

ORIGINAL ARTICLE

Indoleamine 2,3-dioxygenase 1 in coronary atherosclerotic plaque enhances tissue factor expression in activated macrophages

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Abstract

Background: Recent clinical studies have found that changes in the kynurenine (Kyn) pathway of tryptophan (Trp) metabolism are associated with cardiovascular events. However, the roles of the Kyn pathway on vascular wall thrombogenicity remain unknown. Indoleamine 2,3-dioxygenase 1 (IDO1) is a rate-limiting enzyme of the Kyn pathway.

Objective: The present study aimed to localize IDO1 in human coronary atherosclerotic plaques from patients with angina pectoris and define its role in plaque thrombogenicity.

Methods: Immunohistochemical methods were applied to localize IDO1 in coronary atherosclerotic plaques from patients with stable (SAP) and unstable (UAP) angina pectoris. The role of IDO1 in tissue factor (TF) expression was investigated in THP-1 macrophages activated by interferon (IFN) γ and tissue necrosis factor (TNF) α .

Results: We localized IDO1 mainly in CD68-positive macrophages within atherosclerotic plaques, and in close association with TF. Areas that were immunopositive for IDO1, TF, and CD3-positive T lymphocytes were significantly larger in plaques from patients with UAP than SAP. Macrophages activated by IFN γ and TNF α upregulated IDO1 expression, increased the Kyn/Trp ratio and enhanced TF expression and activity, but not TF pathway inhibitor expression. The IDO1 inhibitor epacadostat significantly reduced the Kyn/Trp ratio, TF expression and activity, as well as NF- κ B (p65) binding activity in activated macrophages. Inhibition of the aryl hydrocarbon receptor that binds to Kyn, also reduced Kyn-induced TF expression in activated macrophages.

Conclusion: Indoleamine 2,3-dioxygenase 1 expressed in coronary atherosclerotic plaques might contribute to thrombus formation through TF upregulation in activated macrophages.

KEYWORDS

indoleamin 2,3-dioxygenase 1, aryl hydrocarbon receptor, macrophage, thrombosis, tissue factor

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Essentials

- Indoleamine 2,3-dioxygenase 1 (IDO1) is associated with cardiovascular events.
- Tissue factor (TF) plays a critical role in thrombus formation on disrupted plaques.
- IDO1 plays a critical role in TF expression in human THP-1 macrophages.
- IDO1 might contribute to thrombus formation through TF upregulation in activated macrophages.

1 | INTRODUCTION

Acute coronary syndrome (ACS) is provoked by disruption (rupture or erosion) of coronary atherosclerotic plaques and subsequent thrombus formation.¹ Thrombi produced on disrupted plaques comprise aggregated platelets and considerable amount of fibrin; therefore, a tissue factor-mediated coagulation pathway plays a critical role in thrombus formation that leads to the onset of ACS.²⁻⁴ Tissue factor (TF) expression by macrophages and smooth muscle cells in plaques^{5,6} is induced by proinflammatory cytokines such as interferon- γ (IFN γ), tumor necrosis factor- α (TNF α), lipopolysaccharide, as well as hypoxia,⁵⁻⁷ and its procoagulant activity is inhibited by TF pathway inhibitor (TFPI).⁸

Tryptophan (Trp) is an essential amino acid that is either used for protein synthesis or metabolized to various bioactive molecules via the serotonin pathway or the kynurenine (Kyn) pathway, and the latter pathway is the major route of Trp catabolism in humans.⁹ Catabolites in the Kyn pathway of Trp catabolism are important for regulating the immune system and inflammation.^{10,11} Emerging evidence also indicates that these catabolites play significant roles in cardiovascular pathophysiology.¹²⁻¹⁴

Indoleamine 2, 3-dioxygenase (IDO) is an enzyme that catalyzes the first and rate-limiting step of Trp metabolism along the Kyn pathway⁹ and it has two isoforms. The IDO1 isoform is expressed in various tissues, including dendritic cells, endothelial cells, macrophages, fibroblasts, and mesenchymal stromal cells, and this major isoform contributes to Trp degradation. The IDO2 isoform is primarily expressed in the kidney, brain, colon, liver, and reproductive tract.^{9,14} The expression of IDO2 is apparently basal, whereas that of IDO1 is induced by cytokines, particularly IFN γ .¹⁵ IDO1 is overexpressed in many pathological states such as cancer, autoimmune, cardiovascular, and inflammatory bowel diseases.¹⁶ IDO1 is thought to cause immune escape via local Trp degradation and subsequent inhibition of the T cell response.¹⁷ According to the inflammatory nature of atherosclerotic diseases, the serum Kyn/Trp ratio increases in patients with coronary heart diseases,¹⁸ and plasma or urinary Kyn/Trp ratios or plasma Kyn values are associated with coronary thrombosis and cardiovascular mortality.¹⁹⁻²¹ Plasma levels of TF, Kyn, and Kyn/Trp ratios are significantly increased, whereas TFPI remains unchanged in patients with chronic kidney disease (CKD).²²

These lines of evidence suggest that IDO1 and the Kyn pathway significantly contribute to cardiovascular diseases and thrombus formation. However, IDO1 expression in human coronary arteries

and its role(s) in vascular wall thrombogenicity remains unknown. We therefore evaluated the expression of IDO1 in human coronary atherosclerotic plaques and its contribution to TF expression in macrophages activated using cytokines.

2 | MATERIALS AND METHODS

2.1 | Immunohistochemical staining for IDO1 and TF in human coronary plaque

We immunohistochemically stained human coronary plaques in specimens obtained by directional coronary atherectomy (DCA) from patients with stable (SAP, n = 20) and unstable (UAP, n = 20) angina pectoris to detect IDO1 protein expression. This study proceeded under a protocol approved by the institutional review board of the University of Miyazaki (Approval No. 2015-090). Samples obtained under the protocol were immediately fixed in 4% paraformaldehyde and embedded in paraffin. Serial 4- μ m slices of the samples were stained with hematoxylin and eosin (HE) for immunohistochemical evaluation using mouse monoclonal PG-M1 anti-human CD68 for macrophages (Dako, Glostrup, Denmark), mouse monoclonal anti-human α -smooth muscle actin for smooth muscle cells (Dako), rabbit monoclonal anti-human tissue factor for TF (EPR8986; Abcam, Cambridge, UK), mouse monoclonal anti-human indoleamine 2, 3-oxygenase for IDO1 (ab55305; Abcam), and mouse monoclonal anti-human CD3 for T lymphocytes (F7.2.38; Dako). The sections were stained using Envision Kits (Dako), visualized as horseradish peroxidase activity with 3, 3'-diaminobenzidine tetrahydrochloride and counterstained with Meyer's hematoxylin. The negative controls contained universal negative control mouse immunoglobulins (Dako) instead of primary antibodies. We evaluated immunopositive areas in entire samples by taking two to five microphotographs under $\times 20$ objective magnification depending on the sample size. Histological data are expressed as mean values for each sample, and were verified by two independent investigators (SK and AY) in a blinded manner.

2.2 | Cell culture

The human monocyte THP-1 cell line (Dainippon Sumitomo Pharma, Suita, Japan) was maintained in a subconfluent state (5×10^5 cells/mL) in RPMI-1640 medium supplemented with 10% fetal bovine

serum and 1% Zell Shield (Minerva Biolabs, Berlin, Germany) at 37°C in a humidified incubator. THP-1 cells were differentiated into macrophages by incubation with 100 nmol/L phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, St. Louis, MO, USA) for 24 hours. Non-adherent cells and media containing PMA were removed, then adherent cells were washed three times with PBS. The cells were subsequently activated with 10 ng/mL each of IFN γ (R&D Systems, Minneapolis, MN, USA) and TNF α (Sigma-Aldrich or R&D Systems) or lipopolysaccharide (LPS) (Sigma-Aldrich). We assessed IDO1, TF, and TF pathway inhibitor (TFPI) mRNA or protein expression and procoagulant activity in these macrophages for up to 24 hours. The contribution of IDO1 and aryl hydrocarbon receptor (AHR) to TF expression was evaluated by culturing the macrophages derived from THP-1 cells with or without the IDO1 inhibitor epacadostat, (INCB024360; BioVision, Milpitas, CA, USA) or the AHR inhibitor CH-223191 (Sigma-Aldrich).

The stability of TF mRNA in macrophages stimulated with 10 μ g/mL of actinomycin D, a transcriptional inhibitor (Sigma-Aldrich), was measured using real time PCR.

Human primary monocytes were isolated from cells donated by healthy volunteers using RosetteSep and SepMate (STEMCELL Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's manual. 1.5–3.0 $\times 10^5$ cells in RPMI-1640 supplemented with 10% fetal bovine serum and 1% Zell Shield were seeded into 24 well plates and incubated with 100 ng/mL of macrophage colony stimulating factor (PEPROTECH, Rocky Hill, NJ, USA) for 24 hours. Non-adherent cells and media were removed and washed three times with PBS and remaining adherent cells were regarded as macrophages. Thereafter, RPMI-1640 (1 mL) supplemented with 10% fetal bovine serum and 1% Zell Shield was added to the wells and the cells were activated with IFN γ and TNF α (10 ng/mL each). We assessed TF mRNA expression and evaluated the effects of the IDO1 inhibitor, epacadostat. This study was approved by the institutional review board at the University of Miyazaki (Approval No. O-0272).

2.3 | Trp and Kyn concentrations in cell culture supernatants measured using liquid chromatography/mass spectrometry

Frozen supernatants from THP-1 cell cultures were thawed, maintained at 4°C, deproteinized using the same volume of 6% trichloroacetic acid and kept on ice for 30 minutes. The samples were separated by centrifugation at 5800 g for 15 minutes at 4°C. Levels of Trp and Kyn in the supernatants were measured using liquid chromatography/mass spectrometry (LC-MS) with some modification.²³ A Zorbax stable-bond C8 reversed-phase analytical column measuring 150 \times 4.6 mm with a particle size of 3.5 μ m (Agilent Technologies, Tokyo, Japan) was mounted in a compartment with a thermostat set at 40°C. The mobile phase comprised 95% solution A (650 mmol/L acetic acid) and 5% solution B (acetonitrile). Calibration curves were prepared using L-tryptophan reference material (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and L-kynurenine (K6825; Sigma-Aldrich). Samples (10 μ L)

were injected into the column and detected using Q Exactive (Life Technologies, Carlsbad, CA, USA).

2.4 | Measurement of mRNA expression using real time PCR

Activated THP-1 macrophages in six-well plates were washed with PBS (2 mL) three times and resolved with 1 mL TRIzol (Life Technologies). Total RNA was extracted using RNeasy Mini Kits (Qiagen, Hilden, Germany) and quantified using a Nano Drop 1000 (ND-1000) spectrophotometer (Thermo Scientific, Rockford, IL, USA). Single-strand, complementary DNA (cDNA) was synthesized from the RNA using PrimeScript RT reagent kits (Perfect Real Time, Takara Bio, Shiga, Japan) and used for real time PCR. Gene expression was measured using a LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany), SYBR Premix EX Taq II (Perfect Real Time, Takara Bio) and specific primers with the following sequences: human TF, 5'-TGACCTACCGACGAGATTGTGAA-3' (forward) and 5'-TCTGAATTGTTGGCTGTCCGAGGT-3' (reverse); human TFPI, 5'-TGGATGCTGGGCAATATGA-3' (forward) and 5'-AGGCTGGGAA CCTTGTTGA-3' (reverse); human β -actin, 5'-TGGCACCCAGCACAA TGAA-3' (forward) and 5'-TAAGTCATAGCCGCTAGAAGCA-3'. Gene expression was normalized by β -actin expression.

2.5 | Enzyme-linked immunosorbent assay (ELISA)

Cellular or nuclear and cytoplasmic proteins were extracted using RIPA buffer (Nacalai Tesque, Kyoto, Japan) or NE-PER Nuclear and Cytoplasmic Extraction Reagents (Life Technologies) containing 1% Halt protease and phosphatase inhibitor (Life Technologies). Protein concentrations were determined using Pierce BCA Protein Assay Kits (Life Technologies). Amounts of TF and TFPI expression and NF κ B (p65) activity in THP-1 macrophages were measured using Quantikine ELISA kits for human TF and TFPI (R&D Systems), and NF κ B (p65) Transcription Factor Assay Kits (ab133112; Abcam), respectively.

2.6 | Factor Xa chromogenic activity of tissue factor measurement

Factor Xa chromogenic activity of THP-1 macrophages was assessed using Tissue Factor Human Chromogenic Activity Assay Kits (ab108906; Abcam). Activated THP-1 macrophages in six-well plates were washed with cold PBS (2 mL) three times, then 300 μ L of octyl- β -D-glucopyranoside containing 1% Halt protease inhibitor (Life Technologies) was added and the mixtures were placed at 37°C for 15 minutes. The lysates were collected into 1.5-mL microtubes and stored at -80°C. Chromogenic activity was assayed according to the manufacturer's instructions.

2.7 | Western blotting

The expression of AHR in the nuclei and the cytoplasm of THP-1 macrophages was assessed as follows. The cells were resolved

using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Life Technologies) with 1% Halt Protease and Phosphatase Inhibitor Cocktail (Life Technologies) according to the manufacturer's instructions.

Protein concentrations were determined using Pierce BCA Protein Assay Kits (Life Technologies). Protein (10 µg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 200 V for 30 minutes and transferred to PVDF membranes using X cell II Blot module (Life Technologies). Proteins were detected using anti-AHR antibody (#83200, AhR [D5S6H]), rabbit mAb, (Cell Signal Technology Japan, Tokyo, Japan), mouse monoclonal anti-Lamin B1 antibody (sc-377000; Santa Cruz Biotechnology Inc., Dallas, TX, USA), a mouse monoclonal anti-β-actin (AC-15) antibody (A5441; Sigma-Aldrich) and the specific secondary antibodies, donkey cy3-conjugated affinity pure anti rabbit IgG (H + L) antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for AHR, goat anti-mouse IgG (H&L) and Alexa Fluor 647 (ab150119; Abcam) for Lamin B1 and β-actin, respectively. Fluorescent images were acquired using an ImageQuant LAS-4000 lumino-image analyzer (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK).

2.8 | Statistical analysis

Data were analyzed using JMP 11.2.0. (SAS Institute, Inc., Cary, NC, USA) or GraphPad Prism 6.0f (GraphPad Software, Inc., San Diego, CA, USA) software. Values are expressed as means with standard deviation (SD) or as medians with interquartile ranges when variances were skewed. Continuous variables were compared using Student *t* tests, Mann-Whitney *U* tests, and one-way or two-way ANOVA followed by Bonferroni correction. Categorical variables were compared using Fisher exact tests. Relationships between factors were evaluated using Spearman rank correlation coefficients. Values with *P* < 0.05 were considered to be statistically significant.

3 | RESULTS

3.1 | Characteristics of patients with SAP and UAP

Table 1 shows the clinical characteristics of the patients with SAP (*n* = 20) and UAP (*n* = 20) from whom we obtained DCA samples. Only diabetes was significantly more prevalent in patients with UAP than SAP (*P* < 0.05). Medications did not significantly differ between the groups.

3.2 | Tissue factor and IDO1 in human coronary plaque

We immunohistochemically examined the expression and localization of IDO1 and TF in serial sections of human DCA samples. Figure 1A and B shows microphotographs of hematoxylin and eosin staining and immunohistochemistry for IDO1, TF, macrophages and SMC in coronary plaque from patients with UAP and

TABLE 1 Clinical and immunohistochemical characteristics of patients with SAP and UAP

	SAP (n = 20)	UAP (n = 20)	P value
Clinical characteristics			
Male, n (%)	17 (85)	15 (75)	0.43
Mean age (±SD; years)	62.8 ± 11.6	61.2 ± 12.9	0.69
Body mass index (kg/m ²)	25.8 ± 3.8	25.2 ± 4.7	0.69
Hypertension, n (%)	12 (60)	13 (65)	0.74
Dyslipidemia, n (%)	11 (55)	12 (60)	0.75
Diabetes, n (%)	6 (30)	13 (65)	0.03
Smoking, n (%)	10 (50)	13 (65)	0.34
Family history, n (%)	3 (15)	5 (20)	0.43
Medication			
Aspirin, n (%)	19 (95)	17 (85)	0.29
CCB, n (%)	10 (50)	13 (65)	0.34
ACE-I/ARB, n (%)	9 (45)	8 (40)	0.75
Statin, n (%)	6 (30)	7 (35)	0.74
Anti-diabetics, n (%)	2 (10)	3 (15)	0.63
Immunohistochemical evaluation			
CD 68 positive area (%)	0.75 (0.22-2.5)	1.64 (0.36-5.80)	0.07
SMA positive area (%)	6.4 (2.35-11.92)	7.94 (3.61-10.75)	0.75
T cell number (/mm ³)	6 (2.3-35)	21 (13.8-43.8)	0.03
TF positive area (%)	0.12 (0.02-0.51)	0.28 (0.19-0.96)	0.03
IDO1 positive area (%)	0.06 (0.02-0.55)	0.24 (0.05-4.26)	0.03

ACE-I, angiotensin converting enzyme-inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker; IDO1, indoleamine 2, 3-dioxygenase 1; SMA, smooth muscle actin; TF, tissue factor.

SAP. Although closely localized, IDO1 immunopositive cells were not necessarily associated with TF- and CD68-immunopositive cells. These findings suggested that macrophages are predominant in cell populations that express IDO1 and TF in unstable coronary plaque. Areas that were immunopositive for IDO1, TF, and T lymphocytes were larger in patients with UAP than SAP. Areas containing macrophages also tended to be larger in patients with UAP than SAP, but the difference did not reach statistical significance (Table 1). Areas that were immunopositive for IDO1 in human coronary plaques correlated positively with CD68 (*r* = 0.43, *P* < 0.01, *n* = 40) and TF (*r* = 0.69, *P* < 0.01, *n* = 40) but not with SMA (*r* = 0.23, *P* = 0.16, *n* = 40) (Figure 1C), whereas areas of IDO1 correlated positively with CD68 (*r* = 0.56, *P* < 0.05, *n* = 20) and TF (*r* = 0.51, *P* < 0.05, *n* = 20) in samples from patients with UAP, but not with SAP (Figure 1D and E).

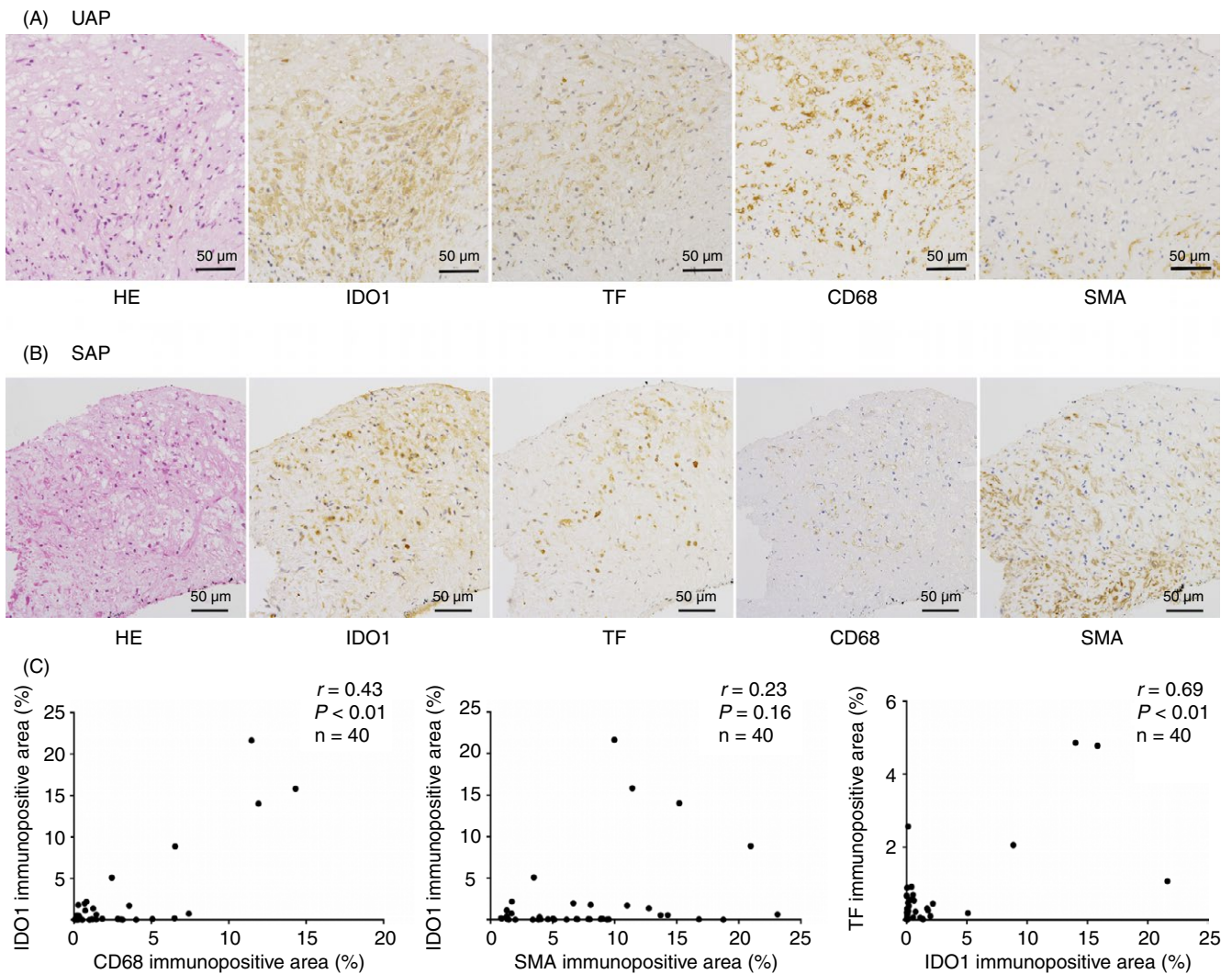


FIGURE 1 Expression of IDO1 and TF in human coronary atherosclerotic plaque. (A and B) Representative histopathological features of serially-sectioned atherosclerotic plaque from patients with unstable (A) and stable (B) angina pectoris. Both IDO1 and TF are expressed in areas immunopositive for CD68, but sparse in SMA. (C) Relationships between IDO1 and areas immunopositive for CD68, SMA and TF in samples obtained from patients by directional coronary atherectomy ($n = 40$). Areas immunopositive for IDO1 positively correlate with those for CD68 and TF, but not SMA (Spearman rank correlation coefficients). (D and E) Relationships between IDO1 and areas immunopositive for CD68 or TF in samples ($n = 20$ each) obtained from patients with UAP (D) and SAP (E) by directional coronary atherectomy (each, $n = 20$). Areas that were positive for IDO1 and CD68 or immunopositive for TF significantly correlated in samples from patients with UAP ($r = 0.56$ and 0.51 , $P < 0.05$), but not SAP. HE, hematoxylin and eosin; IDO1, indoleamine 2,3-dioxygenase 1; SMA, smooth muscle actin; TF, tissue factor

3.3 | Induction of IDO1 and expression of TF in THP-1 macrophages activated using IFN γ and TNF α

Since both IDO1 and TF were detected in macrophage-rich coronary plaques, we examined IDO1, TF, and TFPI expression in macrophages derived from THP-1. Macrophages derived from inactivated THP-1 expressed low levels of IDO1 mRNA, whereas IDO1 mRNA expression at 12 hours was increased 400-fold in macrophages from THP-1 cells activated with IFN γ and TNF α (Figure S1A). The expression of TF mRNA and protein in the activated macrophages was significantly increased for up to 24 hours (Figure S1B and D). In contrast, TFPI mRNA and protein expression decreased

over time in both inactivated and activated macrophages, and the amount of mRNA was significantly reduced at 3, 6, and 12 hours in activated macrophages (Figure S1C and E). The ratio of TF to TFPI protein was higher in activated, than in inactivated macrophages at 24 hours (Figure S1F).

3.4 | IDO1 catalyzes Trp into Kyn, and enhances TF expression in activated macrophages

We measured Trp and Kyn concentrations in cell culture supernatants using LC-MS to determine IDO1 enzymatic activity in activated macrophages. Levels of Trp and Kyn in the supernatant were

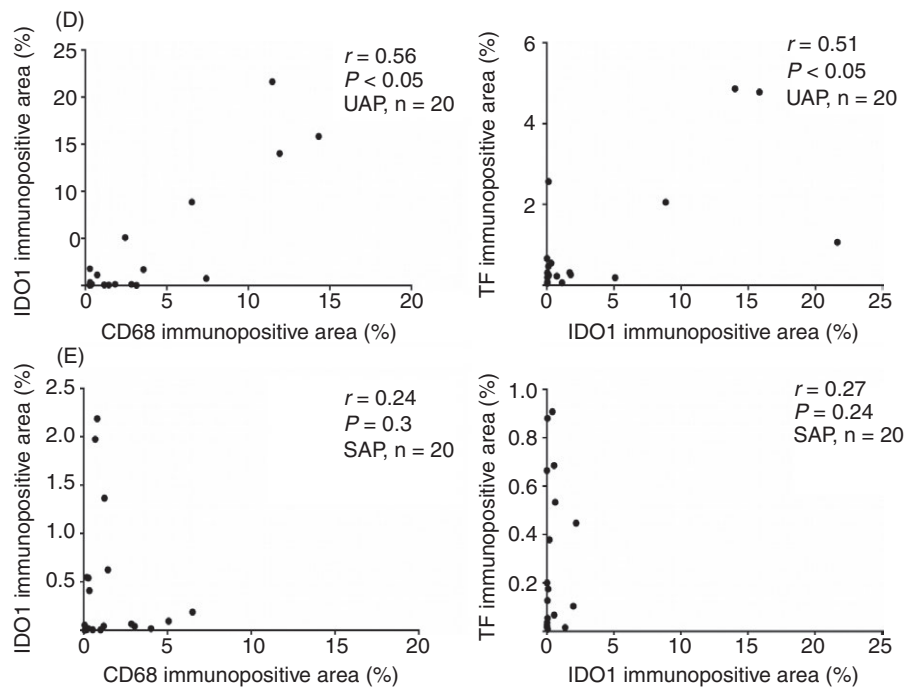


FIGURE 1 (Continued)

reduced and increased, respectively, by inflammatory stimuli, and these processes were inhibited by the IDO1 inhibitor epacadostat (Figure 2A and B). In addition, IDO1 inhibition by epacadostat resulted in significantly reduced TF mRNA and protein expression, and dose-dependently reduced TF activity in activated THP-1 macrophages measured using FXa chromogenic assays (Figure 2C–E, Figure S2). Epacadostat inhibited the TFPI mRNA reduction in the THP-1 macrophages, but did not affect the amounts of TFPI protein (Figure S3A and B), and significantly reduced the ratio of TF to TFPI protein (Figure S3C). Epacadostat also significantly inhibited TF mRNA in THP-1 macrophages activated by IFN γ and LPS (Figure S4). Epacadostat also suppressed NF κ B (p65) binding activity in activated THP-1 macrophages (Figure 2F). Epacadostat did not affect the stability of TF mRNA in activated macrophages incubated with DMSO (control) or actinomycin D (Figure S5). We confirmed the inhibitory effect of epacadostat on TF expression stimulated by IFN γ and TNF α in macrophages derived from human primary monocytes donated by healthy volunteers (Figure S6).

3.5 | Kynurenine partly contributes to cytokine-induced TF expression via the Kyn receptor, AHR

We evaluated TF expression in activated macrophages when endogenous Kyn production was inhibited by epacadostat to determine whether or not the Kyn pathway metabolites kynurenine, 3-hydroxyanthranilic acid, and quinolinic acid contribute to TF expression in macrophages activated with IFN γ and TNF α . Kynurenine dose-dependently increased TF mRNA expression under IDO1 inhibition whereas hydroxyanthranilic and quinolinic acids did not. The range of kynurenine concentrations (10–200 μ g/mL) was selected

based on a previous study.²⁴ However, TF expression did not reach the level of the inflammatory activated control (Figure 3A, Figure S7).

We investigated the nuclear translocation and contribution of AHR to TF expression in activated macrophages and found that kynurenine enhanced the nuclear translocation of AHR under IDO1 inhibition (Figure 3B and C). The specific AHR inhibitor CH223191 modestly, but dose-dependently, decreased the amount of TF mRNA (Figure 4A), and modestly increased the amount of TFPI mRNA (Figure S8) in activated macrophages. The inhibition of AHR by CH223191 significantly inhibited Kyn-induced TF expression in activated macrophages under IDO1 inhibition (Figure 4B).

4 | DISCUSSION

The present study found that IDO1 and TF were closely localized in human coronary atherosclerotic plaque, that IDO1 and TF were predominant in plaques from patients with UAP, and that IDO1 and Kyn contributed to TF expression in macrophages activated with IFN γ and TNF α .

Macrophages in human coronary plaques contained IDO1. The Tampere Vascular Study found that IDO1 expression is elevated in the macrophage-rich cores of advanced atherosclerotic lesions.²⁵ Levels of Kyn that reflect IDO1 activity are increased in human unstable plaques²⁶ and in rabbit macrophage-rich atherosclerotic arteries.²⁷ In addition, IDO1 activity in blood significantly and positively correlates with more advanced degrees of atherosclerosis.²⁸ These findings collectively suggest that the IDO1-mediated Kyn pathway plays a significant role in atherosclerotic lesions. Here, immunostaining detected IDO1 in CD68-positive macrophages

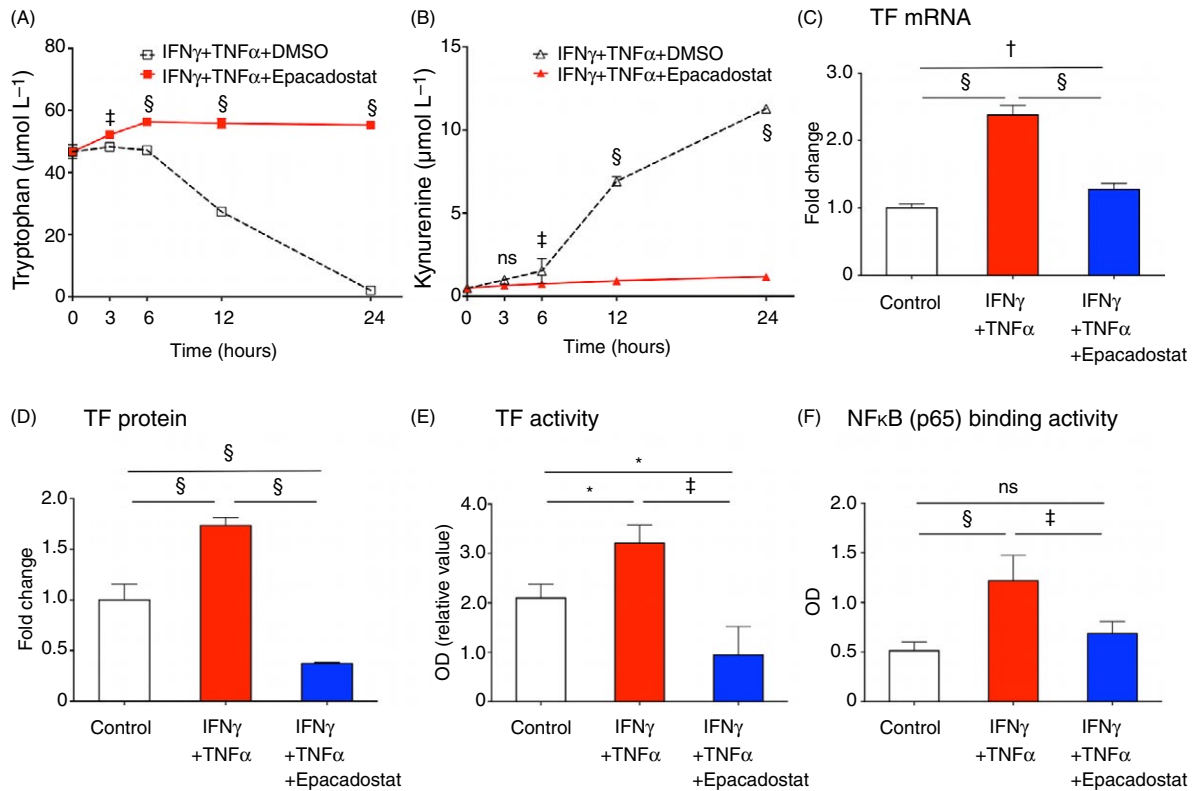


FIGURE 2 Induction of IDO1 catalyzes tryptophan to kynurenine and enhances TF expression in THP-1 macrophages activated by IFN γ and TNF α . Tryptophan and kynurenine concentrations in cell culture supernatants (A and B). THP-1 macrophages induced with PMA were incubated with IFN γ plus TNF α (10 ng/mL each) and epacadostat (100 $\mu\text{g/mL}$), or with DMSO (control) for 24 hours ($n = 5$ each). Tryptophan and kynurenine concentrations in supernatants were measured using LC-MS at indicated times. Inflammatory stimuli reduced levels of tryptophan and increased those of kynurenine in the supernatants, whereas epacadostat reduced kynurenine production under inflammatory stimuli. * $P < 0.001$, † $P < 0.0001$ (two-way ANOVA followed by Bonferroni correction). Effects of epacadostat on expression of TF mRNA, protein and chromogenic activity in activated THP-1 macrophages at 3 (mRNA) or 24 (protein and chromogenic activity) hours ($n = 5$ each; C–E). Effects of Epacadostat on NF κ B (p65) binding activity in activated THP-1 macrophages at 2 hours ($n = 6$ each [F]). * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$, § $P < 0.0001$, one-way ANOVA followed by Bonferroni correction. LC-MS, liquid chromatography-mass spectrometry; ns, not significant; TF, tissue factor

from samples of coronary plaque, and this was more obvious in patients with UAP than SAP. These and previous findings are comparable. However, not all macrophages were positive for IDO1, which might have been due to heterogeneity among macrophages and inflammatory stimuli in human plaques. Although we did not assess cytokine levels, several inflammatory cytokines including IFN γ and TNF α are increased in blood and atherosclerotic plaques from patients with ACS.^{29,30}

The expression of IDO1 positively correlated with TF expression in plaque. The overexpression of TF in atherosclerotic lesions contributes to platelet-fibrin thrombus formation via excess thrombin generation during plaque disruption.^{2,31} We previously correlated TF expression with coronary thrombus size, which is a determinant of the onset of ACS.⁴ Macrophages and smooth muscle cells in plaques express TF, and this is induced by many stimuli, including inflammatory cytokines, hypoxia, oxidized low-density lipoprotein, and C-reactive protein.^{6,32} We localized TF and IDO1 in macrophages from coronary plaques, and found that IDO1 mediated TF expression in THP-1 macrophages activated by IFN γ and TNF α . These results

suggest that TF expression in macrophages within atherosclerotic lesions is linked to the Kyn pathway. Although clinical evidence for a relationship between the Kyn pathway and blood coagulation is scant, Pedersen et al showed that elevated plasma Kyn values or Kyn/Trp ratios predict increased risk of acute myocardial infarction in patients with stable angina pectoris.²¹ Pawlak et al significantly associated TF, TFPI, and prothrombin fragments 1 + 2 with levels of Kyn pathway metabolites in patients with uremia on dialysis.³³ Such evidence suggests that plasma Kyn values or Kyn/Trp ratios could be markers with which to predict cardiovascular events.

Gene expression of TF is regulated by transcriptional factors such as NF κ B, SP-1 and AP-1^{34,35} and/or mRNA stabilization and NF κ B is a major transcriptional factor for inducible TF expression. We found that inhibiting IDO1 activity using epacadostat reduced NF κ B (p65) binding activity in the nuclei of THP-1 macrophages activated using IFN γ and TNF α . Although the precise mechanism(s) remains unknown, the present findings suggest that transcriptional upregulation via NF κ B rather than mRNA stability contributes to IDO1 mediated TF upregulation.

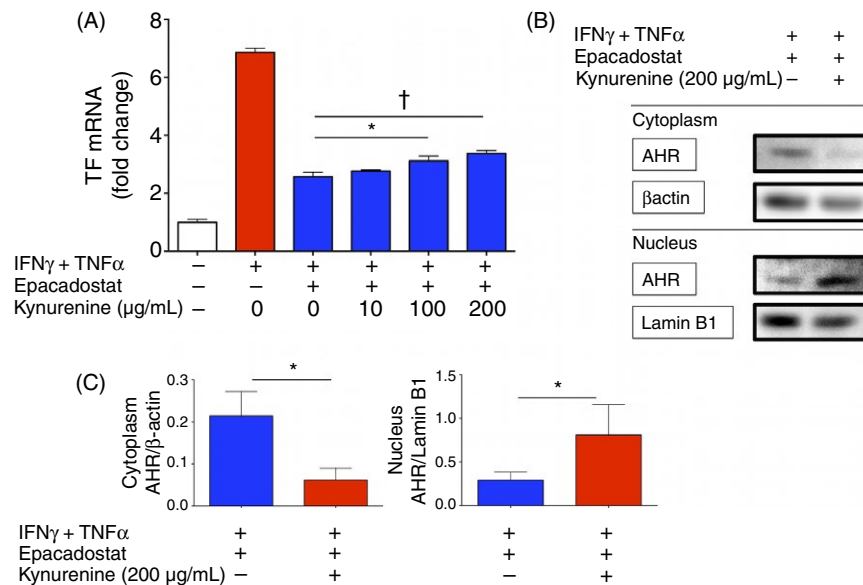


FIGURE 3 Effects of exogenous kynurenine on macrophages activated by IFN γ and TNF α under inhibited IDO1 activity. (A) Various concentrations of kynurenine were added to macrophages derived from THP-1 cells activated with 10 ng/mL of IFN γ and TNF α under IDO1 activity inhibited with epacadostat (100 $\mu\text{g/mL}$). Levels of TF mRNA were measured at 3 hours using real time PCR. Kynurenine dose-dependently increased TF expression. Data are expressed as fold change relative to controls and as means \pm SD ($n = 3$). * $P < 0.01$, † $P < 0.001$, one-way ANOVA followed by Bonferroni correction. (B and C) Representative fluorescent images and signal intensity of Western blots of AHR in cytoplasm and nuclei of activated THP-1 macrophages under IDO1 inhibition with epacadostat (100 $\mu\text{g/mL}$) for 2 hours (B and C). Kynurenine enhanced nuclear AHR translocation under IDO1 inhibition in activated macrophages. Data are expressed as means \pm SD ($n = 3$). * $P < 0.05$, Mann Whitney U test. AHR, aryl hydrocarbon receptor; IDO1, indoleamine 2,3-dioxygenase; TF, tissue factor

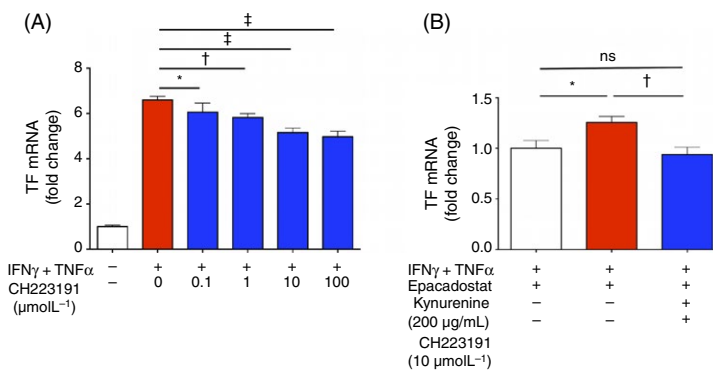


FIGURE 4 Contribution of kynurenine to TF mRNA expression in macrophages activated by IFN γ and TNF α . Dose-dependent effect of AHR inhibitor, CH223191, on TF mRNA expression in THP-1 macrophages activated with 10 ng/mL IFN γ or TNF α at 3 hours (A). Data are shown as values relative to controls and are expressed as means \pm SD ($n = 3$). * $P < 0.05$, † $P < 0.001$, ‡ $P < 0.0001$, one-way ANOVA followed by Bonferroni correction. Effect of AHR inhibition to TF mRNA expression in THP-1 macrophages activated with IFN γ and TNF α (10 ng/mL each) together with kynurenine (200 $\mu\text{g/mL}$) under IDO1 activity inhibited by 100 $\mu\text{g/mL}$ of epacadostat at 3 hours (B). Data are shown relative to control values and are expressed as means \pm SD ($n = 3$). * $P < 0.05$, † $P < 0.01$, one-way ANOVA followed by Bonferroni correction. AHR, aryl hydrocarbon receptor; IDO1, indoleamine 2,3-dioxygenase; TF, tissue factor

The ligand-inducible transcription factor AHR, which mediates the toxic effects of dioxins and uremic toxins, has recently emerged as a pathophysiological regulator of immune-inflammatory conditions and Kyn is an endogenous ligand for AHR.³⁶ Activating AHR promotes both atherosclerosis in mice with an apolipoprotein E deficiency,³⁷ and leukocyte endothelial interaction induced by TNF- α .³⁸ The findings of clinical studies have suggested that AHR contributes to thrombotic complications in patients with chronic kidney

disease.^{39,40} Indolic uremic sulfates, levels of which are increased in patients with chronic kidney disease, enhance TF mRNA and protein expression in endothelial and peripheral blood mononuclear cells via the AHR pathway.³⁹ The AHR receptor regulates TF protein stability via TF ubiquitination and degradation.⁴⁰ The present findings showed that Kyn enhances TF expression via AHR under IDO1 inhibition in activated macrophages. However, the contribution of the Kyn-AHR pathway to TF expression in macrophages is only partial,

because Kyn-induced TF expression did not reach the levels induced by IFN γ and TNF α .

Epacadostat (INCB024360) is a competitive IDO1 inhibitor that suppresses Kyn generation *in vitro* and *in vivo*.⁴¹ Many solid malignancies express IDO1, which can promote tumor escape from host immunosurveillance. Clinical trials of epacadostat combined with other immunomodulatory drugs against several malignancies are ongoing.⁴² The present findings showed that epacadostat not only reduced IDO1 activity, but also suppressed TF expression via NF κ B (p65) binding activity and Kyn production. This suggests that epacadostat could help to prevent coronary thrombosis. However, further studies are required, because whether or not IDO1 can prevent atherosclerosis remains controversial.^{26,43,44}

This study has several limitations. Serum levels of the inflammatory cytokines, Trp, and Kyn were not investigated due to the retrospective design. Therefore, we could not show a relationship between serum parameters and IDO1 expression in coronary atherosclerotic plaques. Because coronary atherectomy samples were obtained from parts of atherosclerotic plaques, the data might not represent entire atherosclerotic lesions derived from patients. We mainly used a human leukemic monocyte THP-1 cell line. Nonetheless, similar to peripheral blood monocytes, IDO1 and TF were expressed after stimulation with cytokines, and the IDO1 inhibitor, epacadostat, reduced TF expression in macrophages derived from this line.

We did not investigate the contribution of IDO1 to TF expression in atherosclerotic plaque and thrombus formation *in vivo*. Further study is required to determine role of IDO1 on atherothrombosis *in vivo*.

In conclusion, enhanced IDO1 expression in coronary atherosclerotic plaque might contribute to atherothrombosis through TF upregulation in activated macrophages.

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RELATIONSHIP DISCLOSURE

None of the authors have any disclosures relevant to this paper.

AUTHOR CONTRIBUTIONS

Y. Watanabe performed experiments, analyzed the data, and wrote the manuscript. S. Koyama, Y. Matsuura, and K. Nishihira supervised the study, designed experiments. A. Yamashita, K. Kitamura, and Y. Asada revised the manuscript for intellectual content.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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