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Paris, 4 May 2019.

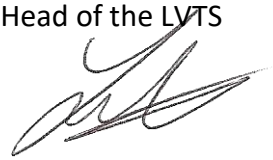
To Whom It May Concern,

We hereby confirm that Dr. Nerea Mendez Barbero has finished today her stay in our laboratory (Laboratory for Vascular Translational Science, INSERM Unit 1148, Bichat Hospital in Paris) as an IAS visiting fellow awardee under Dr Benoît Ho-Tin-Noé's supervision.

Yours sincerely,

Benoît Ho-Tin-Noé

Didier Letourneur
Head of the LVTS



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The work presented is a collaboration between Vascular Pathology Lab (IIS-FJD Madrid Spain) and laboratory of Vascular Translational Science, Dr. Ho Tin Noe (Inserm Unit 1148 - Hôpital Bichat Paris, France) and Dr. Didier Letourneur (Head of the LVTS, Hôpital Bichat).
IAS Visiting Fellowship Award has supported the work to the main author Dr. Mendez Barbero.

HYPOTHESIS

Based on the preliminary results in which we observed by RNAseq that our adhesion regulator protein (we will call it ARP) is augmented in Apoe -/- compared to WT aortas, and in base to the bibliography, our hypothesis for the realization of this collaboration is that; We believe that our protein of interest could have a role in the extravasation of leukocytes and platelets in the wall of the injured artery. Moreover, this protein would be a target protein in the process of atherosclerosis because it would decrease the chronification of the inflammatory response in the injured vessels and the development of the atherosclerosis plaque.

OVERALL OBJECTIVE

This collaborative project with the group of Dr. Ho-Tin-Noé has the main aim; perform intravital microscopy assays in mice deficient in ARP and in vitro adhesion assays in flow chambers. Moreover, we have performed experiments to analyze the possible impact of ARP deletion on the functionality of the immune system cell especially on platelets.

CONCRETE OBJECTIVES

1- Vascular permeability and leukocyte recruitment may be partly also produced by platelets not only by activated endothelial cells, because they also secrete granules with mediators like p-selectin. Therefore, we have also analyzed the nature of the platelets of animals deficient in ARP (counting, capacity of aggregation activation, and expression of p-selectin in its granules).

2- In vitro assays of neutrophils, platelets and monocytes adhesion to a monolayer of WT or ARP-deficient endothelial cells, activated with oxLDL and maintained under physiological conditions.

3- Intravital Microscopy study in Apoe -/- mice with predisposition to development atherosclerosis vs animals Apoe -/- ARP -/- fed with high fat diet, analysis and visualization of the *in vivo* recruitment of platelets, leukocytes and neutrophils to the area of the injured artery.

RESULTS OF THE 3 MONTHS OF STAY (Hospital de Bichat, Inserm, Unit 1148, Paris, France) FEBRUARY-MAY:

1- Analysis of the nature of the platelets of animals deficient in ARP protein vs WT. In addition to the endothelial cells, platelets are cells that have the ability to present internal granules, which release mediators that increase vascular permeability. In this sense, p-selectin that is also released from the platelets increase the recruitment of neutrophils and leukocytes to the vessel wall, increasing extravasation and therefore disease chronification. Thanks to the experience of Dr. Ho-Tin-Noe's group in platelet activation assays we decided to analyze if our ARP would be able to regulate the exocytosis of these granules and therefore decrease vascular permeability. For this we analyzed the expression of p-selectin in platelets of ARP deficient mice and in WT. To analyze this hypothesis, several experiments were carried out:

1- PLATELET ACTIVATION ASSAY USING TROMBINE RECEPTOR ACTIVATING PEPTIDE (TRAP) IN;

a. Total blood from wild type or ARP deficient mice. Animals were anesthetized and blood from WT and from ARP knockout mice was isolated by retro-orbital extraction (method by which the lowest platelet activation is guaranteed). Blood samples were incubated with PBS1x (1mM CaCl) without stimulation or with TRAP stimulation (200uM or 400uM). Subsequently, the expression of p-selectin was analyzed in blood samples by flow cytometry using the antibody p-selectin CD62P rat RB40.34 FITC 0.5 mg / ml (BD 553744 TO 5). The results showed that while platelets without stimulation did not present expression of p-selectin, the incubation of the blood with TRAP caused platelet activation and platelet aggregation, with similar p-selectin activation in blood from WT and animals deficient in ARP. These results

showed that the deficiency of this protein in platelets does not produce differences in the expression of p-selectin. In addition, we observe that the ARP deficiency does not affected platelet-neutrophil aggregates percentage. Moreover, we used oxidized low-density lipoprotein (LDLox) stimulus as more physiological stimulus to try to activate the platelets. However, we did not get any results apparent, indicating that oxidized LDL was not able to stimulate platelet activation in whole blood. (Fig1).

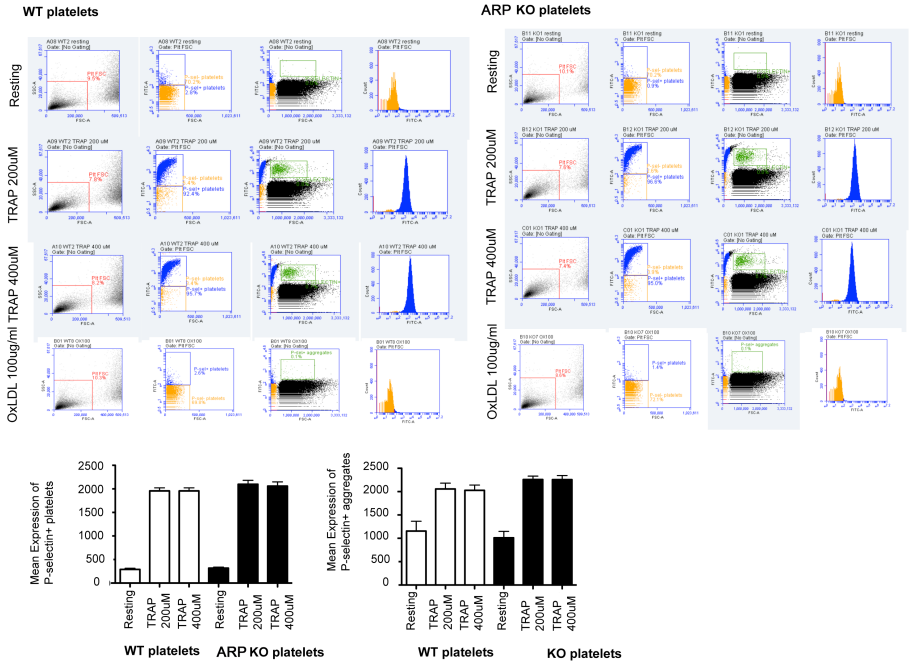

Figure 1: (A-B) Flow cytometry graphs for the selection of positive p-Selectin platelet population in blood of WT animals and deficient of ARP. (C) P-selectin expression (Mean of fluorescence) (D) platelet-neutrophils aggregation expression (mean fluorescence).

b- In platelets purified from blood of wild type mice or ARP deficient mice. Since in the previous experiments we did not observe differences in the activation of platelets among the different genotypes we thought it could be because we had used whole blood and not purified platelets. For that reason, we use a mouse platelet extraction protocol to purify platelets and later stimulate them with TRAP. Briefly the protocol consists; blood was isolated by retro-orbital way to avoid platelet aggregation. Subsequently, the blood is centrifuged at 150g for 5 hours. The serum was collected and taken up to 1ml with buffer of stabilization. Afterwards, it is centrifuged again and platelets were obtained in the pellet. Approximately $2x10^8$ platelets/ml were activated with TRAP in concentrations of 200 and 400 mM for platelet activation assay. As we observed for activation in whole blood, we did not observe differences in the expression of p-selectin between WT or ARP deficient platelets.

2- MODELS OF SKIN AND PULMONARY INFLAMMATION IN ANIMALS DEFICIENT IN ARP AND IN WT ANIMALS FOR ANALYSIS OF INFLAMMATORY CELL RECRUITMENT *IN VIVO*: Since we did not observe differences in platelet activation in the presence or absence of our ARP, we wanted to see if this protein was important then in the differential recruitment of inflammatory cells. For this we carried out several models of inflammatory cells recruitment *in vivo* using acute pro-inflammatory stimuli.

a- Induction of cutaneous dermatitis in WT or mice deficient in ARP; The animals were sensitized by retro-orbital injection of 50ul BSA 2% followed by a subcutaneous injection of 60ug rabbit anti-BSA in the dorsal area. During 4 hours we monitorized the reaction caused in the skin of each animal. After that time the animals were sacrificed and the fragments of skin were obtained. Recruitment of polymorphonuclear cells (PN) to the area of inflammation was studied by peroxidase activity in the tissue (MPO). Moreover, platelet content measure by

the haemoglobin content in tissue was also analysed. The results did not show a significant difference, however there is a trend towards a lower percentage of inflammatory cells recruited and less haemoglobin content in skins of animals deficient in our ARP (Fig2).

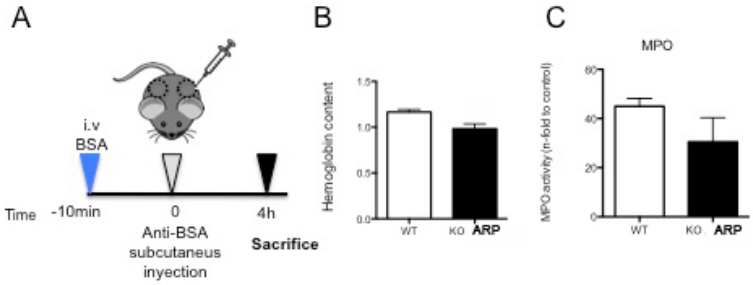

Figure 2: (A) Representative diagram of animal model. i.v (intravenous), BSA (bovine serum albumin). (B) Haemoglobin content in the analyzed skin fragments (B) MPO activity (peroxidase) that indicates the amount of immune cells recruited to the area of inflammation.

b- Induction of pulmonary inflammation by nasal inhalation of LPS in WT mice or deficient in our ARP; The animals were sensitized by nasal inhalation of LPS (20ug in each of the nostrils). We waited 4 hours for the reaction to take place. Later the animals were sacrificed and by pulmonary lavages we obtained the cellular exudate of the lungs. Cell pellet from the pulmonary lavages was centrifuged and cells were stained with specific markers for: leukocytes: CD45, neutrophils: Ly6G, monocytes and granulocytes: CD11b, and macrophages: F4 / 80. The analysis of cellular populations in the exudate was analyzed by flow cytometry. The results obtained showed that there was no biological significance in regarding differences in the migration of inflammatory cells to the inflamed area in term of percentages of neutrophils, monocytes and macrophages between genotypes (Fig 3).

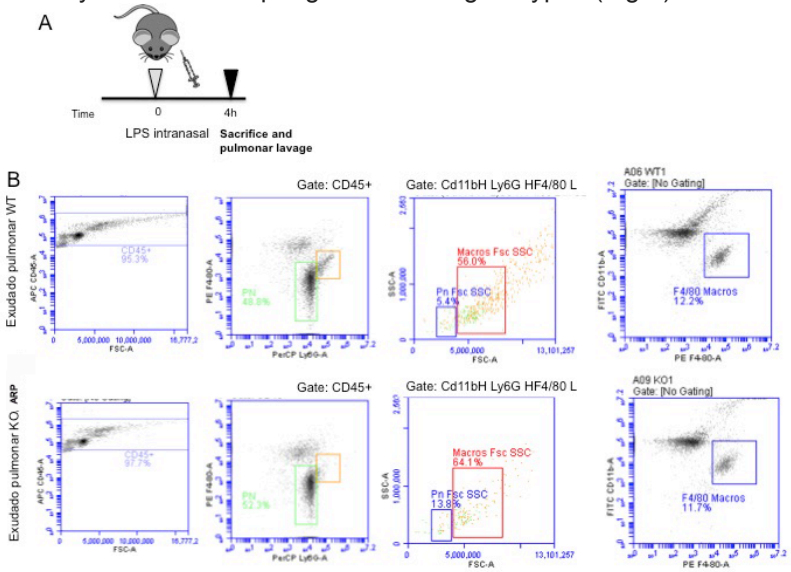

Figure 3: (A) Schematic representation of the animal model. (B) Flow cytometry graphs indicating the different cell populations of pulmonary lavages in WT animals and our ARP knockout animals.

2- ADHESION ASSAYS; Study *IN VIVO* Intravital microscopy and *IN VITRO* Flow chamber.

All the data observed in the previous models indicated that there were no differences between genotypes in platelet activation and in inflammatory cells recruitment at least in these acute models of inflammation.

In this sense we thought to use a more physiological atherogenic stimulus (oxLDL) in vitro, and high fat diet for analysis in vivo of the inflammatory cells recruitment.

1- *IN VITRO*: INMUNE CELLS ADHESION OVER oxLDL ESTIMULATED PRIMARY AORTIC ENDOTHELIAL CELLS OF WT OR DEFICIENT IN ARP UNDER FLOW CONDITIONS. Since these experiments have never been done using primary mouse endothelial cells, we had to set up the assay for its adaptation to mice cultures. The primary aortic endothelial cells were obtained from WT and animals deficient in our ARP using the protocol described in Esteban et al 2017. Briefly, the aortas were isolated and the adventitial layer removed. The aortas were cut into small rings and were incubated 45 min in DMEM without serum with collagenase type II by aorta. The collagenase reaction was stopped after 45 min and rings were seeded. After 5 days a positive selection of the endothelial cells was done using anti-CD102 antibody (or ICAM-2), and secondary antibody (Dylabeads) allows us to separate vascular smooth muscle cells and endothelial cells. For cell adhesion experiments in flow conditions, specific cell culture plates (Vena8 Endothelial Cellix) were treated with Fibronectin. Next day endothelial cells were seeded in each channel of the plate and leave them for a time. After these hours cells were culture under a continuous flow environment using a flow pump that allows mimicking the blood flow (Fig4).

After 24h in which the endothelial cells have been maintained in conditions of constant flow, cells look like similar to how they are in blood vessels, forming a cell monolayer that covers the channels. After that, we added our atherosclerotic stimulus to induce the activation of the endothelium. We infused oxidized low-density lipoproteins (LDLox). The next day we extracted blood from WT animals and animal deficient in our ARP via retro-orbital. These blood samples were incubated with specific antibodies to monocytes (CD68), neutrophils (Ly6G) and platelets (GPIX). Subsequently, and using the fluorescence microscope, we infused blood over the endothelium monolayer using an infusion pump that simulates the venous flow. We analyze the percentage of neutrophils, monocytes and platelets that have been adhered to the endothelial cells monolayer. The first results obtained from these experiments showed that while blood WT on activated endothelium WT present higher amount of platelet aggregation and adhesion of neutrophils, ARP deficient endothelial monolayer are less active to platelet aggregation and neutrophils adhesion. We did not find differences in the percentage of adhered monocytes (Fig5). Although the time of the scholarship has been enough to set up the technique and start elucidate the possible role of ARP in endothelial cells adhesion, currently we are in collaboration with Dr Ho-Tin-Noe's laboratory in Paris and we are that continuing with the experiments to get more robust results.

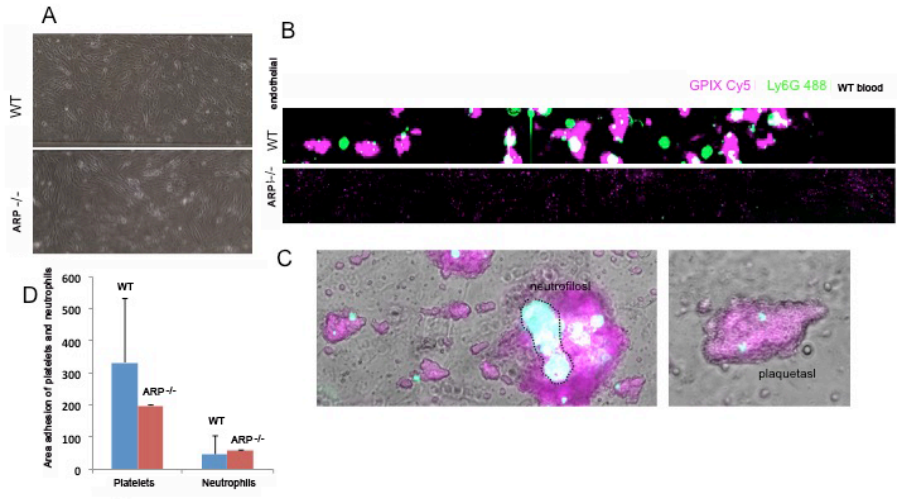

Figure 5: (A) Monolayer images of endothelial cells on fibronectin channels. (B) Fluorescence images of platelet and neutrophils adhesion of WT blood on monolayer of WT cells or ARP knockout under flow conditions (C) Bright field and fluorescence images of platelet aggregate (Pink GPIX Cy5) together with neutrophils (green Ly6G 488) in a monolayer of endothelial cells (D) Quantification of platelet and neutrophils adhesion in flow chamber assays.

2- *IN VIVO*: STUDY OF INTRAVITAL MICROSCOPY IN MOUSE WITH PREDISPOSITION TO DEVELOP ATHEROSCLEROSIS APOE -/- VS APOE -/- ARP -/-

ApoE -/- and ApoE -/- ARP -/- mice of 12 weeks of age were fed with high fat diet to induce recruitment of platelets, leukocytes and neutrophils to the area of the injured artery. Both groups of mice were anesthetized with ketamine / xylazine. Under the microscope and carefully the

carotid artery was isolated avoiding damaging it. Subsequently, we injected retro-orbitally a mix of antibodies for monocyte labelling (CD68), Neutrophils (Ly6G) and Platelets (GPIX). We use a fluorescence microscope (MacroFluo, Leica Microsystems, Nanterre, France) equipped with a heating plate thermostatic and a 20X objective to visualize cell recruitment to the area of the carotid artery. From each animal we obtained videos and high-resolution images with the sCMOS camera (Orca-Flash-4.0, Hamamatsu Photonics, Hamamatsu, Japan). Moreover, both the images and the videos were analyzed with the software Imaje J. Parallel analysis of the recruitment of platelets, neutrophils and monocytes showed that ApoE -/- animals fed a fatty diet presented more platelet aggregation in the carotid walls than mice deficient for the ARP. These results support those observed in *in vitro* experiments of flow chamber in which our ARP knockout animals have a less active endothelium and lower platelet aggregation (Fig6). Currently we are in collaboration with Dr Ho-Tin-Noe's laboratory in Paris to analyze more data from that experiments.

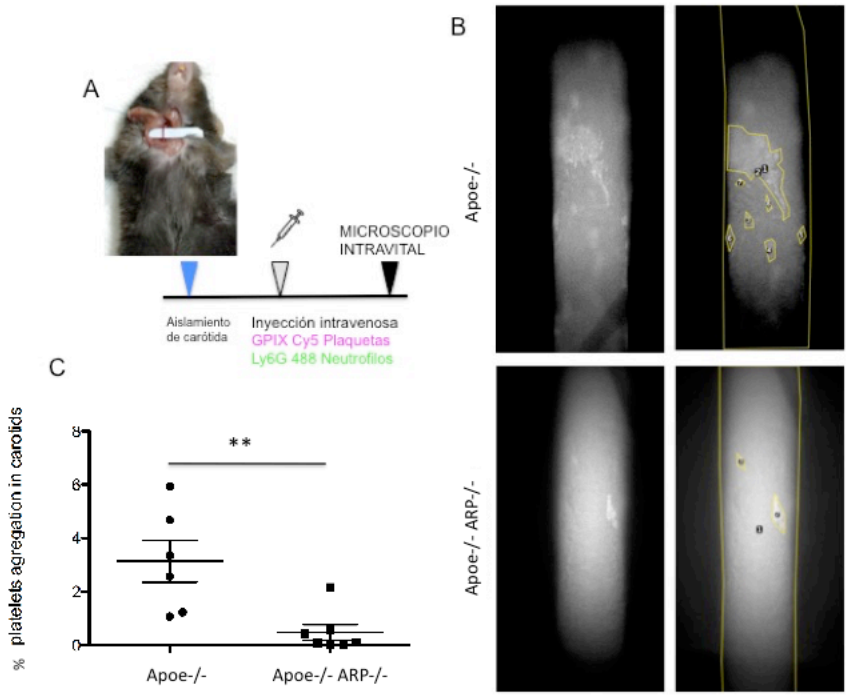

Figure 6: (A) Schematic representation of the animal model. (B) Intravital microscopy image of the isolated carotid area. The marked area represents the positive zone for GPIX Cy5 Platelets in the carotid. (C) Quantification by ImageJ of the platelets labeling in the injured carotid area.

BIBLIOGRAPHY:

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ACKNOWLEDGEMENTS

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