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## **A CD31-derived peptide prevents the development of antibody-mediated lesions in a rat model of aortic allograft**

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## **Introduction**

Accelerated arteriosclerosis of solid organ transplants is mediated by the development of donor-specific antibodies (DSA) directed against the allogeneic HLA molecules expressed by the graft. Immunosuppressive drugs are insufficient to prevent the generation of DSA. As a result, antibody-mediated rejection (AMR) has emerged as a major cause of graft loss.

The development of antigen-specific antibodies requires the activation of antigen presenting cells as well as of T helper and B lymphocytes, the coordination of which is physiologically controlled by cell-surface immunoregulatory molecules. Among the latter, CD31 is particularly interesting as a potential therapeutic target for AMR because it is expressed by all the immune cells and its regulatory functions are engaged by trans-homophilic interaction. CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is a transmembrane Ig-like immunoreceptor tyrosine-based inhibition motif (ITIM) protein expressed on endothelial cells, platelets, and leukocytes. This inhibitory signaling receptor ensures homeostasis at the interface between immune cells and blood vessel cells. Upon strong immune stimulation, the regulatory function of CD31 is lost due to cell activation-induced cleavage of its extracellular portion [1]. However, as CD31 is partially but not completely lost on activated cells, its incomplete cleavage provides the opportunity to rescue its immunomodulatory function by targeting the residual portion of the molecule. In this setting, P8RI, an agonist synthetic peptide that binds to the juxta-membrane amino acid sequence of the ectodomain of CD31, shows an immunosuppressive effect through restoration of the CD31 inhibitory pathway [1-3].

In this work, we hypothesized that CD31 could represent an attractive molecular target in AMR and investigated whether P8RI could prevent the development of vascular antibody-mediated lesions in a murine model of orthotopic aortic allograft (AA).

## **Materials and methods**

Seven-week-old Lewis (LEW; RT11) rats were used as recipients and age-matched male Brown-Norway (BN; RT1n) rats as allogeneic donors in a model of AA, as described [4,5]. All experiments conformed to Directive 2010/63/EU of the European Parliament and approval was obtained from the

Paris Nord Animal Ethics Committee. A 1 cm long segment of the donor BN abdominal aorta was transplanted in orthotopic position by end-to-end anastomosis in the recipient sub-renal aorta. Rats were induced with 4% isoflurane and then maintained on 2% isoflurane. Intraperitoneal injection of buprenorphine was used for analgesia (0.03 mg/kg pre-operatively and post-operatively every 8 hours). P8RI was administered subcutaneously for 28 days at 2.5 mg/kg/day, control animals received subcutaneous injection of PBS.

Twenty-eight days after AA, blood was collected in dry tubes to retrieve serum. The animals were sacrificed by intraperitoneal injection of sodium pentobarbital. Aortas were snap frozen in liquid nitrogen. Longitudinal cryosections were cut at 5  $\mu$ m thickness in optimal cutting temperature (OCT) medium, stained with 4',6-diamidino-2-phenylindole (DAPI) and orcein. Density of nuclei in the media and media surface area were measured in ten fields per aorta at x20 magnification and quantified using a computerized morphometric analysis system (Qwin Software, Leica Microsystems).

Immunofluorescence staining of aortic allografts was performed using the following antibodies: polyclonal alpha-smooth muscle actin (alpha-SMA) and IgG, monoclonal CD68 (clone ED-1).

Apoptosis was identified by the TUNEL assay. Detection of circulating DSA was performed in LEW recipients twenty-eight days after AA by adding LEW sera on fresh BN splenocytes mixed with pre-immune LEW sera. After incubation with IgG-AF647 and CD45R-FITC, IgG MFI was measured on CD45R negative cells, using a LSRII® flow cytometer and BD FACSDiva® software 6.0.

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

The differences between groups were evaluated using Mann-Whitney U test and ANOVA, as appropriate. The differences were considered significant when the *P* value was < 0.05.

## **Results**

### **Antibody-mediated medial remodeling is observed in allogeneic aortic allograft model**

Disappearance of alpha-SMA+ smooth muscle cells (SMCs) was observed in the aortic allografts and was associated with marked apoptosis in the media and ED-1+ macrophage infiltration (Fig 1A). IgG binding to SMCs was observed in the media and colocalized with apoptotic TUNEL+ cells (Fig 1B).

These data validate the allogeneic aortic allograft as an accurate model of AMR, in which antibodies

binding to donor SMCs are responsible for medial apoptosis and remodeling, as previously described [4,5].

### **P8RI reduces DSA titers in an allogeneic aortic allograft model**

Aorta-allografted rats receiving P8RI for 28 days from allograft developed less DSA than aorta-allografted control animals (MFI 344 vs. 741,  $p = 0.03$ ; Fig 1C).

### **P8RI prevents the development of DSA-mediated lesions in the allograft**

Analysis of aortic grafts showed that density of nuclei in the media ( $3.4 \times 10^{-5}$  vs.  $2.2 \times 10^{-5}$  nuclei/ $\mu\text{m}^2$ ,  $p = 0.01$ ; Fig 1D) and media surface area ( $2.33 \times 10^6$  vs.  $2.02 \times 10^6$   $\mu\text{m}^2$ ,  $p = 0.007$ ; Fig 1E) were higher in animals treated with P8RI than in control animals. Intimal surface area was not different between P8RI and control animals ( $0.53 \times 10^6$  vs.  $0.62 \times 10^6$   $\mu\text{m}^2$ ).

## **Conclusions**

CD31 is a protein expressed on endothelial cells, platelets, and leukocytes, ensuring homeostasis at the interface between immune cells and blood vessel cells. As CD31 is partially but not completely lost on activated cells upon immune stimulation, this cleavage provides the opportunity to rescue its immunomodulatory function by targeting the residual portion of the molecule. In this setting, the agonist synthetic peptide P8RI shows an immunosuppressive effect in inflammatory processes, such as those underlying progression of atherosclerosis [3]. In solid organ transplants, the development of DSA and the occurrence of AMR directly contribute to accelerated arteriosclerosis [5]. We herein provide experimental data supporting a therapeutic potential for molecules able to restore the CD31 signaling to fight AMR and transplant arteriosclerosis. P8RI, an agonist synthetic peptide targeting CD31, might not only prevent DSA production but also have a beneficial effect in limiting arterial antibody-mediated lesions, as suggested by our findings in a murine model of aortic allograft. CD31 agonists may become important therapeutic tools to prevent and treat solid organ transplant arteriosclerosis.

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## Figure legends

**Figure 1** P8RI prevents vascular antibody-mediated lesions observed in aortic allograft.

(A) Disappearance of alpha-SMA+ smooth muscle cells (SMCs) was observed in the aortic allograft and associated with marked apoptosis in the media and ED-1+ macrophage infiltration. (B) IgG binding to SMCs was observed in the media, many of SMCs being DAPI negative, and colocalized with apoptotic TUNEL+ cells. (C) Quantification of DSA estimated by the IgG MFI in control (n=6) and P8RI groups (n=7) ( $p < 0.05$  by Mann-Whitney U test). (D) Density of nuclei in the media, measured in ten fields at x20 magnification per animal, was higher in P8RI than in control group ( $p < 0.05$  by ANOVA). (E) Media surface area, measured in ten fields at x20 magnification per animal, was higher in P8RI than in control group ( $p < 0.01$  by ANOVA). \* $p < 0.05$ , \*\* $p < 0.01$ .

