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A peptide antagonist of F11R/JAM-A reduces plaque formation and prolongs survival in an animal model of atherosclerosis

Anna Babinska[a,*], Cristina C. Clement[b,i], Tomasz Przygodzki[c], Marcin Talar[c], Yan Li[d], Marcin Braun[e], Joanna Wzorek[c], Maria Swiatkowska[f], Yigal H. Ehrlich[g], Elizabeth Kornecki[a,h], Cezary Watala[c], Moro O. Salifu[a]

[a] Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, NY, 11203, USA
[b] Department of Pathology, Albert Einstein College of Medicine, New York, 10461, USA
[c] Department of Haemostasis and Haemostatic Disorders, Biomedical Sciences, Medical University of Lodz, 92-215, Lodz, Poland
[d] Department of Neurology, State University of New York, Downstate Medical Center, Brooklyn, NY, 11203, USA
[e] Department of Pathology, Medical Univeristy of Lodz, 92-213, Lodz, Poland
[f] Department of Cytobiology and Proteomics, Biomedical Sciences, Medical University of Lodz, 92-215, Lodz, Poland
[g] Program in Neuroscience, College of Staten Island of the City University of New York, Staten Island, NY, 10314, USA
[h] Department of Cell Biology, State University of New York, Downstate Medical Center, Brooklyn, NY, 11203, USA
[i] Department of Chemistry, Lehman College, CUNY, Bronx, New York 10468, USA

HIGHLIGHTS

- We examined effects of F11R derived peptide 4D (P4D) on cardiovascular health and atherosclerotic plaque of *ApoE*$^{-/-}$ mice.
- The F11R peptide 4D reduced plaque formation and prolonged survival with overall better health in *ApoE*$^{-/-}$ mice.
- P4D inhibited adhesion of platelets to cytokine-inflamed aortic endothelium in *ApoE*$^{-/-}$ mice shown by intravital microscopy.
- P4D represents a potential drug for the prevention and treatment of thrombosis and atherosclerosis triggered by inflammation.

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ABSTRACT

Background and aims: The F11 Receptor (F11R), AKA Junctional Adhesion Molecule-A (JAM-A) (F11R/JAM-A), is an adhesion protein constitutively expressed on the membrane surface of circulating platelets and the luminal surface of inflamed endothelial cells (EC).

Platelet adhesion to an inflamed endothelium is one of the early steps of atherosclerotic plaque formation. Our previous studies, conducted with cultured EC *in vitro*, have demonstrated the expression of F11R/JAM-A on the luminal surface of inflamed EC, platelet adhesion to inflamed EC through F11R/JAM-A interactions, and inhibition of this interaction by the presence of F11R/JAM-A antagonistic peptide (F11Rpeptide 4D). In the present study, we examined *in vivo* the overall health-benefits and cardiovascular effects of long-term treatment of animals prone to atherosclerosis, *ApoE*$^{-/-}$ mice, with F11R-peptide 4D.

Methods: Twenty *ApoE*$^{-/-}$ mice were assigned to daily treatment with peptide 4D and compared to their counterparts control untreated mice. Mice were observed for wellness and survival. Plaque size in the aorta and heart was measured using histological analysis. Effects of peptide 4D (or scramble control) on platelet adhesion to inflamed endothelium were measured using intravital microscopy.

Results: Significant reductions in atherosclerotic plaques number and size, an overall robust health with longer survival were found in the peptide 4D treated group of *ApoE*$^{-/-}$ mice. Intravital microscopic studies conducted in exposed vessels of *ApoE*$^{-/-}$ mice demonstrated significant inhibition by peptide 4D of platelet adhesion to the cytokine-inflamed endothelium.

Conclusions: Our results demonstrate that peptide 4D significantly reduces atherosclerotic plaque formation in *ApoE*$^{-/-}$ mice and inhibits platelet adhesion to the inflamed arterial endothelium.

* Corresponding author. SUNY/Downstate Medical Center, Department of Medicine, Box # 52, 450 Clarkson Avenue, Brooklyn, NY, 11203, USA.
E-mail address: anna.babinska@downstate.edu (A. Babinska).

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1. Introduction

The F11 receptor (F11R), originally identified in human platelets, is considered to be the founding member of the junctional adhesion molecule-A [1]. A protein with close sequence similarities to human F11R was identified in murine tight junctions of endothelial and epithelial cells, and named JAM-A [2], and in this report, we will refer to this protein as F11R/JAM-A. F11R/JAM-A was discovered, purified, sequenced and identified as two proteins differentiated by their degree of glycosylation [3,4]. The gene for F11R/JAM-A was cloned from human platelet mRNA [5]. Our subsequent studies probed the function of F11R/JAM-A *in vitro,* in inflammation, atherosclerosis, and in the initiation of inflammatory thrombosis [6–10]. The involvement of platelets in the early formation and development of atherosclerotic plaques was demonstrated in studies showing that circulating platelets adhered to the luminal surface of the inflamed endothelium [11]. Ozaki et al. [12] have reported that cytokines cause the redistribution of F11R/JAM-A from the tight junctions of endothelial cells to the luminal surface of the inflamed endothelium. In the healthy physiological state of the vasculature, the F11R/JAM-A molecule is constitutively expressed on the surface of circulating platelets and localized within the tight junctions of endothelial cells, locations which prevent adherence of platelets to the vasculature. Under pathophysiological conditions, platelets adherence to the inflamed endothelium initiates atherosclerotic plaque formation, thereby leading to the development of systemic atherosclerosis and inflammatory thrombosis. The critical role of F11R/JAM-A in the adhesion of platelets to inflamed endothelial cells was shown by investigating the adhesion of platelets to endothelial cells (EC) exposed to the proinflammatory cytokines TNFα and INFβ *in-culture*. By utilizing specific F11R-derived peptides with sequences based on active pockets/sites containing amino acid sequences of the N-terminus and Ig fold of F11R, we observed significant blockade in the adhesion of isolated human platelets to cultured, inflamed EC [6,7].

Support for the involvement of F11R/JAM-A in atherosclerotic plaque formation and the initiation of thrombosis in humans has been provided by clinical studies conducted with patients diagnosed with cardiovascular disease. We observed a significantly higher level of F11R/JAM-A expression within the atherosclerotic plaques of arteries derived from coronary artery disease (CAD) patients [7,10]. We also observed a significantly high level of soluble F11R (sF11R) in CAD patients [13], in hemodialysis patients [14] and in patients with hypertension [15].

ApoE$^{-/-}$ knockout mice have served as important animal models in the investigation of atherosclerosis in human cardiovascular disease [16–19]. To demonstrate directly that F11R/JAM-A is involved in the development of atherosclerosis *in vivo,* we examined the effects of chronic administration of F11R peptide antagonist [20], termed F11R-peptide 4D, on blood vessel plaque formation in apolipoprotein E (ApoE) - deficient mice. Subsequently, we explored experimentally, using intravital microscopy, the potential mechanisms by which the F11R antagonist peptide 4D may aid in the prevention of atherosclerosis.

2. Materials and methods

2.1. Synthesis of F11R peptide 4D

Our earlier report [8] originally focused on the use of the F11R-derived peptide, termed peptide 4, a thirteen-amino acid peptide that was found to inhibit the stimulatory monoclonal antibody F11 (M.Ab.F11) -induced platelet aggregation and the adhesion of platelets to cytokine-inflamed endothelial cells in culture [6]. To prevent, and/or delay the hydrolysis of peptides in studies involving their administration to animals *in vivo*, we prepared a form of this peptide with enhanced resistance to proteolysis, termed peptide 4D, which was designed to contain two amino acid substitutions positioned at sites prone to hydrolysis: $_2$HN-(dK)-SVT-(dR)-EDTGTYTC-CONH$_2$ [20]. As a first candidate for use as an F11R/JAM-A antagonist *in vivo*, peptide 4D was utilized in the present study for examination of its effects following its' administration to *ApoE*$^{-/-}$ mice.

2.2. Animals

Experiments involving all *ApoE*$^{-/-}$ mice were conducted with the approval of the State University of New York Downstate Medical Center Institutional Animal Care and Use Committee.

2.2.1. Treatment of *ApoE*$^{-/-}$ mice with F11R peptide 4D: dosage and frequency of peptide 4D administration

Female *ApoE*$^{-/-}$ mice (six weeks old) of a C57BL/6J background, (Jackson Laboratory, Bar Harbor, Me), were divided into two separately-housed groups and injections were started in 8 weeks old mice. Group 1 was treated with peptide 4D by daily, intraperitoneal injections for a 3 (see Ref. [21]) or 4 month period. Group 2 was injected daily with an equivalent amount of diluent vehicle (200 μl of 0.9% saline) for the same 3- or 4-month period.

The dosage of peptide 4D used for the administration of this peptide *in vivo* to *ApoE*$^{-/-}$ mice was based on the concentration of peptide 4D that inhibited the stimulatory monoclonal antibody F11 (M.Ab.F11) (0.3 mg) - induced platelet aggregation and secretion *in vitro*. We determined that inhibition of M.Ab.F11-induced platelet aggregation could be achieved at a concentration of peptide 4D of 670 μg/ml. Based on these results with M.Ab.F11, and taking into account the mouse extracellular volume of 6.44 ml, we calculated that a comparable dose of peptide 4D, of 215 mg/kg body weight/mouse would be needed, as the amount of peptide 4D to be utilized in this study.

2.2.2. Pharmacokinetic analysis of peptide 4D

Based on the dose-response relationship of peptide 4D blockage of M.Ab.F11-induced platelet aggregation, we conducted pharmacokinetic (PK) studies in mice to determine several key parameters (Table 1), i.e. plasma half-life of peptide 4D, and the final plasma concentration of peptide 4D (details in Ref. [21]).

Based on the dose-response relationship of peptide 4D blockage of M.Ab.F11-induced platelet aggregation, we conducted pharmacokinetic (PK) studies in mice to determine several key parameters: maximal concentration (Cmax), maximal time (Tmax), area under the plasma concentration time curve (AUC) and terminal elimination half-life ($T_{1/2}$).

From the PK data obtained, the dosage of peptide 4D used for each injection was calculated as 215 mg/kg, and therefore a concentration of 4.3 mg/dose per animal was administered daily by intraperitoneal injections of either peptide 4D or saline, as a control, for a period of three to four months.

2.2.3. Animal diet

All *ApoE*$^{-/-}$ mice were placed initially on a regular Rodent Diet (Calories provided by protein 28.1%, fat 12.13% and carbohydrates 59.8%) for a two-month period. From our experience, the placement of *ApoE*$^{-/-}$ mice initially on a high fat diet, instead of the regular rodent diet, would have resulted in the early death of a large number of

Table 1
Pharmacokinetic analysis of peptide 4D.

Dose	mg/kg	215.0
C_{max}	ng/ml	9911.0
	nM	6783.7
T_{max}	h	0.3
AUC (0–24h)	mg/ml*h	409.4
$T_{1/2}$	mM*h	588.2
	h	3.8
Dose adjusted AUC (mM*hr/mg)		2.7

animals in this study, and would not have allowed us to conduct a long-term study of these animals. Therefore, after a two-month period, the animals were placed on a high fat, Western-type diet (HTD.88137 Adjusted Calories Diet (42% fat; 0.15% cholesterol, 21% anhydrous milk fat) (Harlan Laboratories Inc.) to induce hypercholesterolemia, for a period of one month [22]. Following 3 (see Ref. # 21) or 4 months of injections with either the F11R peptide 4D or the vehicle, saline, the animals were euthanized.

2.2.4. Dissection and examination of $ApoE^{-/-}$ mice aorta and hearts for atherosclerotic lesions

Following euthanasia, the aortas were prepared for dissection and the aortic arches were photographed. Following dissection, *en face* lesion surface area assay was performed on the aortas and hearts as previously described [23–25]. The dissected aortas were stained with Oil-Red-O stain modified from previously described methods [23,24,26].

For morphometric lesion analysis [22,25,23,24] sections obtained from the heart of each animal were stained with Harris hematoxylin-eosin. The total intimal lesion area and the necrotic core area (NE) per cross section were quantified by taking the average of 6 sections spaced 30 μm apart, beginning at the base of the aortic root. The necrotic core areas, acellular/anuclear areas (negative for hematoxylin-positive nuclei, white areas of lesion) were measured per cross section from the same slides where total intimal lesion area was measured. Images were viewed and captured with a microscope (Nikon) equipped with a color video camera (Motic Images Plus 2.0) attached to a computerized imaging system. Lesion areas or necrotic areas (white areas of lesion) where measured and summarized using software Image-J. Histological and immunohistological analysis of T-Lympocytes, macrophages, smooth muscle cells and collagen were performed using Hematoxylin & Eosin and Masson-trichrome staining as well as histochemical and immunohistochemical specific antibodies. Images were viewed, captured and analysed by light microscopy and by using UltraFast Scanner (Philips IntelliSite Solution, USA) with DigiPath™ Professional Production Software (Xerox, Norwalk, CT, USA). Method details are expanded in the Ref. # [21].

We performed bleeding time (details in Fig. 5) and also obtained blood for measurement of lipid levels (Table 2), blood chemistry and a complete blood count (Supplementary Tables 1 and 2).

$ApoE^{-/-}$ mice were injected daily with either a bolus of saline (200 μl) or peptide 4D (4.3 mg) for a period of 4 months. Blood parameters measured included the following: cholesterol, triglycerides, HDL and LDL. Data are expressed as the mean ± SD. Number of animals in each group (n = 6), significant differences by *t*-test at $p > 0.05$.

2.3. Intravital microscopy

For these intravital microscopic experiments, female B6.129P2-ApoEtm1Unc/J mice (further on referred to as ApoE deficient), aged 12 weeks, were obtained from Charles River Laboratories Italia (Calco, Italy). The studies were approved by the Local Ethical Committee on Animal Experiments, Medical University of Lodz (approval number 31/LB94/2018).

Table 2
Comparison of lipid profile in the plasma of untreated $ApoE^{-/-}$ mice and $ApoE^{-/-}$ mice treated with peptide 4D.

Parameter mg/ml	Control $ApoE^{-/-}$ mice		Peptide 4D treated $ApoE^{-/-}$ mice		*p*-value
	Mean	SD	Mean	SD	
Cholesterol	531.15	77.85	625.01	118.15	0.140
Triglycerides	74.67	28.45	78.25	30.92	0.839
HDL	12.63	3.35	14.99	3.34	0.251
LDL	510.90	84.99	594.86	120.14	0.210

Blood platelet-specific DyLight488-labeled anti-GPIbβ antibodies, used for the labeling of platelets *in vivo,* were purchased from Emfret Analytics (Eibelstadt, Germany). Murine recombinant TNF-α and murine recombinant IFN-ϒ were purchased from MyBioSource (San Diego, USA). The F11R peptide 4D and a scrambled peptide based on a version of the amino acid sequence of F11R peptide 4D were synthesized and obtained from Life Pro Tein LLC, Hillsborough, NJ.

2.3.1. Treatment of $ApoE^{-/-}$ mice with peptide 4D and in vivo imaging by intravital microscopy

To induce an inflammatory phenotype of the vascular endothelium, the mice were injected intraperitoneally with murine recombinant TNF-alpha in a dose of 0.5 μg per mouse (approx. 20 μg/kg body weight) and murine recombinant IFN-gamma in a dose of 5 μg per mouse (approx. 200 μg/kg body weight) 4 h prior to measurements.

Eleven mice received injections of the F11R peptide 4D, intraperitoneally, in a dose of 4 mg/mouse in three consecutive applications, as follows: 24 h prior to measurement, 5 h prior to measurement, with the last injection given 4 h prior to measurement together with the cytokines. Eleven mice in the control group received injections of the control peptide, a scrambled form of peptide 4D, at a dose of 4 mg/mouse in the same regime as mice injected with the F11R peptide 4D.

The mice were anesthetized, injected with platelet-specific fluorescent anti-GPIbβ antibodies at a dose of 0.1 μg/g body weight, and placed on the stage of an upright microscope equipped with saline immersion objectives. The antibodies do not interfere with the interaction between GPIbα and vWF. The mesentery was exteriorized and fixed in a chamber allowing constant superfusion with saline. Imaging was carried-out at a magnification of 200× for 60 s with an exposure time of a single frame of a movie sequence set at 200 ms, in at least three arterioles and three venules in each mouse.

2.3.2. Image analysis and calculations

When the exposition time of a single movie frame was set at 200 ms, only those platelets that were immobilized for at least one movie frame on the vessel wall were visible as circular objects. Platelets which flow in the blood stream were either below the detection limit or were recorded as streaks. For the above reasons, only platelets that were immobilized for at least 200 ms were taken to calculus. Analysis of platelets tethering to the endothelium was performed with the use of TrackMate plugin that is implemented in FIJI software [27]. The displacement of each detected platelet between succeeding frames has been tracked by the software. The length of the track and time in which the distance was covered by the platelets were used for calculations. At first approach, all platelets which were immobilized on at least one frame (200 ms), were calculated, which gave the number of all observed episodes of transient adhesion. In a second approach, only platelets which were immobilized for at least 1 s were calculated. The number of platelets fulfilling these criteria in a given movie sequence was divided by the duration of the movie sequence expressed in seconds, and by the actual area of a vessel wall visualized in this sequence expressed in μm^2. Such calculated values were averaged between all movie sequences recorded in arterioles in a given mouse or in the venules in this mouse. Final results are thus presented as the number of events of platelet adhesion fulfilling the defined criteria per unit of time and area in a particular type of blood vessel [28].

2.3.3. Statistical analysis

Assumption of normal distribution of differences was verified with the use of Shapiro-Wilk test. Unpaired Student's t-test was employed as rationale for determination of the significance of differences when comparing two independent groups with distributions not departing from normality. When the normality assumption was violated, the significance of differences was tested with the Mann–Whitney's *U* test. Due to low sample size, we routinely employed the resampling

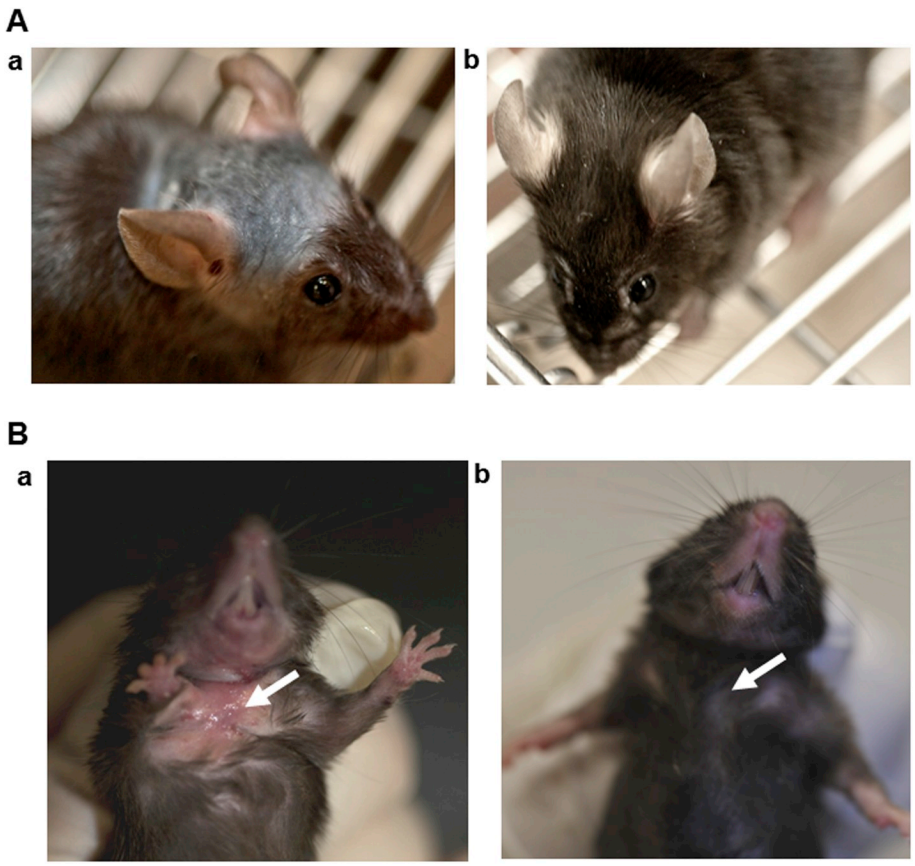

Fig. 1. Comparison of the external appearance of peptide 4D-treated *ApoE*$^{-/-}$ mice injected with peptide 4D versus the appearance of untreated control, *ApoE*$^{-/-}$ mice.
Untreated, *ApoE*$^{-/-}$ mice (a) were injected daily, intraperitoneally with 200 μl of the diluent saline for a period of four months, initiated at six weeks of age, showing loss of hair on their head and neck (A, a) and development of signs of severe skin lesions on their chest, as shown by the arrow (B, a). Peptide 4D-treated, *ApoE*$^{-/-}$ mice (b) were injected daily, intraperitoneally with an equivalent volume of peptide-4D solution. The peptide 4D-treated mice did not demonstrate loss of hair on their head or neck (A, b) and did not develop skin lesions on their chest (B, b) (compare arrows). Photographs are representative of 6 mice in each group.

bootstrap technique (10,000 iterations) for each inference/association test used in this study, in order to assure that the revealed differences were not observed due to pure chance.

3. Results

In this investigation, two groups of atherosclerosis-prone *ApoE*$^{-/-}$ mice were compared over a period of time: group 1 was administered the nonhydrolyzable F11R/JAM-A antagonist, peptide 4D, whereas group 2 consisted of a control group of animals injected with the vehicle, saline, for the same period of time. Both groups of animals were examined for their physical appearance, well-being, overall health, survivability, severity of development of atherosclerotic plaques, and morbidity as developed over time. Within a 3-month period, two of the control mice were unable to continue the study as assessed by their unhealthy physical appearance. They were euthanized, and at the same time, two mice from the peptide 4D-treated group were randomly-selected and euthanized, and their tissues examined in comparison to controls. The analysis of these mice is shown in Fig. 1 in Ref. [21].

Following a 4-month period of injections, the remaining control *ApoE*$^{-/-}$ mice that were not treated with peptide 4D manifested the disease (Fig. 1A, panel a), including large skin eruptions and severe scratching as shown in (Fig. 1B, panel a). However, in comparison, the *ApoE*$^{-/-}$ mice treated with peptide 4D for the identical 4-month period of time demonstrated no signs of disease (Fig. 1A, panel b and Fig. 1B, panel b). As shown in Fig. 2A (panel a), very large accumulations of atherosclerotic plaques were observed within the aortic arches of the 4-month old control animals not treated with peptide 4D, with large accumulations of plaque in blood vessels encompassing the ascending and descending aorta, the right and left subclavian, and the right and left carotid arteries. In comparison, much smaller atherosclerotic lesions were found in the peptide 4D treated mice, as shown in Fig. 2A (panel b). The results of the Oil Red O staining (*en face* assay) of the whole aorta, are presented in Fig. 2B. The control, untreated mice demonstrated a large number of atherosclerotic lesions throughout the entire aorta (Fig. 2B, staining pattern labeled a on the left), as compared to much smaller atherosclerotic lesions identified in the peptide 4D treated mice (Fig. 2B, staining pattern labeled b on the right). The quantitative measurement display, derived from *en face* lesion surface area assays of the atherosclerotic plaques of the entire aortas from both groups of animals, is shown in Fig. 2C. As shown in Fig. 2C, (histogram b), the aorta of peptide 4D treated *ApoE*$^{-/-}$ mice exhibited a significant, 50% decrease in atherosclerotic plaques as compared to the much larger percentage of plaques found in the aorta of control, untreated mice (histogram a). The percentage of the aorta's surface area expressing atherosclerotic lesions in control, untreated mice was calculated as 28.43 ± 9.12% (shown in histogram a), whereas the percentage of the aorta's surface area showing lesions in the peptide 4D-treated group of mice was calculated as 13.23 ± 3.95% (see histogram b).

Fig. 3 shows the results we obtained by hematoxylin-eosin staining of the proximal aorta (aortic root assay) of control, untreated mice (Fig. 3A, panel a), in comparison to the results we obtained for peptide 4D treated mice (Fig. 3A, panel b). The lesion necrotic core areas (NE) visualized in the untreated control mice, are compared to the lesion NE areas detected in peptide 4D-treated mice. We observed that the necrotic lipid core regions of control, untreated mice consisted of large quantities of cellular debris and cellular/fibrous cap, as demonstrated in Fig. 3A (panel a). On the other hand, the NE lesion areas identified in the proximal aorta of the peptide 4D-treated mice were smaller in size, as demonstrated in Fig. 3A (panel b). Large stretches of simple fatty streaks or individual intimal inflammatory cells were also observed adjacent to the complex lesions of the control, untreated animals, and such areas were observed to be smaller in size in the peptide 4D-treated animals. The quantitative display of the results of the aortic root assay of the atherosclerotic lesion area derived from both groups of animals is

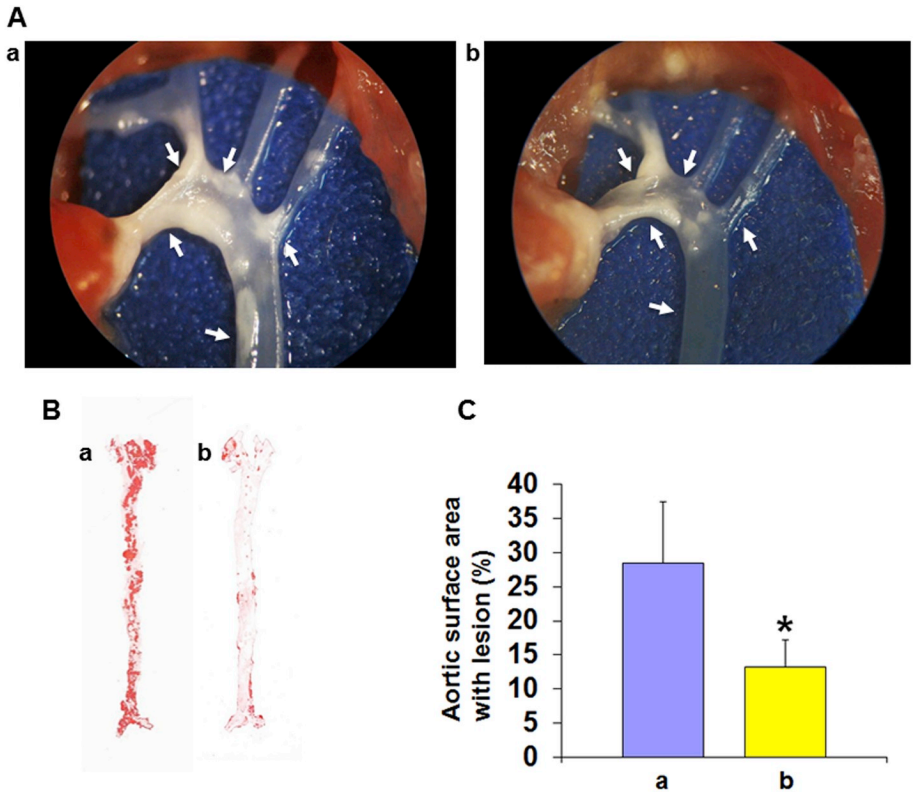

Fig. 2. Comparison of the development of atherosclerotic plaques in the aortic arch and the whole aorta of $ApoE^{-/-}$ mice treated with peptide 4D *versus* control, untreated mice.
Untreated, $ApoE^{-/-}$ mice (a) were injected with saline and peptide 4D-treated $ApoE^{-/-}$ mice (b) were injected with peptide 4D for a period of 4 months. (A) Photographs of the dissected aortic arch. The peptide 4D-treated mice demonstrated a significantly decreased amount and size of atherosclerotic plaques in their aortic arch (A, b) as compared to mice injected with saline (A, a) (shown by the arrows). (B) Oil red O staining pattern of whole aortas (*en face* assay). An intense staining pattern of plaques was evident throughout the entire aorta of control untreated mice (B, a) and demonstrated a reduction in the number and size of atherosclerotic plaques stained along whole aortas in Peptide 4D treated mice (B, b). Each image is representative of 6 mice derived from each group. (C) Comparison and quantitative display of the *en face* lesion surface area of the entire aorta of $ApoE^{-/-}$ mice injected with either saline (a) or peptide 4D (b). Values are the mean (horizontal bars) ± SD for n = 6 mice in each group. A significant reduction of atherosclerotic plaques in the aortae of the peptide 4D-treated mice compared to mice injected with saline was observed. *Significance by *t*-test at level $p < 0.05$.

presented in Fig. 3B. We observed a significant reduction (by 50%) in the size of the lesion area of the aortas of the peptide 4D-treated group (as shown in Fig. 3B, histogram b, value of 33,204 ± 4786), as compared to the size (lesion area) measured in untreated, control mice (see Fig. 3B, panel a, lesion area value of 62,043 ± 5056). Furthermore, the quantitation of measurements conducted by the aortic root assay of the Necrotic core (NE) areas is shown in Fig. 3C. The necrotic core area in mice treated with peptide 4D (shown in Fig. 3C, histogram b) (values of 3645 ± 1135) was reduced by approx. 60%, as compared to the necrotic core area measured in the untreated, control mice (shown in

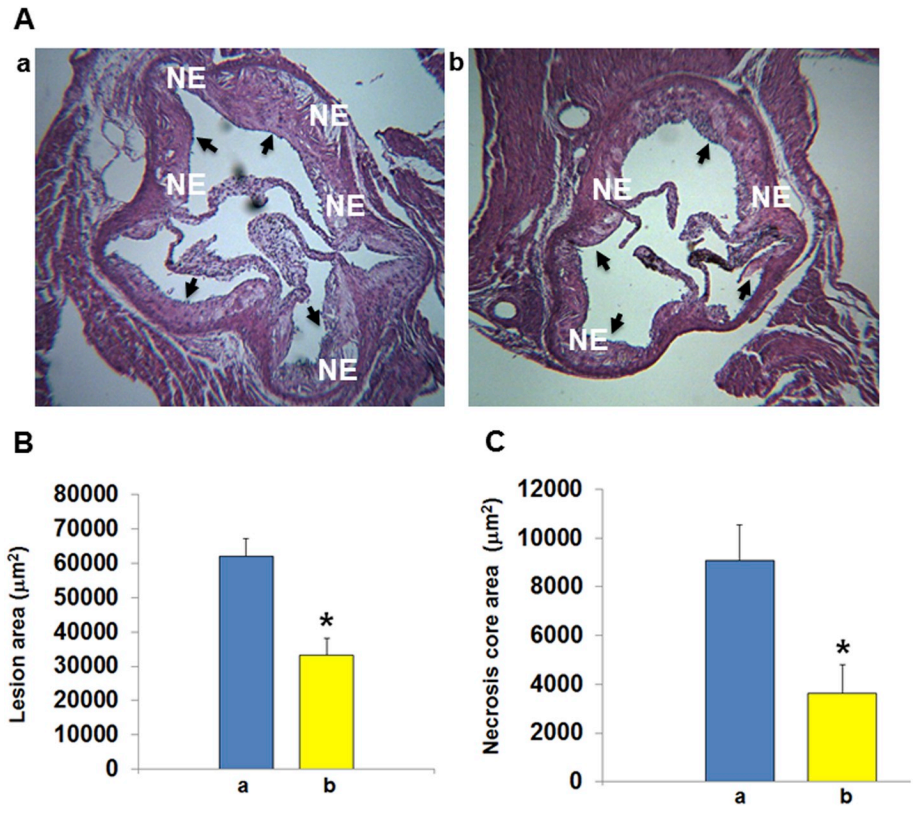

Fig. 3. Comparison of the development of atherosclerotic plaques in the proximal aorta (aortic root assay) of $ApoE^{-/-}$ mice treated with peptide 4D *versus* untreated control $ApoE^{-/-}$ mice.
(A) Representative images of hematoxylin-eosin staining of the of aortic root sections (n = 5–6). Untreated, $ApoE^{-/-}$ mice (a) and Peptide 4D-treated $ApoE^{-/-}$ mice (b) injected for a 4 month period. Lesions are indicated by arrows. NE - indicates the necrotic core area. (B) Quantitative analysis of atherosclerotic lesion size in aortic root sections and (C) quantitative display of the necrotic core area in aortic root sections of $ApoE^{-/-}$ mice injected with either saline (a) or peptide 4D (b) for a 4 month period. Values are shown as means ± SD, n = 6. *Significance by *t*-test at $p < 0.05$.

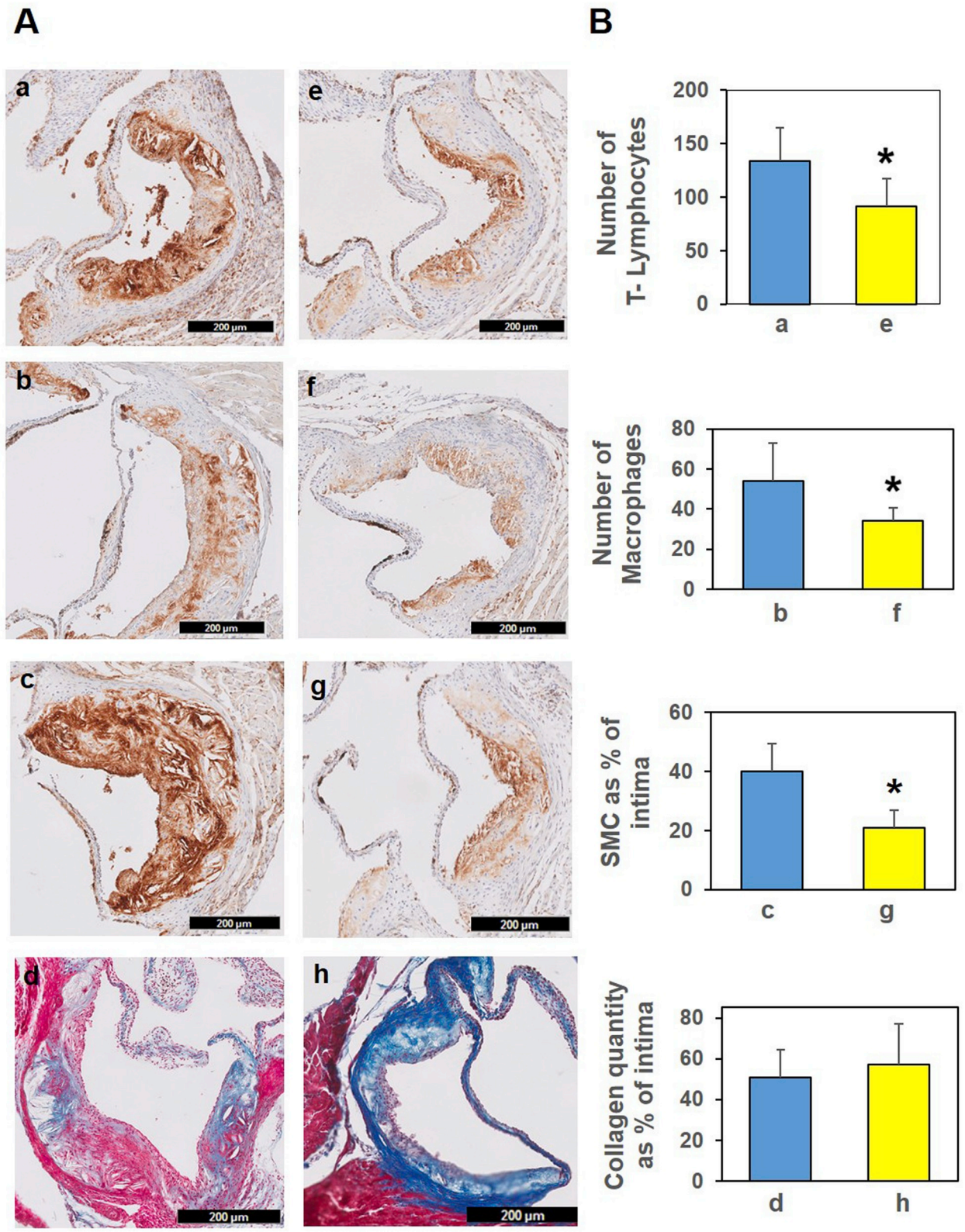

Fig. 4. Comparison of the histopathological analysis and cell composition of atherosclerotic plaques in the proximal aorta (aortic root assay) of *ApoE*$^{-/-}$ mice treated with peptide 4D versus control *ApoE*$^{-/-}$ mice.
(A) Representative images of the immunohistochemical staining of T-lymphocytes (CD3-positive cells) (a, e), macrophages (CD68-positive cells) (b, f), vascular smooth muscle cells (SMA-positive cells)(c, g) and collagen (Masson-trichrome staining) (d, h). Images show the accumulation in lesion areas of control ApoE$^{-/-}$ mice (left panels: a, b, c, d) and peptide 4D-treated *ApoE*$^{-/-}$ mice (right panels: e, f, g, h). Magnification 50×. (B) Quantitation of the content of cells and collagen in the intima of arteriosclerotic plaques of control *ApoE*$^{-/-}$ and peptide 4D-treated *ApoE*$^{-/-}$mice. Quantitative display of the T-lymphocytes, macrophages, vascular smooth muscle cells and collagen in aortic root sections of control *ApoE*$^{-/-}$ mice (a, b, c, d) or peptide 4D treated *ApoE*$^{-/-}$mice (e, f, g, h). Values are shown as means ± SD, n = 5. *Significance by *t*-test at $p < 0.05$. Quantitative analysis was performed using DigiPath™ Professional Production Software (Xerox, Norwalk, CT, USA).

Fig. 3C, histogram a; values of 9084 ± 1,457, $p < 0.005$).

Significant reductions in the size and level of atherosclerotic plaques and lesions were observed in the vessels of peptide 4D treated group of mice. However, the administration of peptide 4D for a 4-month period did not appear to alter liver or kidney function, the level of electrolytes, the serum glucose levels nor protein levels in these animals (see Supplementary Table 1). Notably, the administration of peptide 4D for a 4- month period did not cause a significant change in plasma levels of cholesterol (values provided in Table 2).

Atherosclerotic plaque morphology and cellular composition were performed in *ApoE*$^{-/-}$ mice treated with peptide 4D and control *ApoE*$^{-/-}$. Macrophages, the most prominent type of cells found in atherosclerotic lesions, are associated with inflammation, together with the accumulation of T-cells and smooth muscle cells [29,30]. Fig. 4 compares the immunohistochemical and histochemical staining of atherosclerotic plaques found in both groups of mice. A significant reduction of T-Lympocytes was found in the *ApoE*$^{-/-}$ mice treated with peptide 4D (see Fig. 4A [e] and Fig. 4B [e]) as compared to control, *ApoE*$^{-/-}$ mice (see Fig. 4A [a] and Fig. 4B [a]). Administration of peptide 4D caused a significant reduction in the amount of macrophages (Fig. 4A [f] and Fig. 4B [f]) and smooth muscle cells (Fig. 4A [g] and Fig. 4B [g]) in the *ApoE*$^{-/-}$ mice as compared to the control *ApoE*$^{-/-}$ mice (Fig. 4 A [b] and Fig. 4B [b]) and (Fig. 4 A [c] and Fig. 4B [c]) respectively. Furthermore, the *ApoE*$^{-/-}$ mice treated with peptide 4D demonstrated substantially more collagen as illustrated by the Masson-trichrome stained images (Fig. 4A [h] and 4B [h]) compare to control *ApoE*$^{-/-}$ mice (Fig. 4A [d] and Fig. 4B [d]).

Additional histological assessment of atherosclerosis in mice was performed according to Virmani et al. [31,32], as modified by Hartwig et al. [33]. According to this classification, atherosclerotic plaques developed in all control *ApoE*$^{-/-}$ mice belong to the advanced II and III type, whereas in the peptide 4D - treated *ApoE*$^{-/-}$ mice, the plaques were classified as belonging to the advanced I and II type. These observations indicate that although the cardiovascular disease was classified as advanced in all mice, the inflammatory cellular infiltration was significantly smaller in the atherosclerotic plaques of peptide 4D-treated *ApoE*$^{-/-}$ mice in comparison to the larger inflammatory cell content measured in the plaques of control *ApoE*$^{-/-}$ mice.

Interestingly, a single bolus of peptide 4D resulted in the prolongation of the bleeding time in mice within a 4-day period, with a return to baseline levels within 8 days (Fig. 5A). In contrast, such prolongation of bleeding time that was observed in peptide 4D treated mice was not observed in control animals injected with a bolus of saline (Fig. 5B).

Finally, after a 4-month period, the chronic, daily injections of *ApoE*$^{-/-}$ mice, with either peptide 4D or saline, were terminated, and the animals were continuously observed for the remaining period of time. Two mice from each group were kept for observations, and maintained after their injections were terminated. Both of the control, untreated mice died, the first mouse at 10 months of age, and the second mouse at 11 months of age. Interestingly, in contrast to controls, the two peptide 4D-treated mice, ONLY AT 22 MONTHS OF AGE, showed signs of severe illness and were euthanized at this time. Thus, the peptide 4D-treated animals exhibited a healthy appearance and survived for a much longer period of time, about 10 months longer, than did the control mice (see Fig. 3 in Ref. [21]).

3.1. Examination of potential mechanisms underlying the observed health effects of in-vivo treatment with peptide 4D

To explore a possible mechanism by which the treatment of *ApoE*$^{-/-}$ mice for a 4 month period of time with peptide-4D caused an almost doubling of their life span and an overall healthier state than that observed in the control, untreated *ApoE*$^{-/-}$ mice, we examined and compared the ability of platelets to adhere to the vascular walls of treated and untreated animals *in vivo,* utilizing the intravital

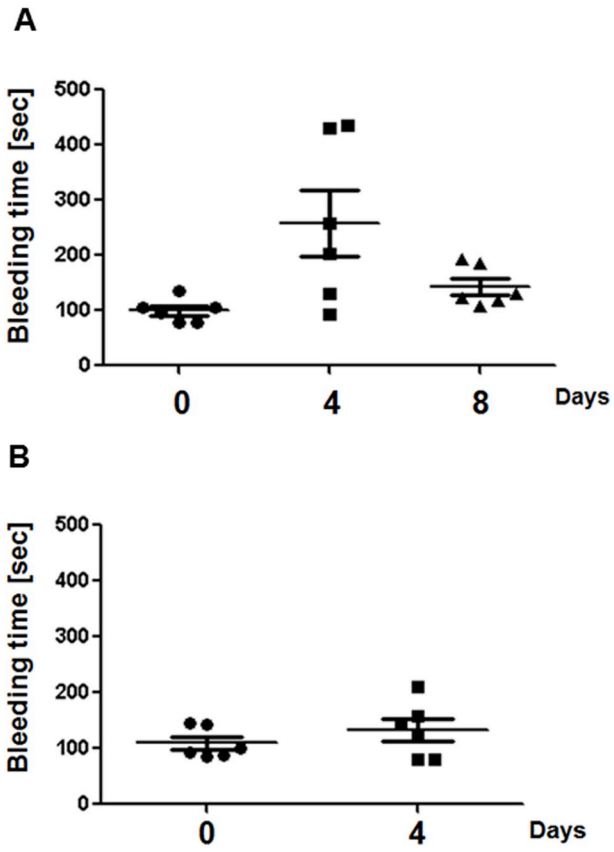

Fig. 5. Comparison of the bleeding time of untreated control *ApoE*$^{-/-}$ mice with *ApoE*$^{-/-}$ mice treated with peptide 4D.
(A) Bleeding times of *ApoE*$^{-/-}$ mice injected with peptide 4D determined at three time points: at zero 0, 24 h prior to a single dose of peptide 4D; at 4, 4 days after peptide 4D injection; and 8, at 8 days following the injection of peptide 4D. Values are the mean ± SD, n = 6 mice, *Significance by *t*-test, $p = 0.022$. (B) Bleeding times of *ApoE*$^{-/-}$ mice injected with saline; 0 time: 24 h prior to injection of bolus of saline, and at 4 days after the injection with saline. Values are the mean + SD, n = 6 mice, Significance by *t*-test, $p = 0.087$.

microscopy system. Both the arterioles and venules of peptide 4D treated mice were examined, and, in comparison, the arterioles and venules of control mice treated with the scrambled form of the peptide.

Arterioles: As shown on Fig. 6A (left panel), the number of platelets adhering to the vascular wall was significantly lower (a 90% decrease in all adhesive events measured) in the arterioles of *ApoE*$^{-/-}$ mice administered the F11R peptide 4D (see histogram 4D), when compared to the platelet adhesion measured in mice injected with the control, scrambled peptide (see histogram labeled “scrambled”). The number of all observed, interacting events, of platelet adhesion to the inflamed arterioles, was significantly lower in peptide 4D-treated mice. Furthermore, in the presence of peptide 4D, a significant decrease of 95% ($p < 0.05$) was observed in the adhesion of platelets to the inflamed arterioles, which lasted longer than 1s (second) (see Fig. 6A, right panel) and see representative movies in Ref. [21]).

Venules: In contrast to the statistically-significant differences measured in platelet-arteriole wall interactions observed between the peptide 4D treated group and the scrambled peptide treated group (control), we observed that peptide 4D treatment did not have a significant effect on the interaction of platelets within venules (Fig. 6B). No statistically-significant differences in the number of interacting platelets were observed between the venules of the peptide 4D treated group and the scrambled treated group (control). No statistically-significant differences in the number of all events of adhesion (Fig. 6B, left panel) as well as in the number of interactions that lasted longer than 1 s (Fig. 6B,

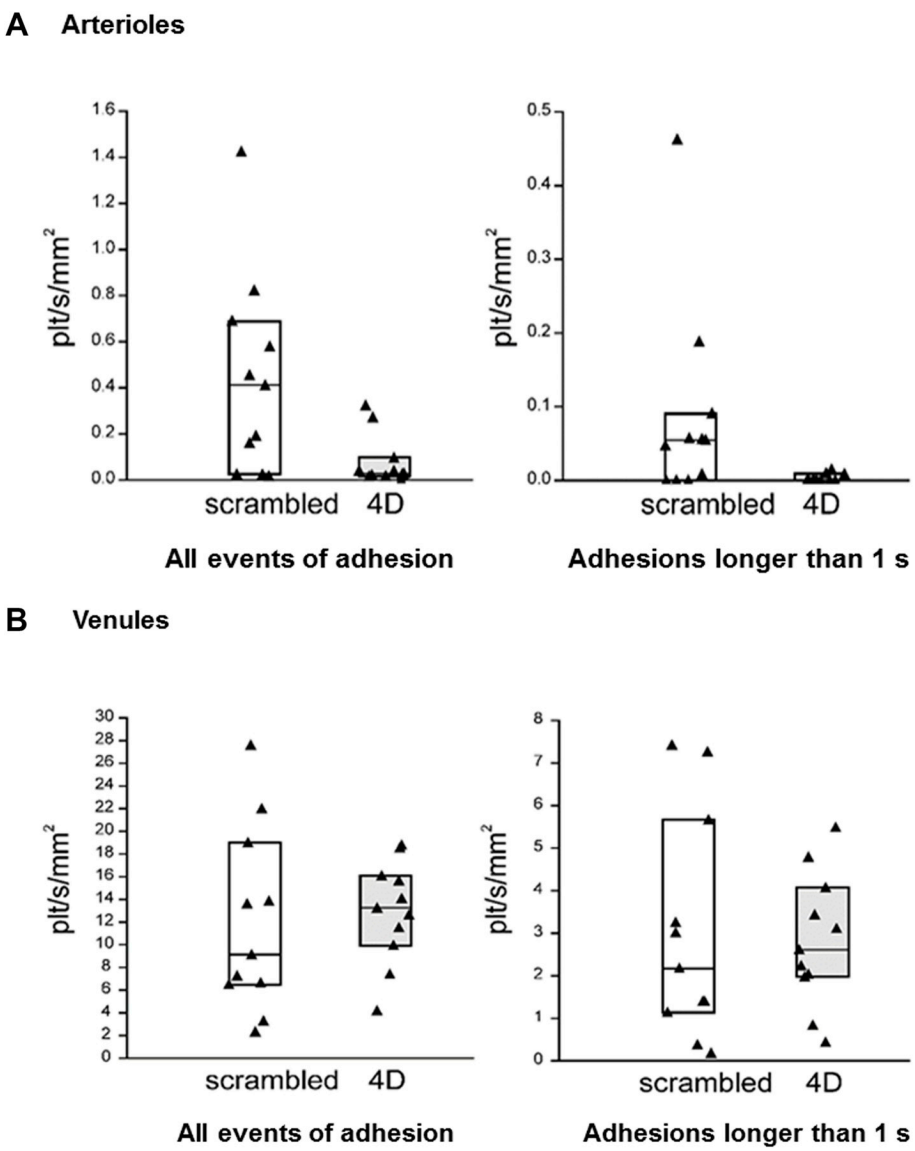

Fig. 6. *In vivo* examination of adhesion of blood platelets to the vascular wall of arterioles and venules of $ApoE^{-/-}$ mice treated with scrambled peptide (control) and peptide 4D with the use intravital microscopy.
Comparison of the adhesion of platelet binding to the endothelium of arterioles (A) and venules (B) of control $ApoE^{-/-}$ mice treated with scrambled peptide (left panels) and of mice treated with peptide 4D (right panels). Results are shown as median (horizontal line) with interquartile range (box). Each point represents average number of adhesions recorded in arteriole or venule of a mouse. $^{****}p < 0.05$ for comparison of scrambled vs. 4D peptide in arterioles by bootstrap-boosted Mann-Whitney *U* test. N = 11.

right panel) were observed between the peptide 4D treated group and the control, scrambled peptide group of mice. (See representative movies in Ref. [21]).

These results demonstrate that peptide 4D significantly inhibits the ability of platelets to interact with the endothelial layer of arterioles.

4. Discussion

This study is the first *in vivo* investigation that reports of the regression of atherosclerosis following long-term treatment of atherosclerosis-prone $ApoE^{-/-}$ mice with an antagonistic-peptide derived from the sequence of the F11R/JAM-A molecule, termed F11R-peptide 4D. While untreated $ApoE^{-/-}$ mice on a high-fat diet develop severe atherosclerotic plaques over time, their peptide 4D-treated counterparts demonstrated a greatly delayed development and a much decreased severity of this disorder. When all of the untreated $ApoE^{-/-}$ mice observed at 3-months of age (or longer) developed severe atherosclerosis, the peptide 4D-treated $ApoE^{-/-}$ mice, treated daily with peptide 4D for the same time period, were robust and healthy and did not exhibit the behavioral signs nor the severe vascular plaque accumulation which developed in the untreated controls.

In an effort to elucidate the mechanism by which treatment with F11R-peptide 4D produces its therapeutic effects, we utilized the technique of intravital microscopy. We directly examined *in vivo* the adhesion of labeled platelets to the exposed, cytokine-inflamed arteriole endothelium of $ApoE^{-/-}$ mice treated with F11R-peptide 4D, in comparison to $ApoE^{-/-}$ mice treated in-parallel with a scrambled form of the peptide. In $ApoE^{-/-}$ mice treated with F11R-peptide 4D we observed a significant reduction in the number of platelets interacting with vascular wall (all observed events) and in interactions longer than 1 s. We conclude that the mechanism by which F11R-peptide 4D antagonizes atherosclerotic plaque-formation is by inhibiting platelet adhesion to the inflamed endothelium of the arterioles. As the inhibitory effect of peptide 4D on the binding of platelets to the endothelium was observed only in the arteriole circulation, it appears that the contribution of F11R/JAM-A as an adhesive force is greater under conditions of high flow and shear rates. Namely, in arterioles rather than in venules. These findings are consistent with the report of Khandoga et al. [34] that the adhesion of JAM-A deficient platelets to hepatic microvenules, following hepatic ischemia and reperfusion injury, was comparable to that of JAM-A wild-type platelets. In previous studies conducted *in culture*, we have observed that F11R/JAM-A expression was significantly higher on human aortic endothelial cells as compared to its expression on human umbilical vein endothelial cells [9]. The higher concentration of F11R/JAM-A on the luminal surface of inflamed arterioles than that of venules, may account for the greater

disruptive power of F11R-peptide 4D in the inhibition of platelet adhesion to inflamed arterioles compared to venules. Several different surface proteins have been implicated as playing a role in the adhesion of platelets to the endothelium [35–37]. The same results here also suggest that while the power of F11R/JAM-A in the adhesion of platelets to the inflamed endothelium of venules is not significantly different to that of other proteins, the power of F11R/JAM-A to the adhesion of platelets to inflamed arterioles is significantly greater than that of other adhesion proteins, as inhibition of F11R/JAM-A alone is sufficient to disrupt this adhesion.

Previous work has demonstrated the important role of platelets in atherogenesis, plaque accumulation and atherosclerosis. Early studies conducted by Massberg et al. [35], demonstrated *in vivo* that the adherence of platelets to the vascular endothelium of carotid arteries of *ApoE*$^{-/-}$ mice occurred prior to the development of manifest atherosclerotic lesions. Also, Huo et al. [38], have demonstrated that circulating activated platelets exacerbated the development of atherosclerosis by binding to the atherosclerotic lesions of *ApoE*$^{-/-}$ mice. The role of JAM-A in atherogenesis was demonstrated by Ostermann and colleagues [39] who reported that JAM-A was upregulated on the early atherosclerotic endothelium of carotid arteries from *ApoE*$^{-/-}$ mice fed an atherogenic diet. By using a soluble form of JAM-A, the recruitment of mononuclear cells to the atherosclerotic endothelium of these arteries could be blocked. Schmitt et al. [40] have shown that the specific knockout of JAM-A in endothelial cells of the hyperlipidemic *ApoE*$^{-/-}$ mice caused a reduction in atherosclerotic lesions, however, the specific knockout of JAM-A in bone marrow cells resulted in an increase in the formation of atherosclerotic lesions.

Studies conducted by Naik et al. [41,42], have shown that knockout-mice genetically-deficient in F11R/JAM-A exhibited a prothrombotic phenotype. They reported that F11R/JAM-A-deficient platelets exhibited an enhanced platelet aggregation in response to low concentrations of physiological agonists. They concluded that F11R/JAM-A functions as an endogenous inhibitor of platelet function. Similarly, studies conducted with JAM-A/F11R-deficient platelets by Karshovska et al. [43], have demonstrated that the deficiency of platelet F11R/JAM-A in *ApoE*$^{-/-}$ mice resulted in platelet hyperaggregability in response to low concentrations of ADP and thrombin, an increase in the adhesion of JAM-A-deficient platelets to the vascular endothelium, and an enhancement in the formation of aortic plaques. An increase in the binding of JAM-A/F11R-deficient platelets to monocytes and neutrophils was observed as well as an increase in the recruitment of platelets and monocytes to the blood vessels of F11R/JAM-A - deficient- *ApoE*$^{-/-}$ mice, all of these events resulting in an acceleration of atherosclerosis. There appears to be an apparent contradiction between our results utilizing the F11R antagonist, peptide 4D, and the results described above, in which mice were made deficient in F11R JAM-A. Our results demonstrate that blockade by peptide 4D led to the prevention of atherosclerosis in *ApoE*$^{-/-}$ mice. Our study has shown that *ApoE*$^{-/-}$ mice treated chronically with peptide 4D, show a decrease in atherosclerotic plaques and favorably alters the plaque composition making it less vulnerable, whereas F11R/JAM-A - deficient ApoE mice demonstrate an enhanced level of thrombosis and atherosclerosis. It must be emphasized, however, that it is wrong to expect that the total elimination of a protein in all cells, and the addition of a specific inhibitor should have identical consequences. It is possible that peptide 4D interacts at a distinct inhibitory site on the F11R/JAM-A molecule, as identified by utilization of the platelet stimulatory monoclonal antibody, M.Ab.F11 [8]. Thus, the interaction of peptide 4D with F11R/JAM-A of platelets and endothelial cells, indeed may be sufficient in blocking platelet adhesion to the inflamed endothelium, and thereby preventing the development of atherosclerotic plaques. Genetic deletion of F11R/JAM-A was reported to cause shortening of tail bleeding time, enhanced thrombosis, enhanced atherosclerosis and hyperaggregability [43] None of these were evident in our present study in the *ApoE*$^{-/-}$ mice chronically treated with peptide 4D.

An interesting finding in the present investigation was that treatment of *ApoE*$^{-/-}$ mice with peptide 4D prevented the severe skin scratching associated with this deficiency. It has been well documented that along with the enhanced degree of atherosclerotic plaques, *ApoE*$^{-/-}$ mice develop cutaneous xanthomatosis which progresses in severity over time, due to the hypercholesterolemia and hypertriglyceridemia [44]. The evidence provided by Lim [45] using *ApoE*$^{-/-}$ mice, shows that the hypercholesterolemia may be due to lymphatic vessel dysfunction associated with profound structural abnormalities in the lymphatic vasculature. JAM-A/F11R has been identified on human lymphatic endothelium [46], and we speculate that JAM-A/F11R may be involved in the pathology of cutaneous xanthomatosis resulting in the scratching of the chest and the severe skin lesions observed in control, untreated *ApoE*$^{-/-}$ mice, but prevented by the administration of peptide 4D. The administration of peptide 4D to *ApoE*$^{-/-}$ mice for a period of 3- to 4-months, resulted in a healthy group of animals that did not exhibit any signs of scratching nor the cutaneous xanthomatosis exhibited by control mice. The peptide 4D-treated group, due to the lack of skin scratching, exhibited a healthy coat of hair throughout the entire period of experimentation. The treatment of animals with peptide-4D appears to have had a profound effect on the prevention of skin disorders.

The decrease in atherogenesis observed in *ApoE*$^{-/-}$ mice treated with peptide-4D appears NOT to be due to the effects of peptide 4D on lipid metabolism, as the levels of plasma lipids, including cholesterol, were found not to be affected by this treatment. In contrast, Statins, the class of drugs currently most frequently used in clinical practice for prevention of thrombosis and heart attacks, exert their preventative effects by influencing lipid metabolism. F11R peptide-4D, on the other hand, was reported to inhibit plaque formation triggered by inflammatory agents [6]. Accordingly, we propose that the preventative treatment of atherogenic processes could be significantly and perhaps synergistically enhanced by the combined use of a F11R/JAM-A -antagonist together with Statins.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

AB, CCC, CW, MS designed the experiments and analysed the data. TP, MT, YL, JW, MB conducted the experiments and contributed to data analysis. AB, YHE, EK, MOS designed the experiments and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.02.014.

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