDO pathway emerge in conditions of moderate hypercholesterolemia that more closely resemble the human condition (27) and play a key role in modulating plaque composition.

Inflammation is an important modulator of plaque vulnerability to rupture (16). Of relevance, loss of IDO in our study significantly increased T-cell and macrophage lesional content. Moreover, in the atherosclerotic lesions of IDO-deficient mice we observed the local up-regulation of the myeloid markers CD11c and CD64 and costimulatory molecules of the B7 family CD80 and CD86. We are aware that murine atherosclerotic pathology is only vaguely representative of human vascular pathology, particularly in terms of plaque vulnerability (28, 29). There are also significant differences between the immune systems of human and mouse (30). Thus, in our laboratory we effectively adopted an ex vivo model of atheroma cell cultures from human carotid arteries, a mixed-cell population where macrophages are the most abundant cell type (19). Treatment of human atheroma cells in vitro with 3,4-DAA also effectively down-regulated cytokine and chemokine production in vitro in human atheroma cells, These data indicate that in both human and murine atherosclerosis, the IDO pathway and

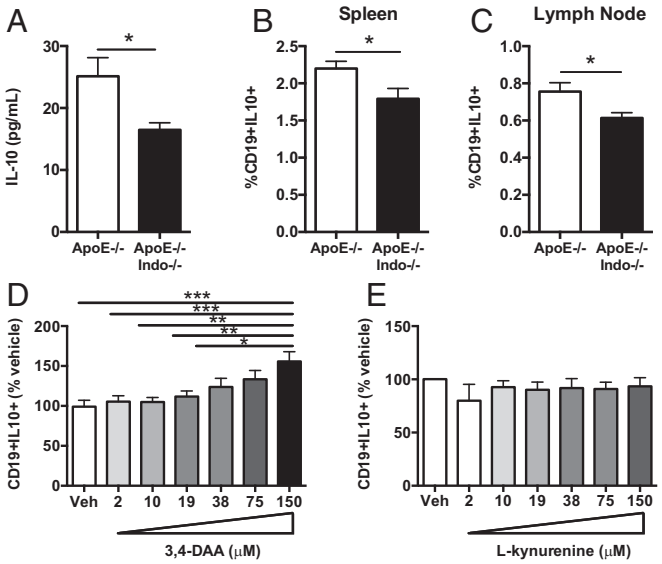

Fig. 4. ApoE$^{-/-}$Indo$^{-/-}$ mice display reduced serum IL-10 and IL-10–expressing B cells. (*A*) IL-10 levels (pg/mL) in serum of 15-wk-old ApoE$^{-/-}$ vs. ApoE$^{-/-}$Indo$^{-/-}$ mice. (*B* and *C*) Percentage of CD19^{+}IL-10^{+} cells in the spleen (*B*) and LN (*C*) of 15-wk-old ApoE$^{-/-}$ versus ApoE$^{-/-}$Indo$^{-/-}$ mice. (*D* and *E*) CD19^{+}IL-10^{+} cells (as percentage of vehicle) in splenocytes from ApoE$^{-/-}$ mice cultured in the presence or absence of 3,4-DAA (*D*) or L-Kyn (*E*) at various concentrations for 48 h. Bars show mean + SEM, (n = 7–12; *P < 0.05, **P < 0.01, ***P < 0.001).

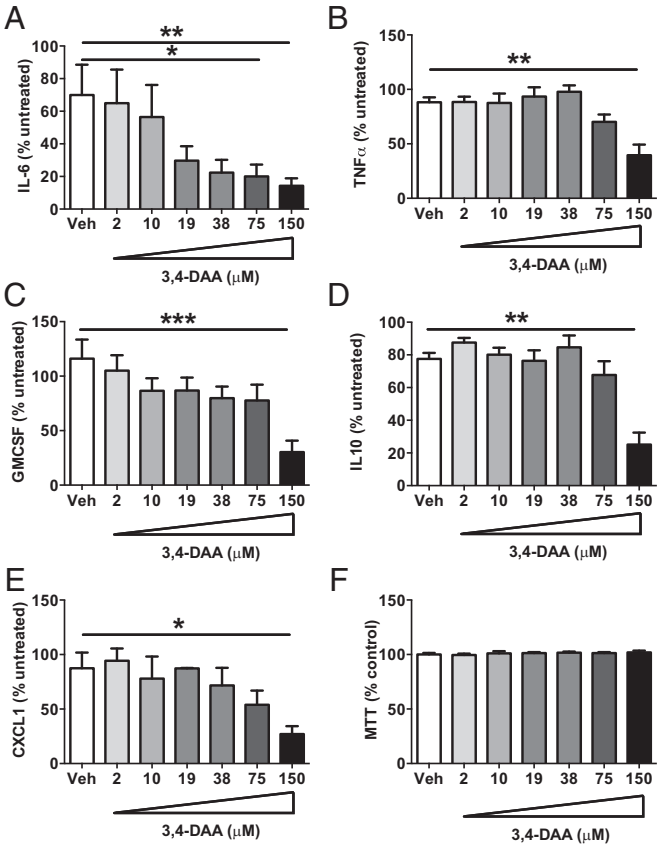

Fig. 5. 3,4-DAA inhibits cytokine production by human atheroma cells. Production of IL-6 (*A*), TNF-α (*B*), GMCSF (*C*), IL-10 (*D*), and CXCL1 (*E*) (as percent untreated) in the supernatants of human atheroma cells cultured in the presence or absence of 3,4-DAA at various concentrations for 48 h as measured by luminex. (*F*) MTT assay to assess cell viability following 48-h treatment with 3,4-DAA. ($n = 5$ donors) Bars show group mean + SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

its downstream metabolites are important for controlling local intraplaque inflammation.

Initial studies attributed the immunoregulatory properties of IDO to Trp starvation of T cells (17) or the generation of Kyns with direct immune-regulatory properties (31, 32). Modulation of plaque size and phenotype in ApoE$^{-/-}$Indo$^{-/-}$ mice was associated with a reduction in serum Kyn but not serum Trp levels, potentially because of replenishment of the amino acid from the diet. This reinforces the concept that the immunomodulatory effect of IDO is mediated by Trp metabolites rather than Trp starvation. However, we cannot exclude the possibility that local changes in Trp levels may occur in ApoE$^{-/-}$Indo$^{-/-}$ mice, which might have a biological effect.

IDO deficiency in ApoE$^{-/-}$ mice reduced IL-10 serum levels and its gene expression in the spleen and LN in our study. We sought to identify mechanisms underlying the protective effect of IDO. Interestingly, we found an increase in IL-10–expressing B cells in the spleens and LN of IDO competent vs. IDO-deficient ApoE$^{-/-}$ mice. Furthermore, treatment of splenocytes with 3,4-DAA in vitro also augmented the numbers of CD19$^+$IL10$^+$ cells, supporting our findings in the ApoE$^{-/-}$Indo$^{-/-}$ mice and indicating that IDO can enhance the production of the anti-inflammatory and antiatherogenic cytokine IL-10 by B cells. We have recently identified a population of IL-10–producing B cells that are expanded in atherosclerosis and confer protection from vascular injury via IL-10 (33), suggesting that IL-10 production by B cells is a potentially antiatherogenic pathway. In infectious disease, studies have shown that the Kyn pathway facilitates the generation and function of Tregs (34, 35). However, in our study IDO deficiency in ApoE$^{-/-}$ mice did not affect CD4$^+$FoxP3$^+$ Treg in the spleen or LN, in agreement with previous studies (25, 26). Furthermore numbers of Tr1 cells and IL-10 production by Tr1 cells were unchanged.

Interestingly, in contrast to 3,4-DAA, the in vitro treatment of splenocytes with the Trp metabolite L-Kyn had no effect on the number of CD19$^+$IL-10$^+$ cells. L-Kyn also did not affect cytokine levels in human atheroma cell cultures. This finding implies that metabolites downstream of Kyn rather than Kyn itself have the most potent effects on IL-10–expressing B cells and thus are likely to be the most athero-protective. Marked differences in the immune effects of different metabolites have previously been described with 3-HAA but not L-Kyn mediating inhibition of antigen-independent proliferation of CD8$^+$ T cells (36). Finally, in vitro 3,4-DAA treatment induces IL-10 up-regulation in splenic murine B cells but decreases IL-10 production in the human atheroma cells, suggesting either cell-dependent effects or human and murine differences in the regulation of IL-10 expression (37). While this manuscript was under review, Metghalchi et al. showed that the production of another Trp metabolite, kynurenic acid, but not L-Kyn may reduce IL-10 production in myeloid cells and increase susceptibility to atherogenesis and colitis (38), suggesting that cell-type–dependent and Trp metabolite-specific effects are at play downstream in the IDO pathway.

Collectively our study in both mice and human lesions indicates that IDO has an important immunomodulatory role in atherosclerosis. This appears to occur through local modulation of myeloid cell activation and systemic induction of IL-10 production, particularly in B cells. Moreover, 3,4-DAA, a synthetic analog of the Trp metabolite anthranilic acid, has the direct ability to reduce lesion formation in mice and inflammation in human atheroma, indicating the possibility of the repurposing of 3,4-DAA and the initiation of a drug discovery program around its derivatives as a treatment for the important inflammatory aspects of CVD.

Materials and Methods

Analysis of Murine Atherosclerosis Development. ApoE$^{-/-}$Indo$^{-/-}$ mice were generated by crossing ApoE$^{-/-}$ mice with Indo$^{-/-}$ mice. ApoE$^{-/-}$Indo$^{-/-}$ double-knockout mice were fertile and exhibited no overt phenotype.

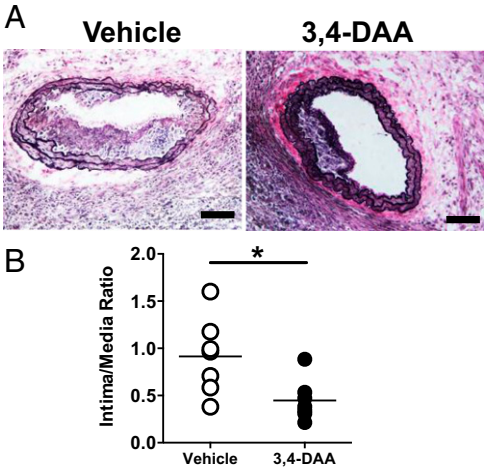

Fig. 6. 3,4-DAA treatment inhibits accelerated atherosclerosis development induced by carotid collar injury. ApoE$^{-/-}$ mice with a perivascular collar on the carotid artery were treated with 3,4-DAA (400 mg/kg) or vehicle. Mice were culled 21 d after collar placement. (*A*) Representative photomicrographs of injured carotid arteries from ApoE$^{-/-}$ mice treated with 3,4-DAA or vehicle stained for elastin. (Scale bars, 100 μm). (*B*) Intima/media ratio (IMR) of carotid arteries 21 d after injury. Data show values for individual mice. Line represents group mean, ($n = 7$; $^*P < 0.05$).

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Animals were housed in specific pathogen-free conditions and all experimental animal procedures were approved by the local ethical review process and performed according to United Kingdom Home Office guidelines. Mice were fed a standard chow diet and killed at 15, 20, or 30 wk of age, as described in *SI Materials and Methods*. Aortic root lesion area was assessed as described in *SI Materials and Methods*.

Treatment of Human Atheroma Cells with 3,4-DAA and L-Kynurenine. Carotid endarterectomies from patients undergoing surgery for carotid disease were obtained at Charing Cross Hospital, London. The protocol was approved by the Research Ethics Committee RREC2989. All patients gave written informed consent, according to the Human Tissue Act 2004 (United Kingdom). Single-cell suspensions of mixed cell types were obtained via enzymatic digestion and cultured as previously described (19). Human atheroma cells were plated immediately after isolation at 10^6 cells per milliliter in RPMI medium containing 5% (vol/vol) FBS in a 96-well plate either alone or in the presence of DMSO or various concentrations of 3,4-DAA [*N*-(3′,4′-dimethoxycinnamonyl) anthranilic acid] or L-kynurenine. Supernatants were collected after 48 h and frozen at −80 °C for batch analysis via Luminex, as described in *SI Materials and Methods*.

Real-Time PCR of Murine Tissues. Total RNA was isolated from murine aortas, spleen, and LN using the Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies). Following preamplification (14 cycles), RT-PCR was performed using either custom TaqMan Array microfluidic cards or individual TaqMan Gene Expression Assays (Table S2) and TaqMan universal PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied BioSystems). PCR amplification was carried out for 40 cycles. Samples were run in duplicate and normalized to 18 s. The 2-ΔΔCt method was used to analyze the relative changes in gene expression.

Perivascular Collar Injury. Because of the need to administer 3,4-DAA daily by oral gavage, a well-characterized model of arterial injury was used in ApoE$^{-/-}$ mice to accelerate lesion formation. At 22 wk of age, male ApoE$^{-/-}$ mice were anesthetized with isofluorane by inhalation, the left carotid artery dissected, and a nonocclusive tygon collar (length, 2.5 mm; internal bore diameter, 510 μm; Cole-Parmer) placed around the carotid artery as described previously (20). Mice received 400 mg/kg 3,4-DAA or vehicle (1% carboxymethylcellulose sodium salt) on the day of operation and then daily by oral gavage starting 3 d after surgery. Twenty-one days following collar placement, mice were killed and lesion development assessed as described in *SI Materials and Methods*.

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