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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

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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).

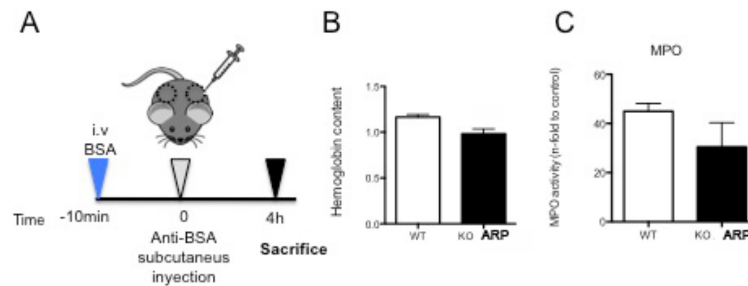

Figure 2: (A) Representative diagram of animal model. i.v (intravenous), BSA (bovine serum albumin). (B) Haemoglobin content in the analyzed skin fragments (B) MPO activity (peroxidase) that indicates the amount of immune cells recruited to the area of inflammation.

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.

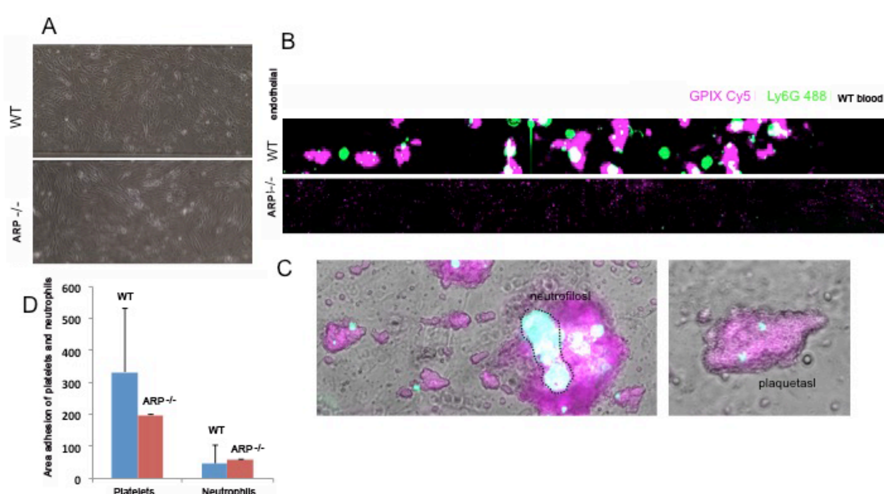

Figure 5: (A) Monolayer images of endothelial cells on fibronectin channels. (B) Fluorescence images of platelet and neutrophils adhesion of WT blood on monolayer of WT cells or ARP knockout under flow conditions (C) Bright field and fluorescence images of platelet aggregate (Pink GPIX Cy5) together 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.

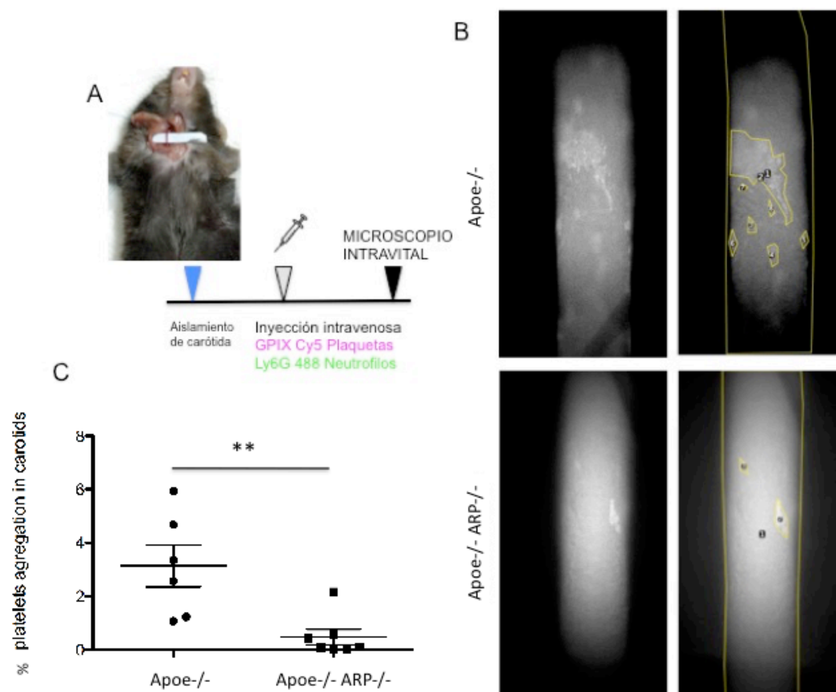

Figure 6: (A) Schematic representation of the animal model. (B) Intravital microscopy image of the isolated carotid area. The marked area represents the positive zone for GPIX Cy5 Platelets in the carotid. (C) Quantification by ImageJ of the platelets labeling in the injured carotid area.

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- Esteban V et al, 2018. Front Immunol. 2017. *Endothelial Regulator of Calcineurin 1 Promotes Barrier Integrity and Modulates Histamine-Induced Barrier Dysfunction in Anaphylaxis.*

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