



KUS121, a novel VCP modulator, attenuates atherosclerosis development by reducing ER stress and inhibiting glycolysis through the maintenance of ATP levels in endothelial cells

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ABSTRACT

Background and aims: Endoplasmic reticulum (ER) stress pathways contribute to atherosclerosis progression. Recently, we developed Kyoto University Substance (KUS) 121, which selectively inhibits ATPase activities of valosin-containing protein (VCP), consequently conserving intracellular ATP consumption and mitigating ER stress. This study evaluated the efficacy of KUS121 in atherosclerosis.

Methods and results: KUS121 was administered daily to *Apoe*^{-/-} mice fed a Western diet (WD) for 8 weeks. KUS121 treatment resulted in a 40–50 % reduction in atherosclerosis progression. Interestingly, we observed that C/EBP homologous protein (Chop), a well-established ER stress marker, was predominantly expressed in plaque endothelium. In human EA.hy926 endothelial cells, KUS121 prevented ER stress-induced apoptosis and down-regulated the Inositol-requiring enzyme 1 alpha (IRE1α)-associated inflammatory pathways. Consistent with these *in vitro* findings, KUS121 treatment significantly reduced endothelial apoptosis, as shown by TUNEL and cleaved caspase-3 staining, and inflammation, as demonstrated by immunostaining of Nuclear factor kappa B (NF-κB) and Intercellular adhesion molecule (Icam) 1 at plaque endothelium. We also demonstrated that KUS121 maintained ATP levels in EA.hy926 cells and atherosclerotic plaque lesions using single-wavelength and the FRET-based fluorescent ATP sensors. Supplementation of intracellular ATP by methyl pyruvate attenuated ER stress-induced apoptotic and inflammatory pathways in endothelial cells, similar to KUS121. Besides affecting ER stress, KUS121 also reduced inflammation even without ER stress by inhibiting glycolysis through increased intracellular ATP levels in LPS-treated EA.hy926 cells.

Conclusions: KUS121 can be a new therapeutic option for atherosclerotic diseases by maintaining intracellular ATP levels, leading to the attenuation of ER stress and glycolysis in plaque endothelium.

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1. Introduction

Atherosclerotic cardiovascular diseases, including cerebral infarction and coronary artery disease, caused by stenosis or occlusion of arteries are the principal cause of mortality worldwide [1]. Over the past few decades, the management of risk factors for atherosclerosis such as

hypertension, dyslipidemia and diabetes has been advanced, and new treatment technologies for atherosclerosis such as stent implantation have been developed. However, there is still no treatment option that directly targets systemically developed atherosclerotic plaques.

The pathophysiology of atherosclerosis is a complex process and affected by several factors [2,3]. Among those, recent evidence has been

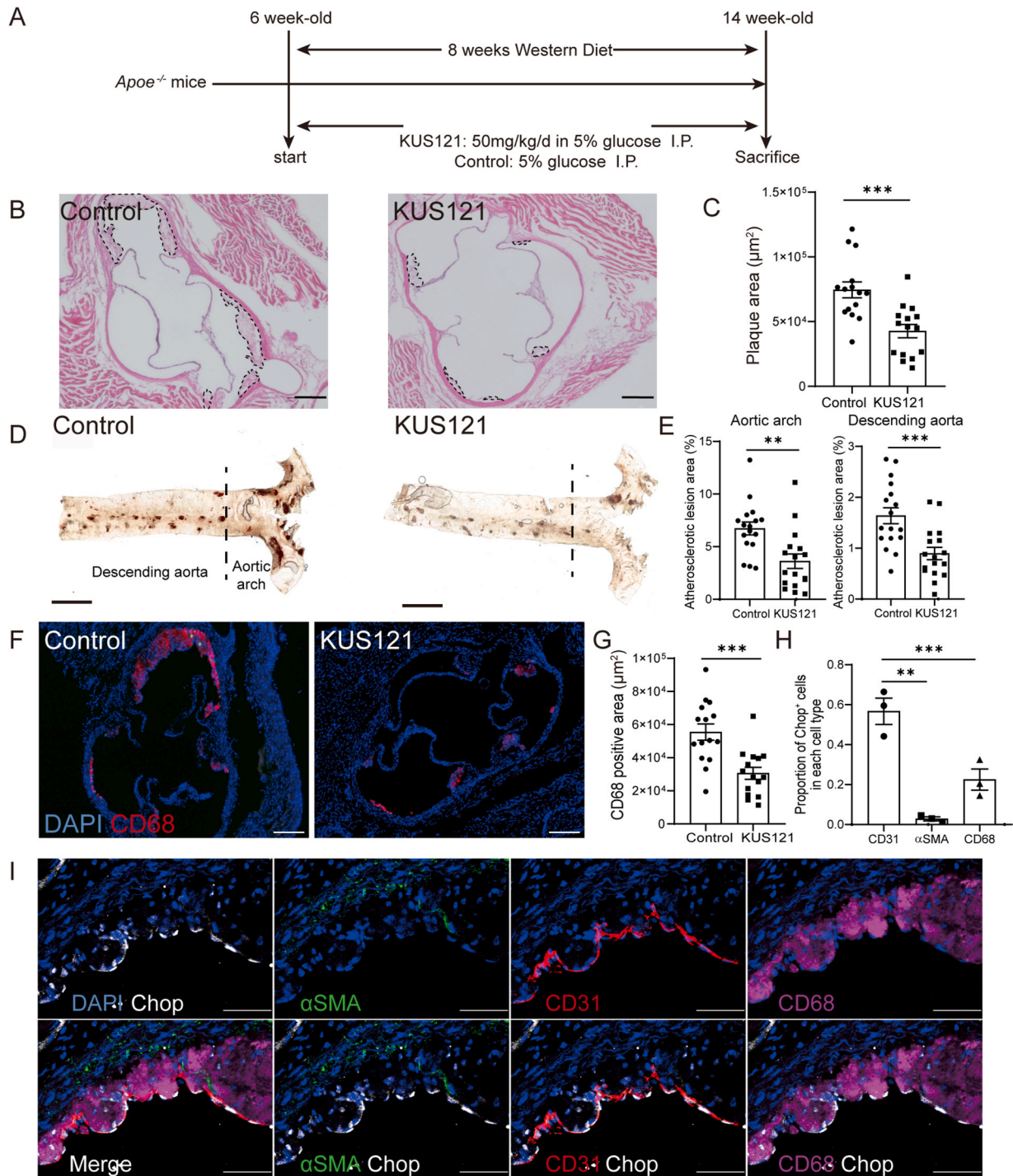


Fig. 1. KUS121 attenuated atherosclerosis progression *in vivo*.

(A) Scheme of experimental protocol to assess the effect of KUS121 on atherosclerosis progression. (B) Representative images of HE staining in aortic sinuses and (C) quantitative analysis of plaque areas ($n = 15$ per group). Scale bar: 200 μ m. (D) Representative images of en face ORO staining of whole thoracic aorta and (E) quantification of atherosclerotic lesions in aortic arches and descending aortas ($n = 17$ per group). Scale bar: 2 mm. (F) Representative images of CD68 staining in aortic sinuses and (G) quantification of CD68-positive areas in atherosclerotic plaques ($n = 15$ per group). Scale bar: 200 μ m. (H) Quantification of the proportions of Chop-positive cells in different cell types and (I) representative images of Chop staining in the atherosclerotic plaques. Scale bar: 50 μ m. All data is presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ by student's *t*-test.

increasingly suggesting that endoplasmic reticulum (ER) stress plays a crucial role in the development of atherosclerosis. Various atherosclerosis-inducing factors, including hyperlipidemia, oxidative stress, and low shear stress, disturb ER homeostasis, subsequently triggering ER stress [3–6]. Chronic ER stress is thought to influence the formation of atherosclerotic plaques via multiple mechanisms. Apoptotic and inflammatory pathways are involved in the downstream of ER stress responses, and accelerate plaque formation and vulnerability [4,7–9].

Kyoto University Substance (KUS) 121 is a small molecule compound specifically designed to inhibit the ATPase activity of valosin-containing protein (VCP). VCP, which belongs to the AAA (ATPases Associated with diverse cellular Activities) family, is a ubiquitously expressed protein found across essentially all cell types. It assumes to perform a range of functions, including the cellular proteasome system, protein degradation related to the endoplasmic reticulum, and facilitating membrane fusion [10,11]. KUS121 does not disturb these cellular functions of VCP [12], but only inhibits its ATP consumption, which indicates that KUS121 works as a “VCP modulator”. In many stressed conditions, KUS121 has been demonstrated to prevent the decline in intracellular ATP levels by inhibiting the ATPase activity of VCP. It has been also demonstrated that KUS121 reduces ER stress and subsequent cell death, and these benefits are expected to be coupled with the maintenance of intracellular ATP [12].

KUS121 showed its efficacy in preventing cell death across various animal disease models, which include several ophthalmic diseases, ischemic stroke and neurodegenerative disorders such as Parkinson's disease [12–18]. Furthermore, its Phase I/II clinical trials have indicated its safety of local administration and potential effectiveness in patients with non-arteritic central retinal artery occlusion [19]. Our group also found that KUS121 reduced infarcted areas in ischemia and reperfusion injury models, and ameliorated cardiac functions in acute and chronic heart failure models [20,21].

Here, we demonstrate that KUS121 is able to attenuate atherosclerosis progression in *Apoe*^{−/−} mice. We also found that ER stress primarily occurred at plaque endothelium. KUS121 effectively preserved intracellular ATP levels and suppressed ER stress responses in endothelial cells, which in turn mitigated endothelial apoptosis and inflammation both *in vitro* and *in vivo*. Moreover, we also demonstrated that increased cellular ATP levels by KUS121 could reduce inflammation through the inhibition of glycolysis in endothelial cells, independent of the attenuation of ER stress. These results suggest not only that KUS121 is a promising candidate for the treatment of atherosclerosis but also that maintenance of intracellular ATP levels in endothelium can be a new therapeutic target of atherosclerosis via attenuation of both ER stress and glycolysis.

2. Materials and methods

2.1. Mice

Apoe^{−/−} (B6.129P2-*Apoe*^{tm1Unc}/J, 002052) mice were purchased from Jackson Laboratories. Mice were fed with normal chow until 6 weeks of age. Then, diet was switched to Western diet (WD) (Research Diets Inc, D12079B) containing 0.21 % cholesterol and 41 % milk fat to accelerate atherosclerotic plaque formation. To evaluate the impact of KUS121 on the progression of atherosclerotic plaques, mice were administered a daily intraperitoneal injection of 50 mg/kg KUS121 as the previous studies [20,22], starting at 6 weeks of age. Control mice were injected a 5 % glucose solution. Mice were sacrificed and samples were collected after 8 weeks of KUS121 treatment (Fig. 1A). Mice were accommodated in specific pathogen-free animal facilities maintained by the Institute of Laboratory Animals within Kyoto University Graduate School of Medicine. All experiment procedures were approved by the Ethic Committee for Animal Experiments of Kyoto University.

2.2. Quantification of atherosclerotic plaque lesions

Atherosclerotic lesions were quantified by Hematoxylin and Eosin (HE) (Wako, 051–06495 and 131–09665) staining of the cryosections of the aortic sinus and by en face analysis of the whole thoracic aorta stained by oil red O (ORO) (Sigma-Aldrich, O0625-25G) [23–25]. After sacrifices, hearts were fixed and embedded in OCT compound for cryosections (10 μm) of aortic sinuses. Sections were then subjected to HE staining for quantification. The average plaque size of 3 sections from each mouse was used as a representative value. For en face analysis, thoracic aortas were fixed and pretreated with 100 % propylene glycol (Sigma-Aldrich, ESK4023) for 15 min, then incubated with ORO solution for 3 h at room temperature (RT). They were washed with 85 % propylene glycol and then with PBS. After staining, thoracic aortas were placed between a cover glass and a slide glass, and imaged by LSM 710 (ZEISS). Then, ORO positive areas of aortic arches and descending aortas were separately measured. The aortic arch was defined as the segment from the aortic root to 3 mm distal to the left subclavian artery. The left part was defined as the descending aorta. Images were analyzed using Image J software.

2.3. Cell culture

EA.hy926 cells (CRL-2922TM, ATCC) and THP-1 (TIB-202TM, ATCC) derived macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 (Nacalai Tesque, 08458 and 30264) supplemented with 10 % FBS and antibiotics (Gibco, 173012 and 10378–016). THP-1 monocytes were differentiated into macrophages by incubating for 3 days with 100 nM PMA (Nacalai Tesque, 27547–14). To induce endoplasmic reticulum (ER) stress, 1 μg/mL tunicamycin (Sigma-Aldrich, T7765) was added to the cultured cells. Cells were also treated with KUS121, Methyl pyruvate (MePyr) (Wako, 137–10152), Lipopolysaccharide (LPS) (Wako, 127–05141), 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (Selleck, S7639), oxidized low-density lipoprotein (ox-LDL) (Thermo Scientific L34357) or Adenosine triphosphate (ATP) (Jena Bioscience, Nu-1010) depending on the experiment.

2.4. Assessment of cell viability *in vitro*

Cells were plated in a 96-well plate, followed by incubation with tunicamycin or LPS, and KUS121. Then, cell viability was assessed by Cell Counting Kit-8 (Dojindo, 347–07621) according to the instruction manual. The absorbance value was measured by 2030 ARVO X (PerkinElmer).

2.5. Quantitative real-time PCR

Total RNA was isolated and purified using TriPure Isolation Reagent (Roche, 11667165001), and cDNA was synthesized from 1 μg of total RNA using Verso cDNA Synthesis Kit (Thermo Scientific, AB-1453/B) in accordance with the manufacturer's instructions. For quantitative real-time PCR (qPCR), specific genes were amplified with THUNDER-BIRD[®] SYBR qPCR Mix (TOYOBO, QKD-201 T) using StepOnePlusTM (Thermo Scientific). Expression levels were normalized using house-keeping gene, *ACTB*. The primer sequences are as follows:

ACTB forward, 5'-AGGCATCTTCCAGCCTTCC -3';
ACTB reverse, 5'-GCACTGTGTTGGCGTACAGG-3';
CHOP forward, 5'-AGCTGTGCCACTTTCCTTTC-3';
CHOP reverse, 5'-CAGAACAGCAGAGGTCACA-3';
BIP forward, 5'-TGTTCAACCAATTATCAGCAAATC-3';
BIP reverse, 5'-TTCTGCTGTATCCTCTTTCACACGT-3';
sXBP1 forward, 5'-CTGAGTCCCGAATCAGGTGCAG-3';
sXBP1 reverse, 5'-ATCCATGGGGAGATGTTCTCG-3';
ATF4 forward, 5'-GTTCTCCAGCGACAAGGCTA-3';
ATF4 reverse, 5'-ATCCTGCTTGCTGTTGTTGG-3';
IL6 forward, 5'-CCTGAACCTTCCAAAGATGGC -3';

IL6 reverse, 5'-TTCACCAGGCAAGTCTCCTCA-3';
IL8 forward, 5'-ACTGAGAGTGATTGAGAGTGAC-3';
IL8 reverse, 5'-AACCTCTGCACCCAGTTTC-3';
IL1B forward, 5'-AGCTACGAATCTCCGACCAC-3';
IL1B reverse, 5'-CGTTATCCCATGTGTGAAGAA-3';
ICAM1 forward, 5'-GGCTGGAGCTGTTTGAGAAC-3';
ICAM1 reverse, 5'-ACTGTGGGGTTCAACCTCTG-3';
VCP forward, 5'-CCCAGCCCAAGATGGATGAA-3';
VCP reverse, 5'-CGTTTGCCGTACTTCACATCAG-3'.

2.6. Western blotting

Western blotting was performed using standard procedures as described previously [26]. A total of 15 µg of protein was fractionated using NuPAGE 4–12 % Bis-Tris Mini gels (Thermo Scientific, NP0322BOX) and transferred to Amersham™ Protran® Western blotting membranes (Cytiva, 10600002). The membrane was blocked using PBS containing 5 % non-fat milk or 5 % bovine serum albumin (BSA) for 30 min and incubated with the primary antibody overnight at 4 °C. After washing by PBS containing 0.05 % Tween-20 (PBST), the membrane was incubated with the secondary antibody for 1 h at RT. The membrane was washed again with PBST and then Pierce™ Western Blotting Substrate (Thermo Scientific, 32132) or Pierce™ Western Blotting Substrate Plus (Thermo Scientific, 32106) was used to detect proteins by LAS-4000 Mini system (Fuji Film).

To extract nuclear and cytoplasmic proteins, cells were harvested after washing with cold PBS and then lysed using the Cytoplasmic Extraction Buffer provided in the LysoPure™ Nuclear and Cytoplasmic Extractor Kit (Wako, 295–73901). After incubation on ice and centrifugation, the cytoplasmic fraction was collected from the supernatant. The nuclear pellet was then resuspended in the Nuclear Extraction Buffer, followed by incubation and centrifugation to obtain the nuclear protein fraction.

The following primary antibodies were purchased and used: anti-VCP (7F3, Cell signaling technology, #2649, 1:200), anti-BIP (Cell signaling technology, #3183, 1:200), anti-CHOP (L63F7, Cell signaling technology, #2895, 1:200), anti-p-JNK (Thr183/Tyr185) (81E11, Cell signaling technology, #4668, 1:200), anti-JNK (Cell signaling technology, #9252, 1:200), anti-Cleaved Caspase-3 (Cell signaling technology, #9664, 1:200), anti-p-NF-κB (Ser536) (93H1, Cell signaling technology, #3033, 1:200), anti-NF-κB (D14E12, Cell signaling technology, #8242, 1:200), anti-p-IRE1α (Ser724) (Novus, NB100-2323, 1:100), anti-IRE1α (14C10, Cell signaling technology, #3294, 1:200), anti-TBP (TATA Binding Protein) (N-12, Santa Cruz, sc-204, 1:500), anti-ACTB (AC-74, Sigma-Aldrich, A5316, 1:200). The secondary antibodies are as follows: anti-Rabbit IgG, HRP-Linked (GE Healthcare, NA934V, 1:500), anti-Mouse IgG, HRP-Linked (GE Healthcare, 931 V, 1:500).

2.7. Knockdown of VCP by small interfering RNAs (siRNAs)

To knock down VCP expression, siRNAs were transfected into endothelial cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific, 13778–075), according to the manufacturer's instructions. The following siRNAs were tested: Silencer Select VCP siRNA s14765, s14766 and s14767. Finally, s14767 was selected due to its highest knockdown efficiency. Silencer Select negative control #1 siRNA (4390843) was used as a control. All the siRNAs were purchased from the Thermo Fisher Scientific.

2.8. Transwell migration assay

The impact of KUS121 on migration of THP-1 cells toward EA.hy926 cells was determined using 24-well transwell systems (8-µm pore size; Corning, 3422). EA.hy926 cells (2×10^5 cells/well) were seeded and treated with or without tunicamycin and KUS121 for 18 h in the bottom chamber. On the other hand, untreated THP-1 cells were stained with

calcein-AM (Dojindo, 341–07901) for 20 min at 37 °C, according to the manufacturer's instructions, before being resuspended in RPMI 1640 supplemented with 1 % FBS. The stained THP-1 cells were added to the upper inserts (1×10^5 cells/well) and incubated with the primed EA.hy926 monolayers for 1 h at 37 °C.

Complete images of each well were captured using a fluorescence microscope (Keyence, BZ-X810) and processed with Image J to quantify the number of migrated THP-1 cells in each well for comparative analysis.

2.9. Immunofluorescence staining

For immunofluorescence staining, sections were blocked with 3 % BSA in the presence of 1 % Triton X-100 for 1 h at RT. Then, sections were incubated with primary antibody overnight at 4 °C. After washing with PBS, sections were incubated with secondary antibody for 90 min at RT. Nuclei were stained using mounting medium with DAPI (Fluoro-Gel II Mounting Medium, Electron Microscopy Sciences, 17985–50). Immunofluorescence images were acquired using BZ-X810 all-in-one fluorescence microscope (Keyence), LSM 710 (ZEISS) or TCS SP8 microscope system (Leica). Images were analyzed using Image J and Imaris software (Oxford Instruments). Following antibodies were purchased and used for immunostaining: CD68 (FA-11, Bio-Rad, MCA1957, 1:200), Actin, α-Smooth Muscle (1A4, Sigma-Aldrich, F3777, 1:500), CD31 (2H8, Merck Millipore, MAB1398Z, 1:200), CD31 (MEC13.3, BD Pharmingen, 550274, 1:400), Icam1 (YN1/1.7.4, BioLegend, 116101, 1:200), Chop (F-168, Santa Cruz, sc-575, 1:100), NF-κB (C-20, Santa Cruz, sc-372, 1:200) and Cleaved Caspase-3 (Asp175, Cell signaling technology, #9661, 1:200). TUNEL staining was performed using In-Situ Cell Death Detection Kit (Roche Applied Science, E-CK-A324), according to the manufacturer's instructions. The sections were co-stained with CD31 to identify Endothelial cells. The number of apoptotic endothelial cells (TUNEL⁺ and CD31⁺ cells) was counted and divided by the total number of endothelial cells.

2.10. Serum data

Blood was obtained by submandibular vein puncture after 8 weeks of KUS121 treatment. Serum was separated by centrifugation at 4 °C and stored at –80 °C. Biochemical examination was performed using Hitachi 7180 Auto Analyzer (Nagahama Life Science Laboratory, Nagahama, Japan).

2.11. Monocytes recruitment

Monocyte recruitment was assessed by counting bead-labeled monocytes in atherosclerotic plaques as previously described [27]. Briefly, 200 µl of clodronate liposomes (Liposoma BV) were intravenously administered 5 days before sacrifice, and then, Ly6C^{hi} monocytes were labeled by intravenous injection of 1.0 µm Fluoresbrite® YG Microspheres (Polysciences, 17154) 2 days before sacrifice.

2.12. Complete blood count and flow cytometry analysis

Peripheral blood was taken by submandibular vein puncture after 7 weeks of KUS121 treatment for the complete blood count and the flow cytometry analysis. Complete blood cell counts were measured using a hematology cell counter (Celltac α, NIHON KOHDEN). In preparation for flow cytometry, red blood cells were lysed using a commercial RBC lysis solution (BD PharmLyse, BD Biosciences, 555899). Samples were then incubated with antibodies for 30 min at 4 °C. Following antibodies were purchased and used for flow cytometry analysis: CD45 (30F-11, BD Bioscience and Biolegend, 103138, 1:200), CD11b (M1/70, Biolegend and eBioscience, 101257, 1:200), Ly6G (1A8, Biolegend, 127618, 1:200), CD115 (AFS98, eBioscience, 135506, 1:200), Ly6C (HK1.4, Biolegend, 128032, 1:200), B220 (RA3-6B2, Biolegend, 103222, 1:200),

CD3e (145-2C11, eBioscience,100306, 1:200).

For the *in vitro* apoptosis assay, EA.hy926 cells were stained with Alexa Fluor 647-Annexin V (BioLegend, 640943) and Helix NP™ Green (BioLegend, 425305) at RT for 15 min in the dark for flow cytometry analysis. The samples were analyzed using a BD FACSAria II flow cytometer.

2.13. Qualification of intracellular ATP levels in vitro

The single-wavelength fluorescent sensor (iATPSnFR^{1.0}) was transduced into EA.hy926 cells using lentiviral transfection [28]. ER stress was induced by 1 μg/mL tunicamycin and the cells were also incubated with KUS121 or DMSO as control. After 48 h of incubation, the cells were washed with PBS and fluorescence images were taken by LSM 710 (ZEISS). The cells were also incubated with Methyl pyruvate or 2-Deoxy-D-glucose (2DG) (Wako, 046-06483) depending on the experiments. The fluorescence intensity of the ATP sensor was analyzed using Image J. The intracellular ATP levels were also assessed by flow cytometry. In brief, to eliminate the influence of detachment of cells on intracellular ATP levels, the iATPSnFR^{1.0} transduced cells were cultured in floating state for 6 h with 1 μg/mL tunicamycin and KUS121 at 37 °C. Before flow cytometry analysis, the cells were washed with PBS and resuspended in PBS supplemented with 2 % FBS and 1 mM of EDTA. FlowJo (BD Biosciences) was used to analyze the fluorescence intensity.

To measure ATP levels in LPS-treated endothelial cells, we used ATP assay kit (Toyo B-net, CA2-10) because the cell number did not change compared to the control wells. Briefly, LPS-treated EA.hy926 cells were washed with PBS and then incubated with the cell-dissolving solution of the kit at room temperature for 10 min. The Relative Light Unit (RLU) was measured by 2030 ARVO X (PerkinElmer).

2.14. Quantification of lactate concentration in media

The supernatant of LPS-treated EA.hy926 cells and THP1 derived macrophages were collected, and lactate concentration was quantified by Lactate Assay Kit-WST (Dojindo, L256) according to the instruction manual. The absorbance value was measured by 2030 ARVO X (PerkinElmer).

2.15. Quantification of ATP levels at atherosclerotic plaques using Go-ATeam2 mice

The Go-ATeam2 mice, which carry a fluorescence resonance energy transfer (FRET)-based ATP biosensor (donor fluorophore: green fluorescent protein (GFP); acceptor fluorophore: orange fluorescent protein (OF)) ubiquitously, were kindly given by Dr. Masamichi Yamamoto. Intracellular ATP concentration induces conformational change in this biosensor, and higher ATP levels result in a stronger FRET signal, allowing for the quantitative measurement of intracellular ATP concentration by the FRET/GFP ratio *in vivo*. To assess ATP levels at atherosclerotic plaques, Go-ATeam2 mice were crossed with *Apoe*^{-/-} mice and fed with WD for 12 months. The mice were anesthetized with intraperitoneal injection of 3 types of mixed anesthetic agents (medetomidine; 0.3 mg/kg, midazolam; 4.0 mg/kg, butorphanol; 1.0 mg/kg). Then, the common carotid artery was exposed, and fluorescence images of FRET and donor channels were taken by Olympus SZ16 microscope with the attached Reflected Fluorescence Illuminator (Olympus, SZX2-RFA16). FRET signal was excited by using BP460-495 filter and observed by BP545-580 filter (FRET channel) and by BP460-480 filter (donor channel: GFP). The FRET/GFP emission ratios were analyzed using Image J.

2.16. Statistical analysis

Measured data were presented as mean ± standard error of the mean (SEM). For statistical comparisons between 2 groups, Shapiro-Wilk test

Table 1
Serum profile of *Apoe*^{-/-} on WD for 8 weeks with or without KUS121 treatment.

	Control (n = 4)	KUS121 (n = 5)	P value
TP (g/dL)	5.75 ± 0.64	5.66 ± 0.713	0.8498
ALB (g/dL)	2.575 ± 0.236	2.86 ± 0.467	0.3067
BUN (mg/dL)	17.6 ± 1.506	37.82 ± 45.303	0.6032
Na (mEq/L)	142.5 ± 0.577	144.4 ± 6.025	0.555
K (mEq/L)	4.625 ± 0.55	5.22 ± 0.729	0.22
Cl (mEq/L)	103.75 ± 2.217	97.2 ± 7.294	0.1305
Ca (mg/dL)	8.225 ± 0.45	8.56 ± 0.321	0.1746
IP (mg/dL)	9.45 ± 1.786	9.54 ± 1.618	0.939
AST (IU/L)	58 ± 3.916	58 ± 7.616	>0.9999
ALT (IU/L)	32 ± 3.742	23.6 ± 7.956	0.0952
LDH (IU/L)	771.5 ± 222.675	1197.8 ± 371.934	0.0848
AMY (IU/L)	2333 ± 206.215	2260 ± 101.811	0.5063
T-CHO (mg/dL)	1320.75 ± 161.56	1102.2 ± 196.424	0.1171
TG (mg/dL)	238.5 ± 85	388.8 ± 366.316	0.5079
LDL-C (mg/dL)	292.5 ± 57.006	276.2 ± 101.986	0.7849
HDL-C (mg/dL)	22.5 ± 5.323	22.4 ± 8.414	0.9444
T-BIL (mg/dL)	0.07 ± 0.024	0.126 ± 0.073	0.188
GLU (mg/dL)	379.25 ± 50.803	314.4 ± 63.41	0.1415

All data are presented as mean ± SEM. P values were calculated by student's *t*-test or Mann-Whitney *U* test based on the distribution of each parameter.

was performed to assess the normality of distribution. When the data was normally distributed, unpaired Student's *t*-test was conducted. Otherwise, Mann-Whitney *U* test was conducted. For statistical analysis of three or more groups, 1-way analysis of variance (ANOVA) with the Tukey's multiple comparisons test was used. To analyze the time-dependent graph, 2-way ANOVA with the Tukey's multiple comparisons test was performed. A p-value of <0.05 was considered as statistically significant.

3. Results

3.1. KUS121 attenuates atherosclerotic progression in *Apoe*^{-/-} mice

At first, we assessed the effect of KUS121 on atherosclerosis progression using *Apoe*^{-/-} mice fed with WD (Fig. 1A). The daily administration of KUS121 significantly attenuated atherosclerosis formation at aortic root by 40.9 % (Fig. 1B and C). We also measured ORO-positive areas as atherosclerotic lesions in the aortic arches and the descending aortas separately, which showed a significant reduction in the KUS121-treated group by 46.2 % in the aortic arches and by 49.9 % in the descending aortas (Fig. 1D and E). Macrophage accumulation assessed by CD68 staining was also reduced by KUS121 treatment (Fig. 1F and G). We also analyzed peripheral blood cell counts in those 2 groups. Interestingly, we observed a significant reduction in the number of peripheral neutrophils and Ly6C^{hi} inflammatory monocytes in the KUS121-treated group (Fig. S1). On the other hand, there were no significant differences in body weight and chow consumption between the control and KUS121-treated groups (Fig. S2A–B). Moreover, KUS121 treatment did not affect the serum profile including the cholesterol levels (Table 1).

As mentioned above, KUS121 has been demonstrated to reduce the ER stress [12]. Then, we assessed the distribution of ER stress at atherosclerotic plaques in *Apoe*^{-/-} mice on WD for 8 weeks by the staining of C/EBP homologous protein (Chop), which is a well-known marker of ER stress, and found that Chop was primarily expressed by plaque endothelial cells. Macrophages also expressed Chop, but at much lower levels compared to endothelial cells. On the other hand, smooth muscle cells barely expressed Chop (Fig. 1H–I and S2C). These findings indicate that endothelial cells can be a potential primary target of KUS121.

3.2. KUS121 protects endothelial cells from apoptosis under ER stress

ER stress is known to trigger apoptosis, contributing to the progression of atherosclerosis [6]. Thus, we evaluated the impact of

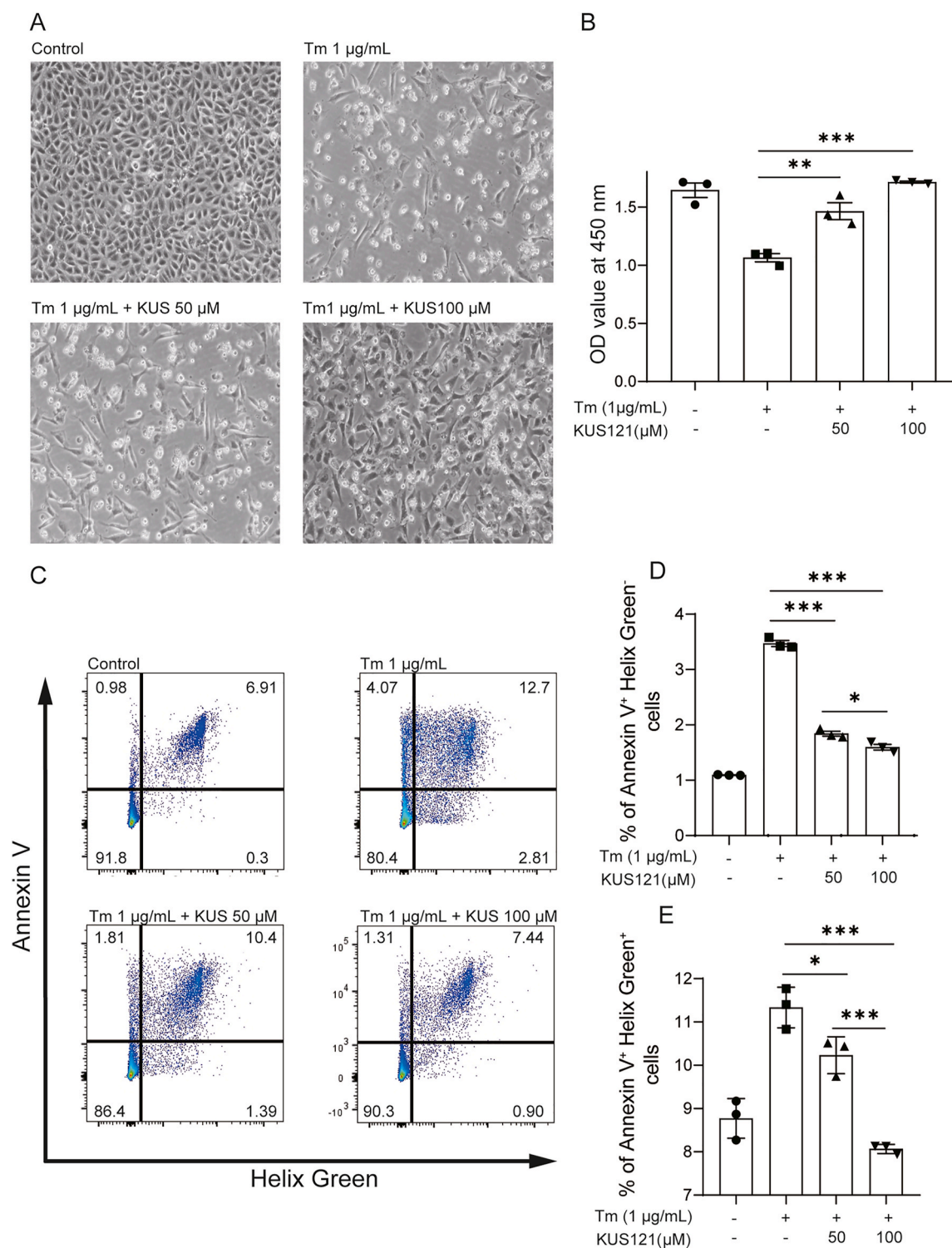
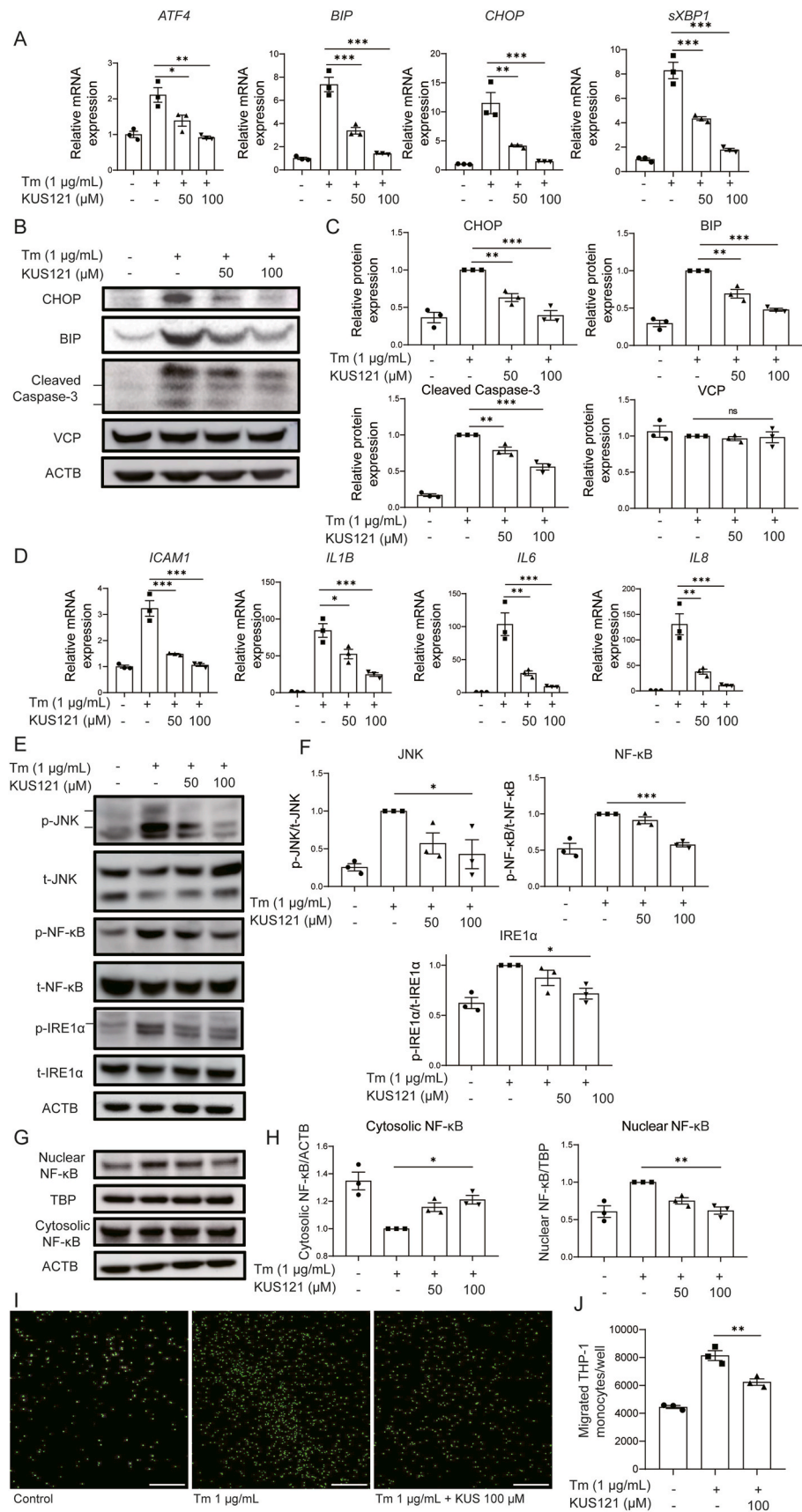


Fig. 2. KUS121 protected endothelial cells from apoptosis

(A) Representative images of EA.hy926 human endothelial cells cultured with 1 µg/mL tunicamycin (Tm) for 48 h treated with different concentrations of KUS121 (50 and 100 µM) or DMSO as control and (B) quantification of cell viability ($n = 3$ per group). (C) Representative dot plots of tunicamycin-induced apoptosis in EA.hy926 cells treated with different concentrations of KUS121 (50 and 100 µM) or DMSO as control. Quantification of (D) apoptosis (Annexin V⁺ Helix Green⁻) and (E) cell death (Annexin V⁺ Helix Green⁺) ($n = 3$ per group). The experiments were independently repeated 3 times. All data are presented as mean $\pm$ SEM. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$ by 1-way ANOVA with the Tukey's multiple comparisons test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



(caption on next page)

Fig. 3. KUS121 mitigated not only the ER stress-induced apoptosis pathway but also the inflammatory pathways in human endothelial cells

(A) Quantitative real-time PCR analyses of the key genes in an ER stress response in EA.hy926 cells stimulated by 1 $\mu\text{g/mL}$ tunicamycin for 48 h treated with different concentration of KUS121 (50 and 100 μM) or DMSO as control ($n = 3$ per group). (B) Representative western blotting images of ER stress-induced apoptosis marker CHOP, BIP and Cleaved Caspase-3, and VCP in EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ tunicamycin for 48 h treated with different concentration of KUS121 (50 and 100 μM) or DMSO as control, and (C) quantification by densitometry ($n = 3$ per group). (D) Quantitative real-time PCR analyses of the inflammatory genes downstream of the ER stress pathway. EA.hy926 cells were cultured with 1 $\mu\text{g/mL}$ tunicamycin for 48 h with different concentration of KUS121 (50 and 100 μM) or DMSO as control ($n = 3$ per group). (E) Representative western blotting images of ER stress-induced phosphorylation of IRE1 α , JNK and NF- κ B in EA.hy926 cells cultured with tunicamycin for 48 h with different concentration of KUS121 (50 and 100 μM) or DMSO as control and (F) quantification by densitometry ($n = 3$ per group). (G) Representative western blotting images of nuclear and cytosolic NF- κ B in EA.hy926 cells cultured with tunicamycin for 48 h with different concentration of KUS121 (50 and 100 μM) or DMSO as control and (H) quantification by densitometry ($n = 3$ per group). (I) Representative fluorescence images of the migrated calcein-AM stained THP-1 monocytes towards the EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ tunicamycin for 18 h with or without 100 μM of KUS121 and (J) quantification of migrated THP-1 monocytes in each well ($n = 3$ per group). Scale bar: 200 μm . The experiments were independently repeated 3 times. All data is presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by 1-way ANOVA with the Tukey's multiple comparisons test.

KUS121 on cellular viability and apoptosis of endothelial cells. An ER stress inducer, tunicamycin reduced the viability of a human endothelial cell line, EA.hy926. However, this effect is reversed by KUS121 in a dose-dependent manner (Fig. 2A and B). Then, we assessed the anti-apoptotic effect of KUS121 in endothelial cells by the Annexin V-binding assay. As expected, KUS121 treatment showed a dramatic reduction in apoptotic cells (Annexin V⁺ Helix Green⁻) (Fig. 2C and D). Moreover, the dead cells (Annexin V⁺ Helix Green⁺) were also significantly decreased (Fig. 2E), which is consistent with the result of the viability assay in Fig. 2A and B.

3.3. KUS121 can attenuate both apoptotic and inflammatory pathways of ER stress in endothelial cells

Next, given the central role of ER stress in plaque formation [29,30], we further substantiated the effect of KUS121 on ER stress. To this end, we examined the effect of KUS121 on the signaling pathways of ER stress using EA.hy926 cells treated with tunicamycin. As anticipated, KUS121 effectively mitigated the upregulation of key genes in an ER stress response, such as Activating Transcription Factor 4 (ATF4), CHOP, Binding Immunoglobulin Protein (BIP), and spliced X-Box Binding Protein 1 (sXBP1) at mRNA levels (Fig. 3A). To further validate the effect of KUS121 attenuating ER stress-mediated apoptosis, we assessed the protein expressions of CHOP and BIP which are the direct upstream of apoptosis-inducing signaling in the ER stress response, as well as the apoptosis marker, cleaved caspase-3, and confirmed they were also reduced by KUS121 treatment. On the other hand, KUS121 treatment did not affect the expression of VCP itself (Fig. 3B and C).

The ER stress pathway is also known to induce inflammation, a pivotal player in atherosclerosis [31,32]. Thus, we hypothesized that the inhibitory effects of KUS121 on ER stress could concomitantly attenuate inflammation. However, it was still unknown whether KUS121 also attenuates ER stress-induced inflammation. To test this hypothesis, we first assessed the transcriptional changes of the inflammatory genes in EA.hy926 cells under ER stress. Our results revealed that KUS121 treatment significantly counteracted the upregulation of the Intercellular Adhesion Molecule1 (ICAM1), interleukin (IL) 1 β (IL1B), IL6, and IL8, driven by tunicamycin-induced ER stress, providing the evidence of its anti-inflammatory effects (Fig. 3D). Since the Inositol-requiring enzyme (IRE) 1 α -c-Jun N-terminal kinase (JNK)- Nuclear factor kappa B (NF- κ B) signaling is the main inflammatory pathway induced by ER stress [31], we assessed the phosphorylation of these proteins as the markers of their activation. Tunicamycin induced IRE1 α phosphorylation, which in turn triggered the phosphorylation of downstream JNK and NF- κ B, and KUS121 successfully suppressed the activation of this inflammatory pathway under ER stress (Fig. 3E and F). To further confirm the effect of KUS121 on the NF- κ B pathway, we separately measured NF- κ B protein levels in the nucleus and cytoplasm. The result indicated that KUS121 reduced NF- κ B nuclear translocation, thereby alleviating cellular inflammation (Fig. 3G and H). We also performed a transwell migration assay to assess whether KUS121's anti-inflammatory effect was functional. The results showed that

KUS121 treatment effectively reduced THP-1 monocyte migration toward endothelial cells under tunicamycin-induced ER stress, suggesting its potential to inhibit endothelial activation and attenuate inflammation (Fig. 3I and J). Taken together, KUS121 is thought to be able to reduce the ER stress-induced endothelial apoptosis and inflammation.

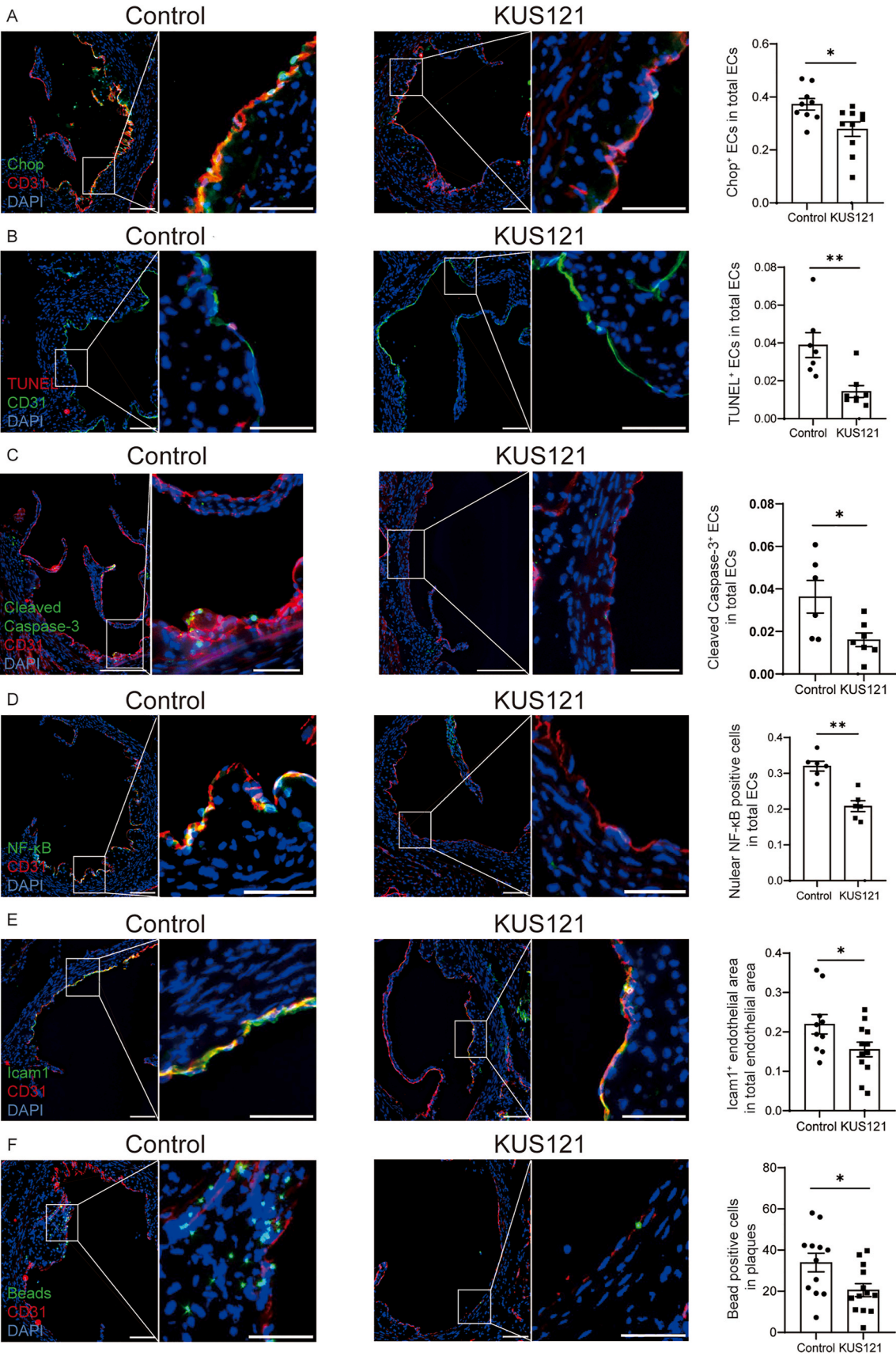
On the other hand, we also observed some ER stress responses in plaque macrophages (Fig. 1H). Considering the possibility that the inhibition of ER stress by KUS121 in macrophages may also contribute to the reduction of atherosclerosis, we next assessed the effect of KUS121 on macrophages. However, KUS121 did not affect the viability of the THP-1 derived macrophages and murine primary peritoneal macrophages under tunicamycin-induced ER stress (Fig. S3A). Moreover, KUS121 could not attenuate the ER stress-induced CHOP and IL8 expression in THP-1 derived macrophages (Fig. S3B).

3.4. KUS121 can reduce ER stress induced apoptosis and inflammation at endothelium in vivo

To validate the impact of KUS121 on endothelial cells *in vivo*, we first assessed the endothelial Chop expression in atherosclerotic plaques of the KUS121-treated group by immunostaining and found that KUS121 treatment significantly reduced the Chop-positive endothelial cells, as compared to the control group (Fig. 4A). Since Chop induces apoptosis, we also assessed apoptotic endothelial cells in plaques by TUNEL and cleaved caspase-3 staining, and confirmed that KUS121 reduced apoptosis in plaque endothelial cells (Fig. 4B and C). Next, to verify the anti-inflammatory effect of KUS121 on endothelial cells, we investigated the impact of KUS121 on the translocation of NF- κ B to the nuclei in plaque endothelial cells. Consequently, KUS121 treatment significantly reduced the nuclear translocation of NF- κ B in endothelial cells during atherosclerosis progression (Fig. 4D). We also demonstrated that Icam1, which is the critical adhesion molecule for recruitment of monocytes, was significantly reduced in plaque endothelial cells of the KUS121-treated group, as compared to the control group (Fig. 4E–S3C). Consistent with this result, monocyte recruitment into atherosclerotic plaques assessed by counting bead-labeled monocytes in the plaques was also reduced in KUS121-treated group, as compared to the control group (Fig. 4F). The reduction of peripheral neutrophils and Ly6C^{hi} inflammatory monocytes may also contribute to the reduction of monocyte recruitment (Fig. S1). These data further corroborate that KUS121 can attenuate atherosclerosis progression by reducing ER stress-induced endothelial apoptosis and inflammation.

3.5. KUS121 can maintain intracellular ATP levels under ER stress and counteract ER stress response in endothelial cells

KUS121 is also known to maintain intracellular ATP levels in several pathological conditions [12,20,21]. To confirm this effect in endothelial cells, we employed the fluorescent ATP sensor, iATPsnFR^{1.0} by which cytosolic ATP levels can be assessed as a fluorescence intensity of GFP [28]. As a result, KUS121 successfully rescued the reduction of intracellular ATP under tunicamycin-induced ER stress (Fig. 5A and B).



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Fig. 4. KUS121 reduced ER stress-induced endothelial apoptosis and inflammation *in vivo*

(A) Representative endothelial Chop expression in the aortic sinuses of the control group (left) and the KUS121-treated group (middle), and the proportion of Chop-positive endothelial cells in the total endothelial cells (right) ($n = 9\text{--}10$ per group). $*p < 0.05$ by Mann-Whitney U test. (B) Representative TUNEL-positive cells in the aortic sinuses of the control group (left) and the KUS121-treated group (middle), and the proportion of TUNEL-positive endothelial cells in the total endothelial cells (right) ($n = 7\text{--}8$ per group). $**p < 0.01$ by student's t -test. (C) Representative Cleaved Caspase-3-positive cells in the aortic sinuses of the control group (left) and the KUS121-treated group (middle), and the proportion of Cleaved Caspase-3-positive endothelial cells in the total endothelial cells (right) ($n = 6\text{--}7$ per group). $*p < 0.05$ by student's t -test. (D) Representative endothelial NF- κ B expression in the aortic sinuses of the control group (left) and the KUS121-treated group (middle), and the proportion of nuclear NF- κ B-positive endothelial cells in the total endothelial cells ($n = 6$ per group). $**p < 0.01$ by student's t -test. (E) Representative endothelial Icam1 expression in the aorta sinuses of the control group (left) and the KUS121-treated group (middle), and the proportion of the Icam1-positive area in the total endothelial area. ($n = 10\text{--}12$ per group). $*p < 0.05$ by student's t -test. (F) Representative bead-labeled cells in the aortic sinuses of the control group (left) and the KUS121-treated group (middle), and the number of bead-labeled cells in atherosclerotic plaques ($n = 12\text{--}13$ per group). $*p < 0.05$ by student's t -test. All data is presented as mean $\pm$ SEM. Scale bar of low magnification images: 200 μ m and high magnification images: 50 μ m.

KUS121 also reverted the ATP reduction by the treatment of 2DG (Fig. S3D–E).

Extracellular ATP is reported to induce vascular inflammation to promote atherosclerosis via P2Y2 receptor [33]. However, it is still unclear how intracellular ATP levels affect atherosclerosis progression especially in endothelial cells. Since an ER stress consumes intracellular ATP (Fig. 5A and B) and KUS121 was designed to inhibit ATPase activity of VCP to save intracellular ATP, we speculated that the maintenance of intracellular ATP levels by KUS121 could directly attenuate ER stress. To confirm this, we adopted methyl pyruvate (MePyr) to increase intracellular ATP levels specifically, which is a membrane-permeable pyruvate and known to be converted to ATP in mitochondria. It actually increased intracellular ATP levels in endothelial cells under tunicamycin-induced ER stress (Fig. 5C). Moreover, we found that MePyr treatment successfully attenuated ER stress-induced apoptosis and inflammatory pathways (Fig. 5D and E). On the other hand, addition of extracellular ATP did not reduce ER stress responses (Fig. S3F), which indicates that intracellular and extracellular ATP work differently for endothelial cells.

Finally, to confirm whether ER stress reduction by KUS121 is specifically dependent on VCP, we knocked down VCP expression using siRNA under tunicamycin-induced ER stress in EA.hy926 cells. We tested three siRNAs and determined the most efficient one (Fig. S3G). The knockdown of VCP mostly abolished the ER stress-attenuating effect of KUS121 treatment (Fig. 5F).

To assess whether the attenuation of ER stress is the sole mechanism behind the anti-atherosclerotic effect of maintaining intracellular ATP levels by KUS121 in endothelial cells, we determined the concentration of LPS that induces inflammation without triggering an ER stress response or cell death (Fig. 5G–S3H). Surprisingly, KUS121 still reduced inflammatory gene expression even in the absence of ER stress (Fig. 5H). Since cell death was not induced, we measured the intracellular ATP levels in each well and found that LPS treatment decreased ATP levels, but these were rescued by KUS121 (Fig. 5I). This suggests that maintaining intracellular ATP levels by KUS121 attenuates inflammation through a pathway other than mitigating ER stress. The activity of 6-phosphofructo-1-kinase (PFK-1), a key enzyme in the glycolytic pathway, is known to be inhibited by ATP [34]. Glycolysis is considered a pro-inflammatory factor in endothelial cells and is thought to accelerate atherosclerosis progression [35]. Therefore, we speculated that the increase in intracellular ATP levels by KUS121 could inhibit glycolysis to attenuate inflammation. To test this hypothesis, we measured the concentration of lactate produced by glycolysis in the media of EA.hy926 cells treated with or without LPS and KUS121. Consequently, LPS enhanced lactate production by endothelial cells, and it was successfully reverted by KUS121 (Fig. 5J). To confirm that glycolysis inhibition reduces endothelial inflammation, we tested a well-known glycolysis inhibitor, 3PO, which suppresses glycolytic flux by targeting PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3) and indirectly inhibiting PFK-1. Pretreatment with 3PO also attenuated glycolysis and inflammation in EA.hy926 cells (Fig. S3I–K). Moreover, we assessed the effect of KUS121 on glycolysis in macrophages. Consequently, KUS121 did not suppress the LPS-induced increase in glycolysis

and inflammation in THP-1 derived macrophages (Fig. S3L–N). Taken together, KUS121 maintains intracellular ATP levels in endothelial cells, thereby attenuating inflammation via inhibition of both ER stress and glycolysis.

3.6. KUS121 can restore ATP levels in atherosclerotic plaque lesions

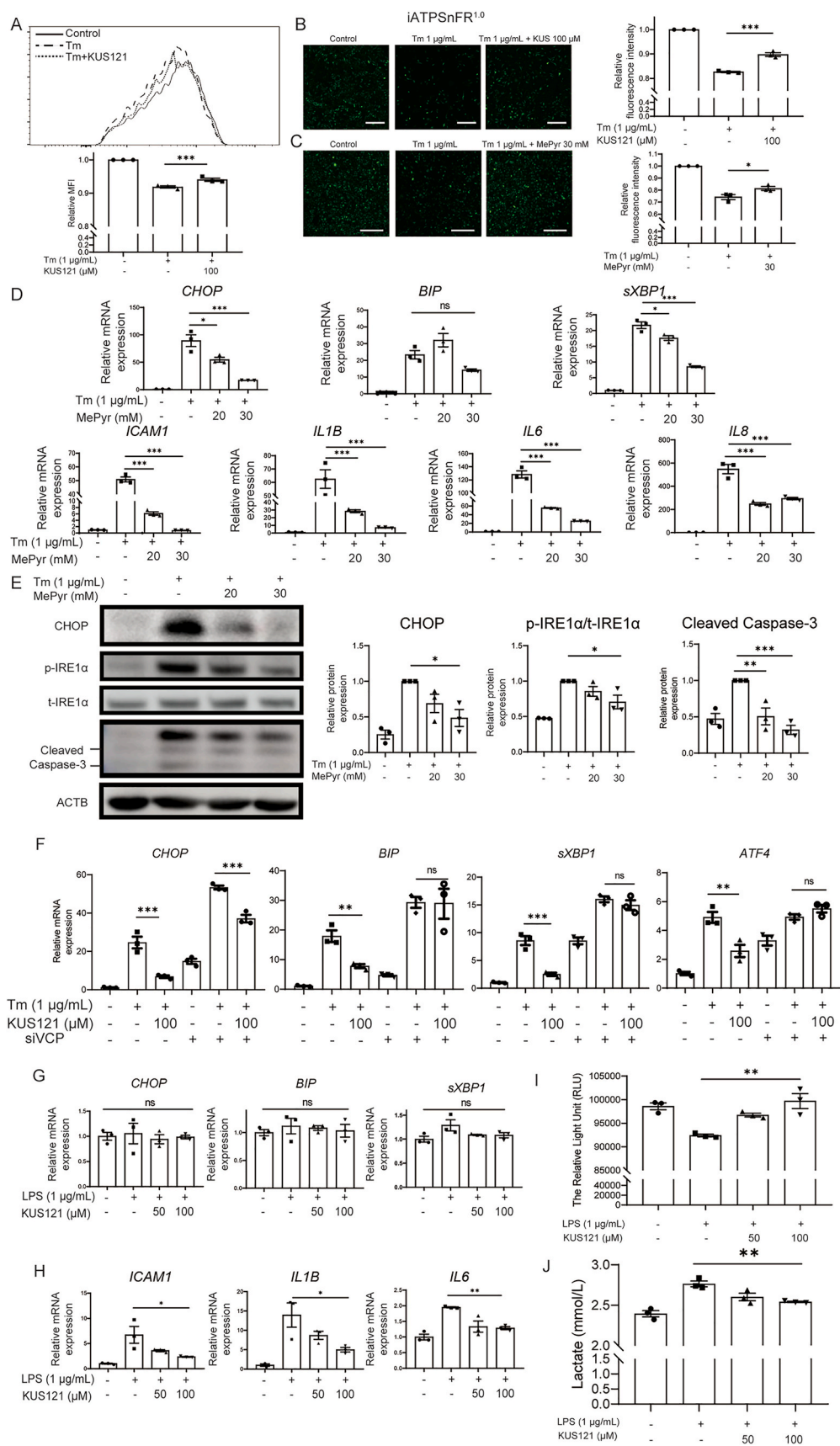
Next, we aimed to confirm that KUS121 can increase ATP levels at atherosclerotic plaques. To assess ATP levels *in vivo*, we employed GO-ATeam2 *Apoe*^{−/−} mice, which carry a genetically encoded FRET biosensor (donor fluorophore: GFP; acceptor fluorophore: OFP) [21]. They were fed a WD to induce the formation of atherosclerotic plaques in the common carotid arteries. Under anesthesia, the common carotid arteries were exposed, and 50 mg/kg of KUS121 or 5 % glucose as control was injected intraperitoneally. The FRET signal of plaque sites was excited using GFP filter, and the images of FRET (OFP) channel and donor (GFP) channel were taken (Fig. 6A and B) at several time points. The FRET ratios (FRET signal/donor signal) were calculated both in the KUS-treated and the control groups (Fig. 6C), which demonstrated that KUS121-treated mice maintained higher ATP levels in atherosclerotic lesions compared to the control group.

Moreover, to investigate ATP dynamics in endothelial cells during the early stages of atherosclerosis, when endothelial injury commonly occurs, we conducted an *in vitro* experiment using EA.hy926 cells treated with ox-LDL, a key pro-atherogenic stimulus. We observed a significant reduction in intracellular ATP levels upon ox-LDL exposure. Notably, treatment with KUS121 effectively restored ATP levels (Fig. 6D). These findings suggest that KUS121 not only helps maintain ATP levels within atherosclerotic plaques but may also play a protective role in counteracting the ATP depletion in endothelial cells during early atherogenesis.

4. Discussion

We had previously demonstrated that KUS121 attenuated ER stress-induced apoptosis in several disease models [12,20,21]. Given the unfavorable effect of ER stress on atherosclerosis [29], it was expected that KUS121 can mitigate atherosclerosis. Actually, the daily treatment of KUS121 reduced atherosclerosis progression by 40–50 %. Interestingly, Chop, an established ER stress marker, was mainly expressed in plaque endothelium. We also observed some Chop expression in plaque macrophages, but KUS121 had no effect on the ER stress in macrophages. It has been thought that ER stress responses in all major cell populations in atherosclerotic plaques, such as macrophages, endothelial cells and smooth muscle cells contribute to the exacerbation of atherosclerosis progression although less is known about smooth muscle cells [3,29,36]. Another report indicated partial activation of ER stress in vascular smooth muscle cells during atherosclerosis progression [37]. However, we could hardly detect Chop expression in smooth muscle cells. Therefore, we considered that the effect of KUS121 on endothelial cells contributes most significantly to the reduction of atherosclerosis.

The onset of atherosclerosis involves the injury to the vascular endothelial cells, which can provoke endothelial apoptosis. Numerous conventional risk factors such as oxidized low-density lipoprotein (ox-



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Fig. 5. KUS121 maintained endothelial ATP levels under ER stress, which attenuated ER stress responses *in vitro*

(A) Representative histogram of the fluorescence intensity of the iATPSnFR^{1.0} vector transfected into EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ tunicamycin for 6 h with or without 100 μM of KUS121 (left) and quantification of relative mean fluorescence intensity (right) ($n = 3$ per group). (B) Representative microscopic fluorescence images of the iATPSnFR^{1.0} transfected EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ tunicamycin for 48 h with or without 100 μM of KUS121 (left) and quantification of relative fluorescence intensity (right) ($n = 3$ per group). Scale bar: 50 μm . (C) Representative microscopic fluorescence images of the iATPSnFR^{1.0} transfected EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ tunicamycin for 24 h with or without 30 mM Methyl Pyruvate (MePyr) (left) and quantification of relative fluorescence intensity (right) ($n = 3$ per group). Scale bar: 50 μm . (D) Quantitative real-time PCR analyses of the key genes in an ER stress response and the inflammatory genes downstream of the ER stress pathway in EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ tunicamycin for 24 h supplemented with different concentration of MePyr (20 and 30 mM) or DMSO as control ($n = 3$ per group). (E) Representative western blotting images of CHOP, Cleaved Caspase-3 and phosphorylation of IRE1 α in EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ tunicamycin for 24 h with different concentration of MePyr (20 and 30 mM) or DMSO as control (left) and quantification by densitometry (right) ($n = 3$ per group). (F) Quantitative real-time PCR analyses of the key genes in an ER stress response in EA.hy926 cells treated with tunicamycin and KUS121, with or without knockdown of VCP. Quantitative real-time PCR analyses of (G) the key genes in an ER stress response and (H) the downstream inflammatory genes in EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ LPS for 24 h supplemented with different concentration of KUS121 (50 and 100 μM) or DMSO as control ($n = 3$ per group). (I) Cellular ATP levels and (J) lactate concentrations in the media in EA.hy926 cells cultured with 1 $\mu\text{g/mL}$ LPS for 24 h supplemented with different concentration of KUS121 (50 and 100 μM) or DMSO as control ($n = 3$ per group). The experiments were independently repeated 3 times. All data is presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by 1-way ANOVA with the Tukey's multiple comparisons test.

LDL), low shear stress, etc. are known to induce endothelial apoptosis [29]. Thus, apoptosis of endothelial cells could be an incipient event in the pathogenesis of atherosclerosis, fostering the initiation and progression of atherosclerotic lesions. Therefore, anti-apoptotic effect of KUS121 was initially considered as a potential reason for the attenuation of atherosclerosis progression by KUS121 treatment. Indeed, we successfully demonstrated that KUS121 can protect endothelial cells from ER stress-induced apoptosis both *in vitro* and *in vivo*.

Atherosclerosis is essentially a chronic inflammatory disease, and inflammation is thought to fuel atherosclerosis progression. This concept was further supported by the recent clinical studies of inhibiting IL-1 β by Canakinumab (CANTOS) or administering colchicine (COLCOT and LoDoCo2). Those anti-inflammatory therapies did not lower atherogenic lipids but reduce cardiovascular events [38–40]. An ER stress response is also known to activate inflammatory signaling pathways. Specifically, IRE1, one of the ER stress sensors, induces inflammatory responses by interacting TRAF2 to activate NF- κ B which is a principal transcriptional regulator of pro-inflammatory pathways. In addition to the activation of NF- κ B, the IRE1 α -TRAF2 complex can also drive inflammation through the JNK pathway [31]. Especially, the NF- κ B pathway is believed to play a central role in the inflammation of endothelial cells during atherosclerosis progression [41]. Even though previous studies have demonstrated that KUS121 protect cells from apoptosis by an ER stress response [12,17,21], it was still unclear if KUS121 can also attenuate ER stress-induced inflammatory pathways. In this study, we demonstrated that KUS121 can mitigate the ER stress-induced IRE1 pathway, which subsequently reduces the activation of the NF- κ B and JNK pathways, and the downstream inflammatory gene expressions. It is notable that the reduced expression of Icam1, a cell adhesion molecule, in plaque endothelium by KUS121 treatment may account for the reduction of monocyte recruitment into atherosclerotic plaques. We also observed a lower count of peripheral neutrophils and inflammatory Ly6C^{hi} monocytes in the KUS121-treated group, which might be due to the attenuation of systemic inflammation. This reduction of Ly6C^{hi} monocytes in circulation may also devote to the less recruitment of monocytes into plaque.

Original function of KUS121 is to inhibit ATPase activity of VCP to save intracellular ATP consumption [12,20,21]. We confirmed this effect also in endothelial cells at single cell levels *in vitro* by employing a novel fluorescent ATP sensor, iATPSnFR^{1.0} [28]. It was reported that depletion of ATP occurred in advanced plaques, coupled with glucose and oxygen deficiency as observed by bioluminescence imaging of fresh frozen sections [42]. In this study, we used a FRET based animal model (Go-Ateam2 mouse) to assess the ATP levels at atherosclerotic lesions *in vivo* and successfully observed KUS121 maintained intracellular ATP levels at the plaque lesions, although it remains uncertain whether the ATP variations in plaque endothelium are consistent with the overall plaque lesion.

Administration of extracellular ATP was reported to promote the

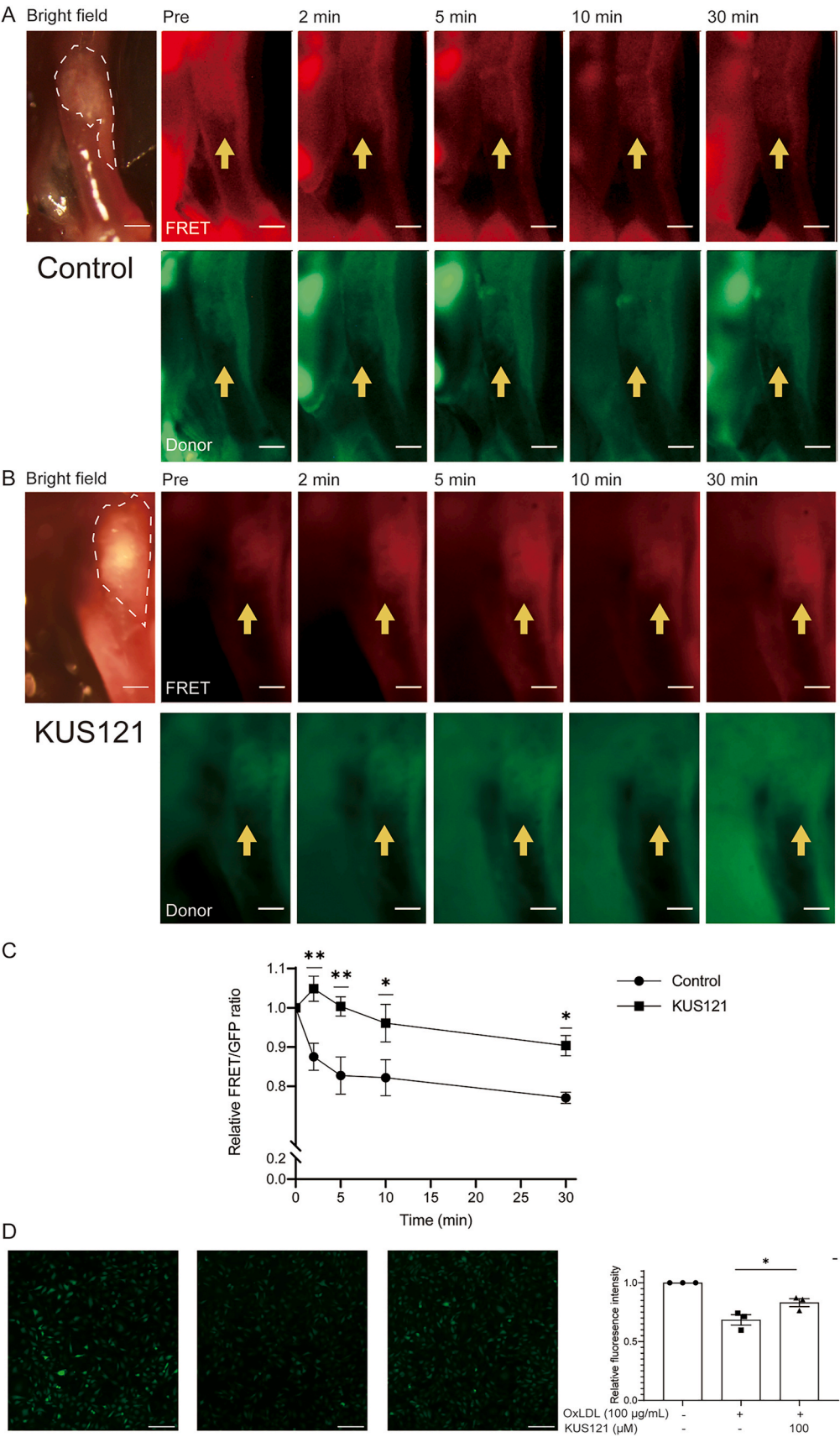
progression of atherosclerosis via P2Y2 receptor [33]. However, it still remains to be elucidated how intracellular ATP levels affect atherosclerosis progression especially in endothelial cells. Several steps in an ER stress response require ATP as a source of energy [43] and we actually observed that intracellular ATP levels were reduced under ER stress at single cell levels, which led to think the idea that KUS121 reduces ER stress by keeping intracellular ATP levels. To clarify this idea, we used methyl pyruvate to selectively increase intracellular ATP levels, and confirmed that maintenance of intracellular ATP levels under ER stress-inducing conditions inhibited both apoptotic and inflammatory pathways of ER stress responses in endothelial cells. These effect of KUS121 could lead to attenuation of atherosclerosis progression. On the other hand, however, addition of ATP extracellularly did not reduce apoptotic or inflammatory gene expressions, rather tended to increase them, which is consistent with the previous reports [33,44].

KUS121 was originally developed to target VCP. However, our VCP knockdown experiment further confirmed that the ER stress-reducing effect of KUS121 is indeed dependent on VCP.

Moreover, we also found that an increase in intracellular ATP levels by KUS121 in endothelial cells engages an additional pathway to reduce inflammation, which may further contribute to the athero-protective effect of KUS121. At the concentration of LPS that did not induce ER stress but reduced intracellular ATP levels, inflammatory gene expression was still enhanced. Even under this condition, KUS121 restored the intracellular ATP levels and attenuated inflammation. Many atherosclerosis-related factors activate endothelial inflammation through glycolysis, and the reduction of glycolysis leads to an attenuation of inflammation [35]. The conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by PFK-1 is the first committed step of glycolysis. PFK-1 activity is inhibited by ATP, citrate, and fatty acids, with ATP being the most potent negative regulator. ATP directly inhibits PFK-1, resulting in a reduction of glycolysis [34]. Therefore, we hypothesized that KUS121 reduces glycolysis by maintaining intracellular ATP levels, thereby reducing inflammation. Consistent with this hypothesis, LPS treatment increased lactate production, a glycolytic product, in the media, but this increase was successfully reverted by KUS121.

In summary, KUS121 preserves intracellular ATP levels in endothelial cells, reducing both ER stress and glycolysis. This, in turn, leads to the suppression of apoptosis and inflammation, contributing to the attenuation of atherosclerosis progression (Fig. 7).

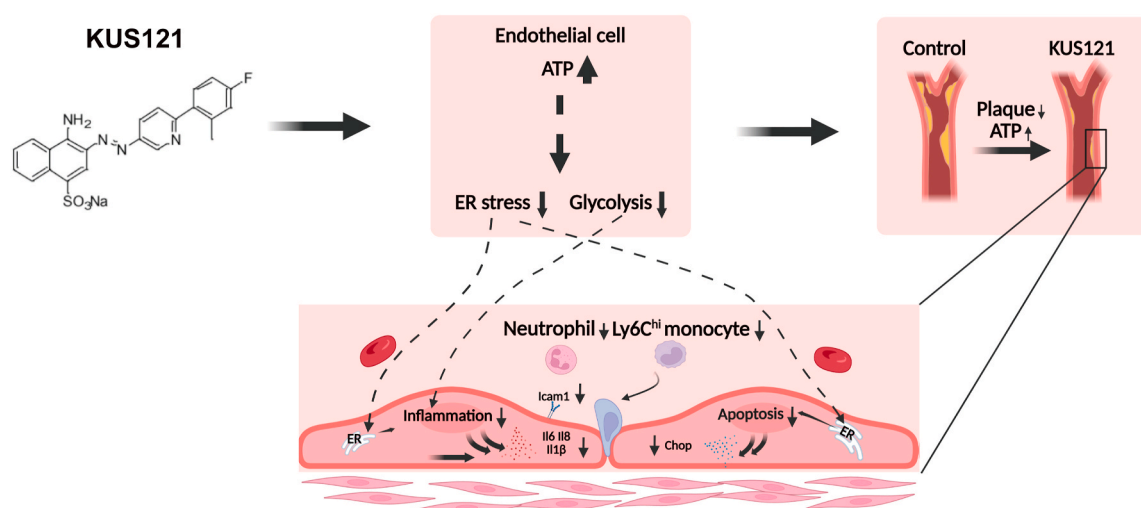
The first limitation of this study is that the cell specificity and potential off-target effect of KUS121 have not been completely elucidated. In the previous studies, KUS121 consistently protected cells [12,20,21], but in the present study, we found that KUS121 did not protect macrophages from ER stress-induced apoptosis or inflammation. It may be simply because a consumption of ATP by VCP or a response against a maintenance of intracellular ATP levels under ER stress is different in each cell population, but it is also possible that KUS121 might have the



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Fig. 6. KUS121 increased the ATP levels at atherosclerotic plaque lesions

Representative time course fluorescence images in FRET (upper) and donor (lower) channels at atherosclerotic plaque lesions of common carotid arteries in Go-Ateam2 *Apoe*^{-/-} mice on WD for 12 months pre and post (A) 5 % glucose solution as control and (B) KUS121 (50 mg/kg) administration. (C) The relative FRET/GFP ratios of KUS121 and control groups at each timepoint (n = 3 per group). The atherosclerotic plaque lesions are marked by the dashed lines in the bright field images or the yellow arrows in the fluorescence images. Scale bar: 200 μ m **p* < 0.05 ***p* < 0.01 by 2-way ANOVA with the Tukey's multiple comparisons test. (D) Representative microscopic fluorescence images of the iATPSnFR^{1.0} transfected EA.hy926 cells cultured with 100 μ g/mL ox-LDL for 24 h with or without 100 μ M of KUS121 (left) and quantification of relative fluorescence intensity (n = 3 per group) (right). Scale bar: 50 μ m **p* < 0.05 by 1-way ANOVA with the Tukey's multiple comparisons test. Representative All data is presented as mean $\pm$ SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Fig. 7.** The mechanism by which KUS121 attenuates atherosclerosis.

off-target effect working just in macrophages. Secondly, the effect of KUS121 on smooth muscle cells might also contribute to a reduction of atherosclerosis. However, given the low expression of Chop in smooth muscle cells, its contribution is likely much lower than that of endothelial cells. Further studies are necessary to answer those questions.

In conclusion, our findings suggest that KUS121 offers a new therapeutic option for atherosclerosis by reducing both apoptosis and inflammation, although further studies are needed toward the future clinical application. There are several other ER stress repressors, such as 4-phenyl butyric acid (PBA) and tauroursodeoxycholic acid (TUDCA), and some of them have been explored for the treatment of atherosclerotic disease [3]. However, their mechanisms are different from KUS121 that specifically inhibits ATPase activity of VCP, not only attenuating ER stress but also reducing glycolysis. Thus, it is possible that KUS121 can be used with other ER stress repressors to treat atherosclerotic disease, possibly in a synergistic manner. Moreover, previously we demonstrated that KUS121 has protective effects against ischemic heart disease and heart failure. Those diseases are often comorbid with atherosclerosis. Therefore, KUS121 has a potential to treat these diseases simultaneously.

CRediT authorship contribution statement

Fuqian Zou: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization. **Osamu Baba:** Conceptualization, Methodology, Validation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Takahiro Horie:** Methodology, Writing – review & editing. **Yasuhiro Nakashima:** Methodology. **Shuhei Tsuji:** Methodology. **Tomohiro Yamasaki:** Methodology. **Chiharu Otani:** Methodology. **Sijia Xu:** Methodology. **Miyako Imanaka:** Methodology. **Kazuki Matsushita:** Methodology. **Keita Suzuki:** Methodology. **Eitaro Kume:** Methodology. **Hidenori Kojima:** Methodology. **Qiuxian Qian:** Methodology. **Kayo Kimura:** Methodology. **Naoya Sowa:** Methodology. **Akira Kakizuka:**

Writing – review & editing. **Koh Ono:** Writing – review & editing, Supervision, Funding acquisition.

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Declaration of competing interest

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for this journal and was not involved in the editorial review or the decision to publish this article.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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Appendix A. Supplementary data

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