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Macrophage CD31 Signaling in Dissecting Aortic Aneurysm

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ABSTRACT

BACKGROUND The authors recently found that a CD31 agonist peptide reaches macrophages in injured aortas and exerts beneficial effects on apolipoprotein E-knockout (Apo $E^{-/-}$) mice subjected to angiotensin (Ang) II infusion, a model of experimental acute aortic dissection and intramural hematoma (ADIM).

OBJECTIVES The purpose of this study was to evaluate the therapeutic potential of a drug-suitable agonist peptide in experimental ADIM.

METHODS P8RI, a retro-inverso sequence of the best candidate identified by functional in vitro screening of a peptide library, passed an absorption, distribution, metabolism, excretion and toxicology analysis. Apo $E^{-/-}$ mice (male, 28-week-old) implanted with Ang II-releasing pumps received P8RI (2.5 mg/kg/day) or vehicle from day 14 ($n = 10$/group). Leukocytes were analyzed by flow cytometry. Healing features of human and mouse dissected aortic segments were assessed by histology and immunofluorescence. The effect of CD31 on macrophages was evaluated using cells from $CD31^{-/-}$ mice and P8RI, in vitro.

RESULTS Human and experimental ADIM were characterized by the infiltration of proinflammatory macrophages. The absence of CD31 enhanced the proinflammatory polarization of macrophages, whereas the CD31 agonist P8RI favored reparative macrophages both in vitro and in vivo. The administration of P8RI after the occurrence of ADIM prevented aneurysmal transformation by promoting the resolution of intramural hematoma and the production of collagen in dissected aortas in vivo, associated with enrichment of M2 macrophages at the site of injury.

CONCLUSIONS CD31 signaling promotes the switching of proinflammatory macrophages to the reparative phenotype and favors the healing of experimental dissected aortas. Treatment with a drug-suitable CD31 agonist may facilitate the clinical management of ADIM. (J Am Coll Cardiol 2018;72:45–57) © 2018 by the American College of Cardiology Foundation.

Acute aortic dissection and intramural hematoma (ADIM) (dissecting aneurysm) is a life-threatening disease with a mortality of 50% within the first 48 h. In the absence of involvement of the ascending aorta, the clinical management is essentially palliative and is directed at reducing the systemic blood pressure and heart rate as much as possible, which limits the propagation of the false

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ABBREVIATIONS AND ACRONYMS

ADIM = acute aortic dissection and intramural hematoma

Ang II = angiotensin II

Apo E$^{-/-}$ = apolipoprotein E knockout

BMDM = bone marrow–derived macrophage

IL = interleukin

iNOS = inducible nitric oxide synthase

US = ultrasound

lumen and prevents end-organ damage and risk of rupture (1).

Due to the lack of specific therapeutic agents aimed at favoring rapid tissue healing, 25% to 30% of ADIM patients require subsequent intervention because of aneurysmal expansion, progressive dissection, and other complications from the unresolved dissection process (2). Interestingly, the rate of recurrent events is independent of the treatment strategy (open surgery, endovascular repair, or aggressive antihypertensive treatment) and occurs more frequently in patients affected by connective tissue disorders (3), highlighting the importance of an appropriate extracellular matrix response for the healing process and long-term prognosis.

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The outcomes of tissue healing after an acute injury essentially depend on the resolution of the initial inflammatory phase, and macrophages play a crucial role in this process. Immediately after entering the wound site, circulating monocytes contribute to the demolition phase of wound healing by acquiring a proinflammatory phenotype; however, for appropriate tissue healing, the monocytes must exert fundamental functions including the acquisition of a reparative phenotype (4). Importantly, the wound healing process may be consistently delayed and can even remain unachieved in the presence of blood-derived elements, as in the case of dissected aortas, which prevent the macrophages from switching from the proinflammatory to the reparative phase (5).

In this work, we sought to assess the role of CD31, an immunoregulatory receptor, in macrophage polarization and ADIM outcome in apolipoprotein E-knockout (Apo E$^{-/-}$) mice subjected to chronic angiotensin (Ang) II infusion, an experimental model of ADIM (6).

METHODS

IDENTIFICATION OF A DRUG-SUITABLE CD31 AGONIST PEPTIDE. The drug-suitable CD31 agonist used in this study was selected from 2 peptide libraries, as detailed in Online Table 1.

The absorption, distribution, metabolism, excretion, and toxicology analysis was performed on the retro-inverso sequence of the best candidate, termed P8RI, and included the measurement of potassium currents mediated by the human ether-a-go-go-related gene channel; bacterial reverse mutation test; blood half-life (pharmacokinetic studies) after intravenous, oral, and subcutaneous administration in C57BL/6 mice; and in vivo toxicity evaluated during a 14-day subcutaneous dose-range study in C57BL/6 mice, as detailed in Online Appendix 1.

ANALYSIS OF MOUSE MACROPHAGE POLARIZATION IN VITRO. Bone marrow–derived macrophages (BMDMs) were prepared and analyzed as described in Online Appendix 1 from the femurs of 10-week-old male CD31$^{+/+}$ and CD31$^{-/-}$ (7) mice derived in our animal facility by breeding CD31$^{+/-}$ mice (C57BL/6 background), which were generously provided by Dr. Debra K. Newman (Blood Center of Wisconsin, Milwaukee, Wisconsin).

ANG II INFUSION IN APO E$^{-/-}$ MICE. Twenty-eight-week-old male Apo E$^{-/-}$ mice (B6.129P2-ApoEtm1Unc/Crl, Charles River Laboratories, Saint Germain Nuelles, France) were maintained on a regular chow diet under standard conditions. Ang II (#A9525, 1 mg/kg/day, Sigma-Aldrich, St. Louis, Missouri) was continuously infused into the experimental mice via osmotic pumps (Model 2004, Alzet, Charles River Laboratories). All investigations conformed to the Directive 2010/63/EU of the European Parliament, and the local animal ethics committee (Comité d'éthique Bichat–Debré) granted formal approval.

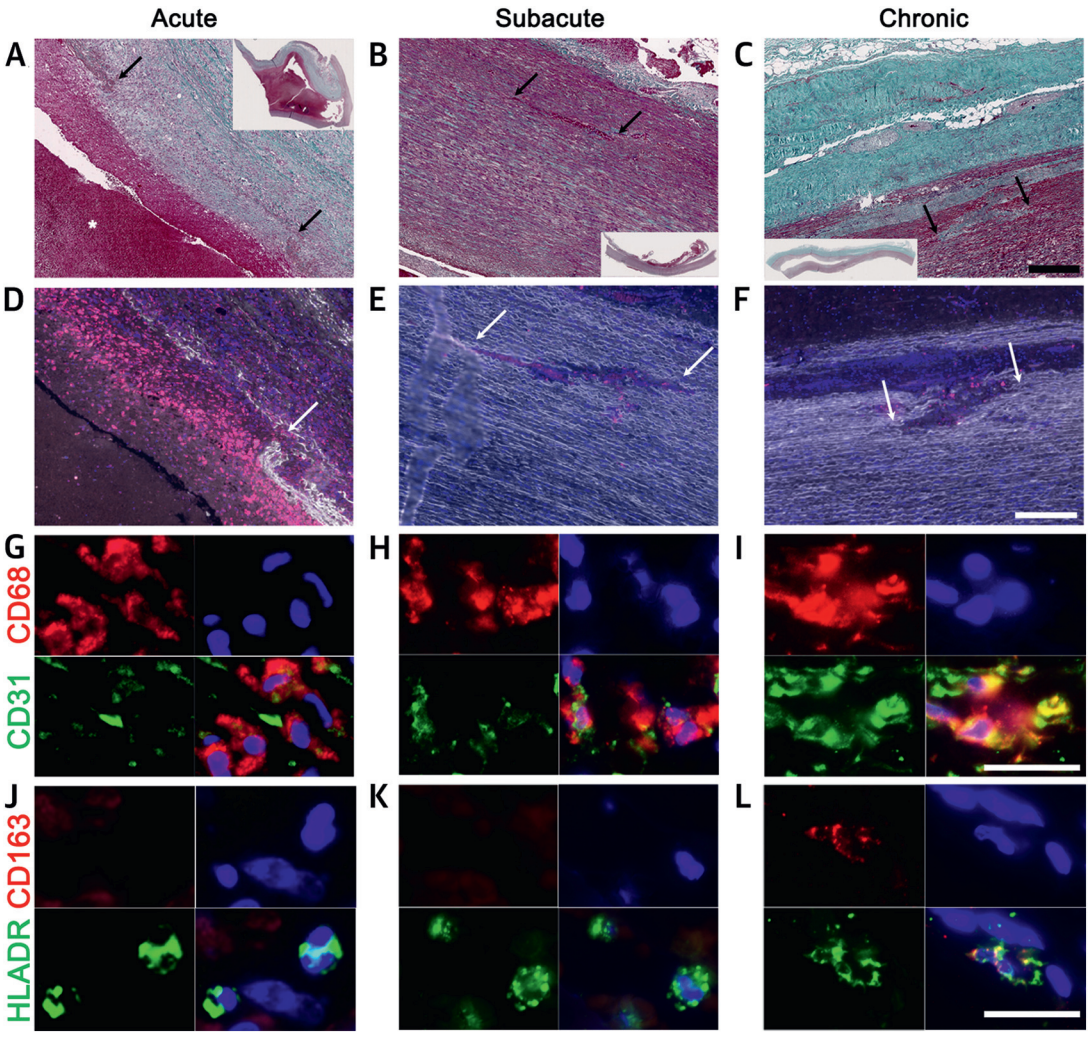
ULTRASOUND IMAGING, HISTOLOGY, IMMUNOFLUORESCENCE, AND FLOW CYTOMETRY. Ultrasound imaging (US) was used to monitor aortic diameter changes over time and heart function in experimental mice. Microscopy and flow cytometry analyses were performed as detailed in Online Appendix 1.

STATISTICAL METHODS. The results are expressed as mean ± SEM. The differences among groups were evaluated by 1-way analysis of variance with Fisher post hoc tests or by Mann-Whitney nonparametric tests, as appropriate. Any differences between groups were considered to be significant when p values were <0.05. All analyses were performed with JMP 6.0 Software (SAS Institute Inc., Cary, North Carolina).

RESULTS

PROINFLAMMATORY MACROPHAGES LACKING CD31 EXPRESSION ACCUMULATE AT THE SITE OF INJURY IN ADIM. The sites of arterial wall dissection (detected as sites of elastin rupture) were consistently associated with the presence of proinflammatory M1 macrophages in both human (**Figure 1**) and experimental ADIM (Online Figure 1), further supporting the key role played by macrophages in the pathophysiology of aortic wall injury. Of note, CD31

FIGURE 1 **Phenotype of Macrophages at Sites of Arterial Wall Disruption in ADIM**

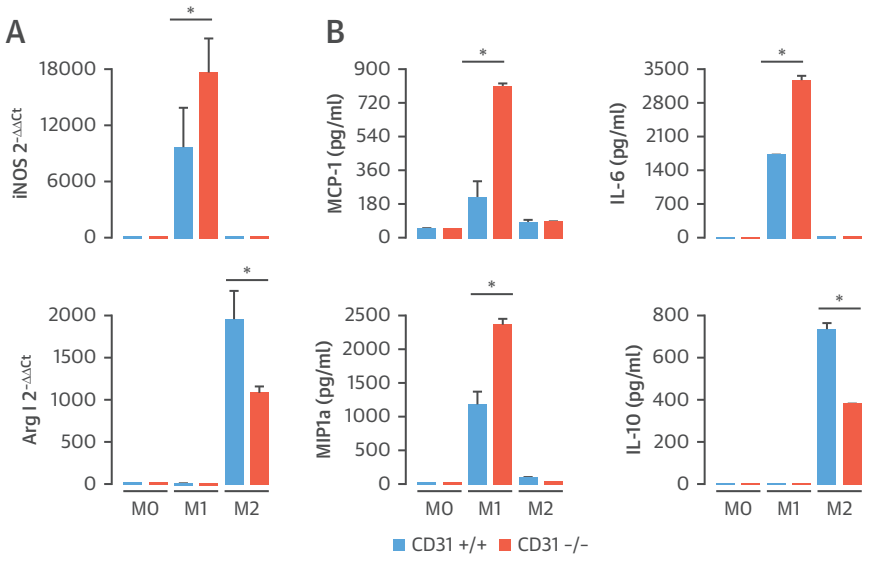


Representative examples of histological and immunofluorescence analysis of macrophages in 3 human aorta samples (of 7) obtained from surgery at different stages of healing after dissection. **(A to C)** Masson's trichrome staining, the whole section is shown in the inset. *Intramural hematoma. **Light green** indicates collagen; **deep red,** fibrin and cytoplasm; and **purple,** nuclei and elastin. **(D to F)** Immunofluorescence of the same samples (consecutive section). Macrophages are identified by positive CD68 signals **(pink)**; nuclei are counterstained in **blue**, and elastin (autofluorescence) appears **white**. **Arrows** indicate borders of elastin disruption. Scale bars: 400 μm. (**G to I)** CD31 expression **(green)**, which is virtually absent at the surface of macrophages **(red)** infiltrating acute **(G)** and subacute **(H)** dissected aortas, appears at the surface of the reparative macrophages that are present at more advanced stages of aortic healing (**I,** corresponding to the dense collagen scars evident in **C** and at the site of elastin disruption evident in **F**). Scale bar: 40 μm. **(J to L)** The phenotype of macrophages associated with acute **(J)** and subacute **(K)** stages of aortic dissection is prevalently proinflammatory, as indicated by the dense expression of human leukocyte antigen-DR (HLA-DR) **(green)** and the virtual absence of CD163 **(red)**. More advanced healing in chronic stages **(L)** is characterized by a polarization switch toward the reparative phenotype, as indicated by the progressive decrease of HLA-DR and the concomitant acquisition of CD163. Scale bar = 40 μm.

was virtually absent at the surface of the proinflammatory macrophages that had infiltrated at the site of acute injury, but its expression was readily detectable on the macrophages that had switched to the M2 phenotype of human samples taken at more advanced healing stages (Figure 1).

ABSENCE OF CD31 SIGNALING PROMOTES THE DEVELOPMENT M1-LIKE MACROPHAGES. Because CD31 expression varied according to the polarization of macrophages in vivo, we wanted to analyze the effect of CD31 on macrophage polarization in vitro. To this end, BMDMs were obtained from $CD31^{+/+}$ and

FIGURE 2 Effect of CD31 Deficiency on Macrophage Polarization



Bone marrow–derived macrophages from CD31$^{+/+}$ and CD31$^{-/-}$ mice were differentiated in vitro with M-CSF (M0) and subsequently polarized into M1 and M2 macrophages. **(A)** In the absence of CD31 signaling, macrophages are more likely to polarize toward an M1 phenotype, as indicated by a stronger expression of inducible nitric oxide synthase (iNOS) (an M1 marker). Instead, the expression of CD31 favors M2 polarization, as reflected by the expression of arginase (Arg) I (an M2 marker). **(B)** Analysis of soluble cytokines released in the supernatant from cultured macrophages showed that CD31$^{-/-}$ macrophages produced more proinflammatory cytokines (MCP-1, interleukin [IL]-6, and MIP1a) than did CD31$^{+/+}$ M1 macrophages. In contrast, the production of IL-10 in CD31$^{-/-}$ M2 macrophages was diminished compared to that in CD31$^{+/+}$ macrophages. n = 4/condition, *p < 0.05, the experiment was repeated 3 times and yielded similar results.

CD31$^{-/-}$ mice and polarized toward M1-like (lipopolysaccharide + interferon-γ) or M2-like (interleukin [IL]-4) phenotypes.

We found that the M2-polarized macrophages from CD31$^{-/-}$ mice had lower arginase I expression than did the M2 macrophages from CD31$^{+/+}$ mice. Accordingly, the expression of inducible nitric oxide synthase (iNOS) was higher in M1 macrophages in the absence of CD31 signaling than in macrophages from CD31$^{+/+}$ mice (Figure 2A).

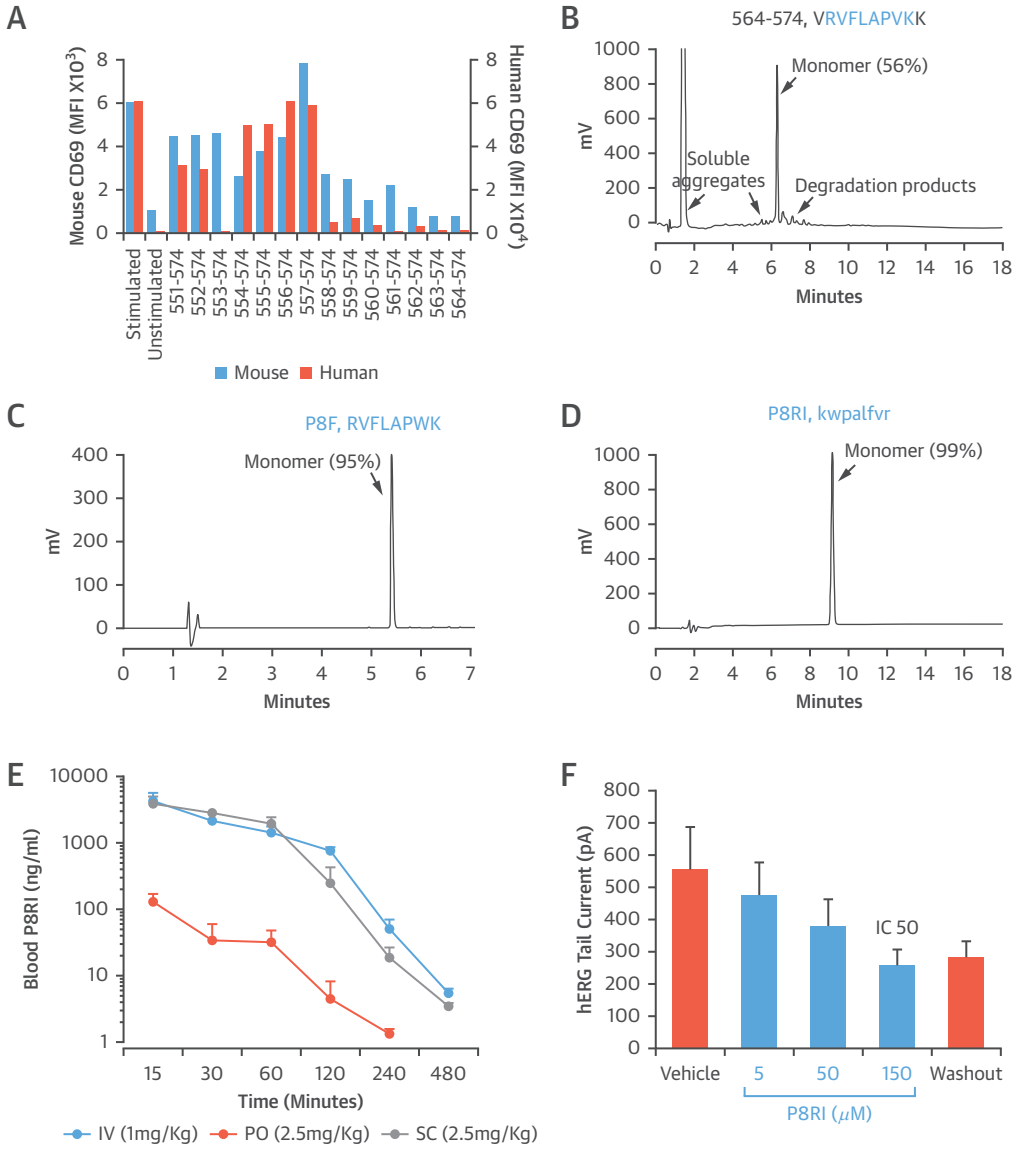
Importantly, the levels of inflammatory cytokines produced by M1 macrophages, such as MCP-1, IL-6, and MIP1α, were significantly up-regulated under M1-polarizing conditions in the absence of CD31 expression. In contrast, IL-10 secretion was reduced in the absence of CD31 expression in M1 macrophages (Figure 2B).

Therefore, the absence of CD31 potentiates and accentuates the inflammatory phenotype during M1 polarization, while it attenuates the phenotype of M2 macrophages under M2-polarizing conditions.

IDENTIFICATION OF A DRUG-SUITABLE CD31 AGONIST PEPTIDE. Because of its poor solubility in water, the parent 23-mer peptide of CD31 was not suitable for further development as a candidate drug molecule for clinical use. To identify a drug-suitable CD31 agonist peptide, we screened 2 peptide libraries derived from the human and mouse parent sequences (Figure 3A). The best candidate was found within the most proximal extracellular sequence of the CD31 molecule (aa 564-574) (Figure 3B), which is highly conserved between mice and humans. Within this conserved sequence, we identified an 8-mer peptide (P8F, L-amino acids, "forward" sequence) that was more readily soluble in water with minimal aggregated/degraded products (Figure 3C). The corresponding 8-mer retro-inverso peptide (P8RI, D-amino acids, inverse order sequence) displayed very high water solubility and a satisfying HPLC profile in saline solution (Figure 3D). P8F and P8RI peptides were as effective as the parent 23-mer peptide in reducing the extent of T-lymphocyte activation (8). Therefore, we chose peptidase-resistant P8RI for further development and preclinical studies in mice.

Based on the efficacy of the parent peptide in a previous study (9), we decided to use the same dose range and schedule (daily subcutaneous injection of a 2.5 mg/kg dose) with the newly derived sequence. The calculated bioavailability in the blood of a single

FIGURE 3 **Identification and Characterization of a Drug-Suitable CD31 Agonist Peptide**

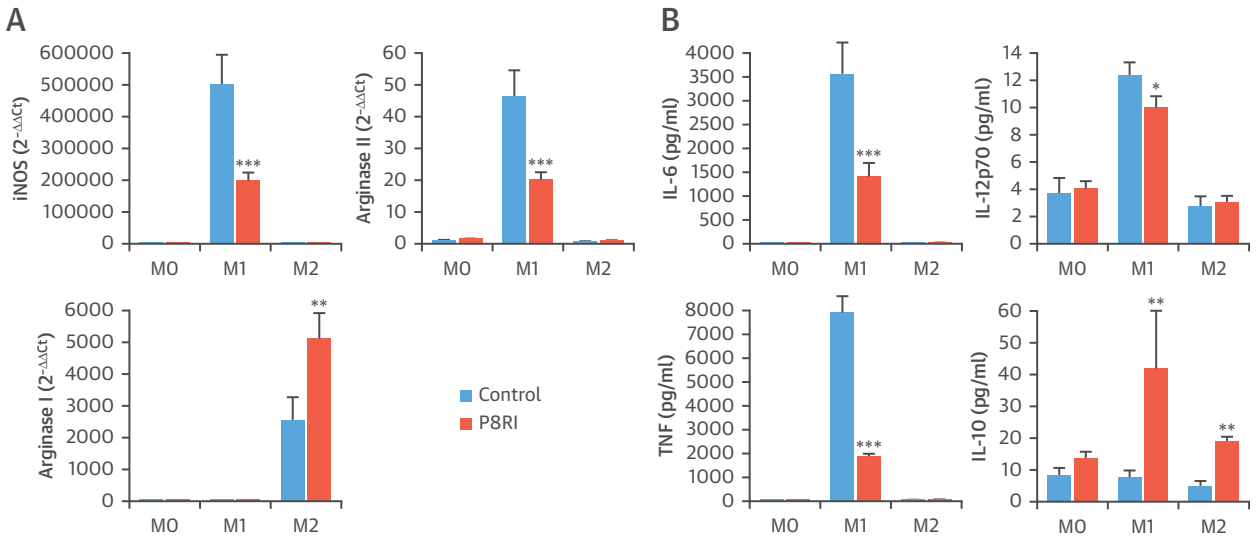


(A) The best candidate was selected from the "truncation" peptide library (see the Methods section) based on its ability to reduce both mouse and human leukocyte activation (CD69 expression). The best candidate comprised a sequence spanning from amino acid (aa) 564 to 574 (VRVFLAPVKK). **(B)** HPLC analysis of the aa 564 to 574 peptide selected in **A** displayed poor water solubility (56% monomer) and formed aggregates of variable sizes. **(C)** From the "scanning" library, 8-mer forward (P8F) sequence (aa 565-573, RVFLAPVK) derived from VRVFLAPVKK, as highlighted by the **blue letters**, displayed excellent water solubility (95%). **(D)** The corresponding retro-inverso sequence (P8RI, kwpalfvr) showed an even greater solubility (99%). **(E)** Pharmacokinetic analysis of P8RI after a single intravenous (IV), subcutaneous (SC), or oral administration (per os [PO]), at the indicated doses, in C57BL/6 mice. n = 3/route. **(F)** In vitro analysis of the human ether-a-go-go-related gene (hERG) tail current amplitude on a stable cell line showed that the IC50 of P8RI was 150 μmol/l, corresponding to 40-fold the area under the curve following an SC injection at a 2.5 mg/kg dose in mice.

subcutaneous injection was 66% with a maximal detectable concentration (Cmax) of 3.8 μg/ml (Online Table 2). The injected peptide was detectable in the plasma for up to 8 h after injection (**Figure 3E**), which supported the daily administration of the peptide.

From the perspective of preclinical and drug development studies, P8RI was subjected to absorption, distribution, metabolism, excretion, and toxicology analysis. As shown in **Figure 1F**, the IC50 of P8RI based on the human ether-a-go-go-related gene

FIGURE 4 Impact of the CD31 Agonist Peptide P8RI on Macrophage Polarization



Bone marrow–derived macrophages from CD31+/+ mice were differentiated in vitro with M-CSF and subsequently left untouched (M0) or were polarized into M1 and M2 macrophages. **(A)** The presence of P8RI significantly increased the expression of Arginase I (an M2 marker), whereas it decreased the expression of iNOS and arginase II (M1 markers), as detected by reverse transcription polymerase chain reaction analysis of adherent macrophages. **(B)** The concentrations of the soluble proinflammatory cytokine IL-6, IL-12p70, and TNF-α were significantly reduced, whereas the concentration of the pro-reparative cytokine IL-10 was increased in the supernatant of macrophages that were polarized in the presence of P8RI. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 4/condition. The experiment was repeated 3 times and yielded similar results each time. Abbreviations as in Figure 2.

tail current amplitude was 150 μg/ml, which represents an ≈40-fold Cmax of the drug in the plasma of subcutaneously injected mice. Of note, at the concentration of 5 μg/ml, which is higher than the Cmax in the blood after subcutaneous injection, the peptide has no effect on the tail amplitude of the potassium channel (Figure 3F). The product is stable in solution for up to 100 days at room temperature (Online Figure 2) and showed no genotoxicity in vitro (Online Appendix 2). The toxicology profile in vivo was excellent: no signs of systemic toxicity (blood test biochemistry) in vivo, even at doses >10-fold of those used for the experimental study (30 mg/kg/day) administered subcutaneously, daily, over 14 days (Online Appendix 3).

CD31 PEPTIDE INFLUENCES MACROPHAGE POLARIZATION TOWARD THE M2 PROFILE. We next wanted to assess whether activating CD31 signaling using peptides has an effect on the polarization of BMDMs from $CD31^{+/+}$ mice. As shown in Figure 4, P8RI significantly affected the polarization of macrophages, resulting in a dramatic reduction in M1 markers such as the expression of iNOS and arginase II and the production of IL-6, IL-12p70, and TNF-α, whereas it enhanced the M2 phenotype, as detected by an increased expression of arginase I and production of IL-10 (Figure 4).

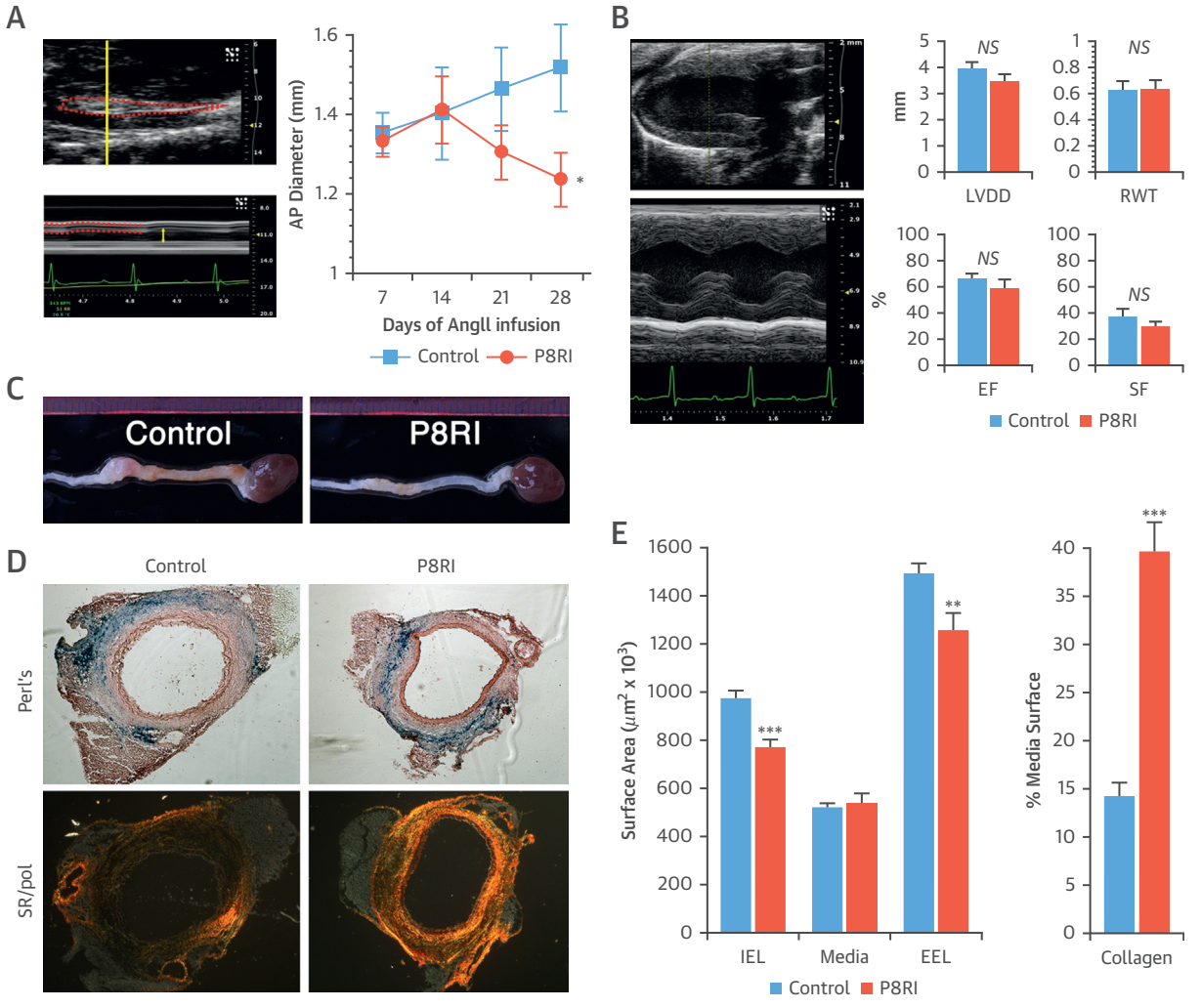
These findings prompted us to evaluate the potential therapeutic effect of CD31 agonists, such as P8RI, in the healing of experimental ADIM.

CURATIVE TREATMENT WITH P8RI PROMOTES THE HEALING OF EXPERIMENTAL DISSECTING ANEURYSM IN ATHEROSCLEROTIC MICE. All mice were subjected to abdominal aorta US imaging weekly starting from day 7 after the implantation of the osmotic pump delivering Ang II. During the last 14 days of Ang II infusion (i.e., well after the establishment of aortic dissection, which occurs within the first 7 days [10,11]), mice displaying signs of acute dissection and intramural hematoma (Figure 5A) were randomly assigned to 1 of 2 groups (n = 10 mice/group) to receive a daily subcutaneous injection of either P8RI (2.5 mg/kg/day) or the vehicle ("control").

Heart function evaluated by US imaging on day 28 was not affected by the treatment (Figure 5B). This enabled us to specifically study the overall effect of the peptide on aortic tissue repair and clinical outcome of experimental ADIM.

Aneurysmal transformation was significantly reduced by P8RI treatment, as illustrated by

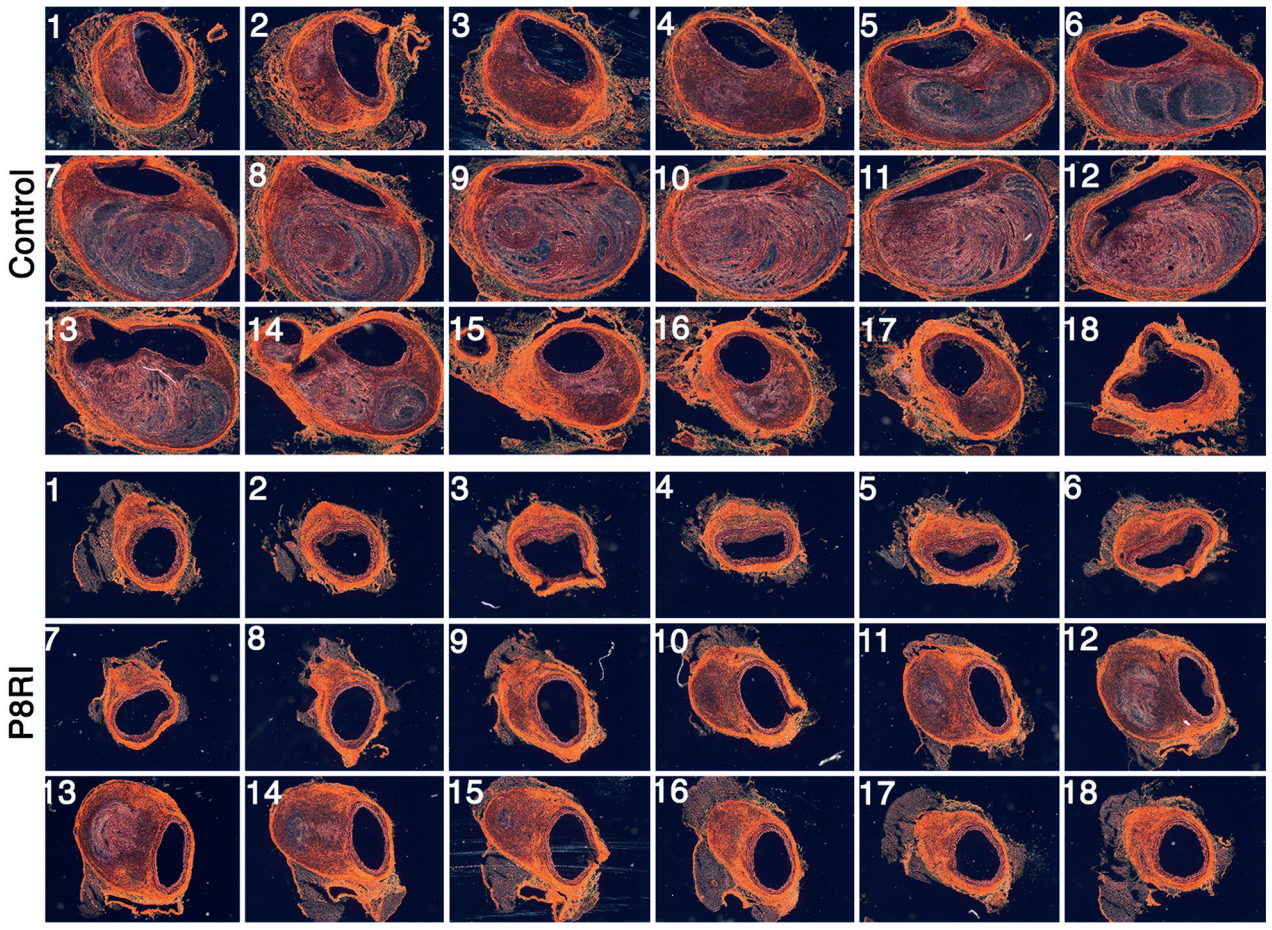
FIGURE 5 Therapeutic Potential of P8RI: Functional and Morphometric Analysis on the Outcome of Experimental ADIM



(A) Noninvasive ultrasound (US) suprarenal aorta imaging in experimental mice. **Red dashed line** indicates intramural hematoma. The anteroposterior (AP) diameter of dissected aortas was measured in time mode (TM) along the **yellow line** traced on the 2-dimensional (2D) inset, as indicated by **the yellow double arrow in the bottom panel**. Quantitative analysis at the indicated time points showed that the dissected aorta progressively enlarged from days 7 to 28 in the control mice **(black squares)** whereas it was halted by the administration of P8RI **(green circles)**, which was started at day 14 **(black arrow). (B)** US evaluation of cardiac function at day 28 was carried out using TM measures in parasternal long-axis windows as shown in the representative 2D and TM examples. Quantification of the data showed no differences between the 2 groups in terms of left ventricle diastolic diameter (LVDD), relative wall thickness (RWT), ejection fraction (EF) (modified quinones equation), and shortening fraction (SF). **(C)** Representative examples of the macroscopic analysis (day 28). ADIM segment appears thinner and limited to the suprarenal aorta in P8RI-treated mice, whereas the whole aorta appears remodeled and the bulging due to the intramural hematoma still persists at the dissection site in the control mice. **(D)** Cross sections of aortic dissecting aneurysm stained with Perl's (site of intramural hematoma) and with Sirius Red (SR) (collagen analysis). SR staining was evaluated by polarized (pol) light microscopy and computer-assisted analysis. Collagen content **(red staining)** was quantified within the media, defined as the area between the internal elastic laminae (IEL) and the external elastic laminae (EEL). **(E)** The surface area within the IEL (lumen) and EEL (lumen + media) were significantly smaller, confirming the beneficial effect of P8RI in reducing the dilative remodeling of the dissected segments. More collagen, reflecting advanced healing, was detected in the P8RI group. n = 10/group. Cumulative data from 18 sections from each mouse were used as continuous variables for the statistical evaluation of differences among the groups. **p < 0.01, ***p < 0.001.

macroscopic, US, and computer-assisted morphometric analysis (Figures 5A to 5C). Of note, at sites showing a similar degree of intramural hematoma, as indicated by Perl's blue-stained ferritin deposits, a greater extent of collagen bundles was evident in the P8RI group, and quantitative analysis showed that this improved arterial wall healing was associated with the prevention of aneurysmal enlargement of

FIGURE 6 Morphological Appearance of Dissecting Aortic Segments in Experimental Mice



Representative examples of SR/pol analysis across the entire pseudoaneurysm (consecutive cross sections cut at 200-μm intervals and numbered from 1 to 18). The relative paucity of collagen was associated with the persistence of the intramural hematoma, which was consistently bigger and multilayered (reflecting growth by iterative blood entry and coagulation phases) in the control mice compared with that in P8RI-treated mice. The experiment was repeated 4 times (n = 10 mice/group in each experiment) and yielded similar results each time.

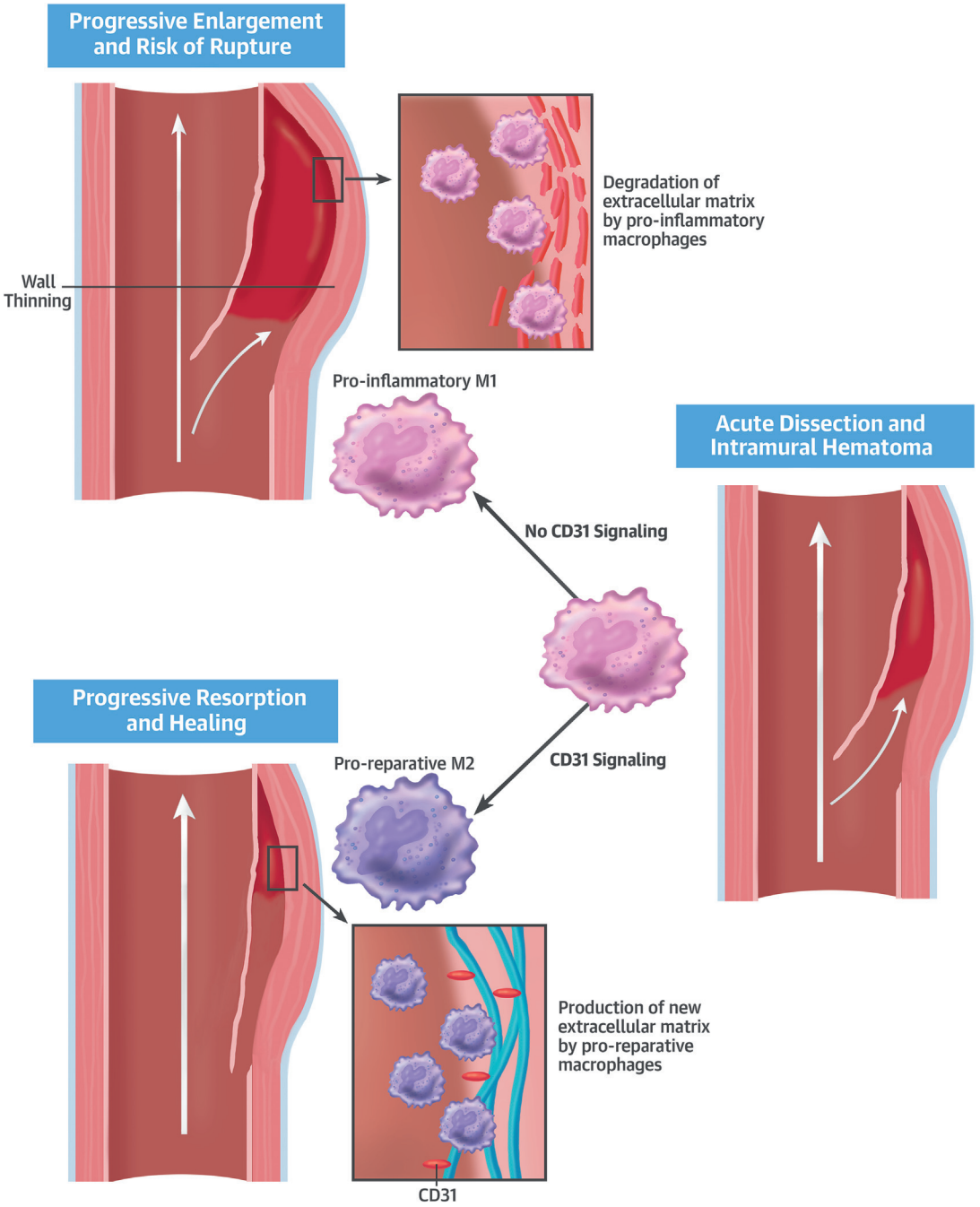
dissected aortas, as evidenced by morphometric analysis (**Figures 5D to 5E**).

Furthermore, blood analysis showed limited signs of internal hemorrhage (relatively higher red blood cell count and hemoglobin concentration) (Online Table 3), and the morphological analysis of Sirius Red–stained microscopic slides showed that more extended and multilayered residual intramural hematomas within the dissected segment were consistently observed in control mice. Overall, these observations support a favorable effect of CD31 agonist molecules on the resorption of injured tissues and the limitation of blood entry in the false lumen following the occurrence of dissecting aneurysms (**Figure 6, Central Illustration**).

Plasma biomarkers of renal (creatinine, urea) or of heart (creatinine kinase-MB) damage were not affected by the treatment (Online Table 3).

TISSUE HEALING PROPERTIES OF P8RI ARE ASSOCIATED WITH THE M2-TYPE OF MONOCYTES/MACROPHAGES. Blood formula revealed a global reduction of the neutrophil count in P8RI-treated mice, in agreement with a blunted inflammatory response (Online Table 3). Proinflammatory M1 monocytes/macrophages are essential for the initiation of wound healing, but these cells must switch to a pro-reparative phenotype for successful tissue repair. Circulating monocytes and resident macrophages, which contribute to these sequential tissue healing phases, were analyzed at the end of the study period,

CENTRAL ILLUSTRATION **CD31 Signaling Favors the Switch of Injury-Associated Macrophages From the Proinflammatory M1 to the Reparative M2 Phenotype**



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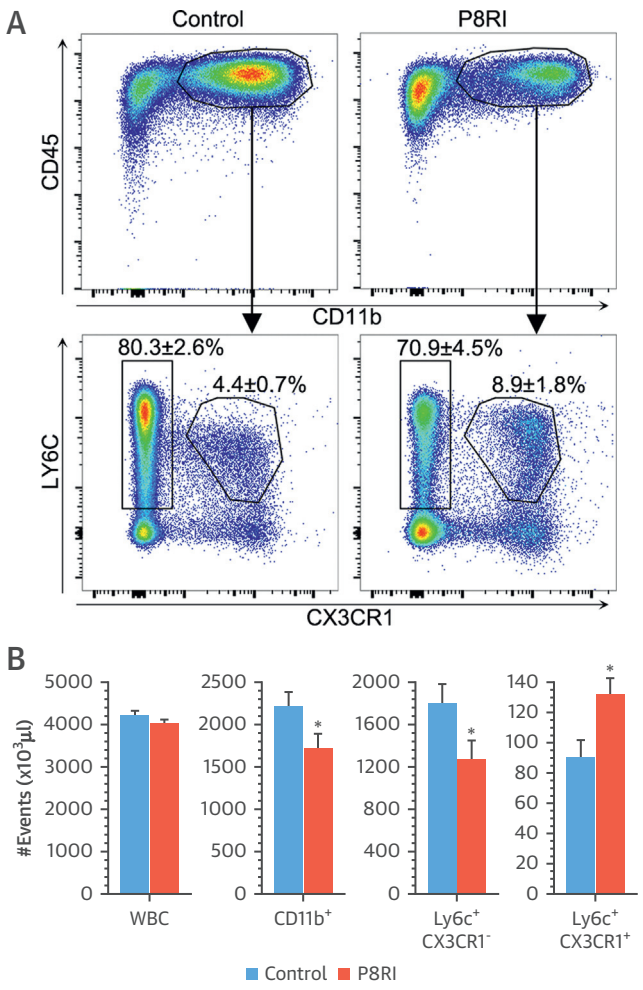
Upon the occurrence of an acute aortic dissection and intramural hematoma, the stabilization of the aortic wall by prompt cessation of matrix-degrading processes and by the production of new extracellular matrix is essential for preventing the thinning of the outermost wall layer, which can rupture due to the pressure of the blood flow in the false lumen. The degradation of the extracellular matrix **(red, hashed wavy lines)** is driven by the action of pro-inflammatory **(violet)** macrophages during the early "demolition" phase of tissue healing. Upon completion of this phase, the production of new extracellular matrix **(green wavy lines)** requires the transition of injury-associated macrophages to a reparatory phenotype **(blue)**. Our in vitro data suggest that this transition is hampered by the invalidation of CD31 signaling, but it is favored by suitable CD31-agonist drugs. The latter species were able to promote aortic wall healing and limit aneurysmal transformation in an experimental model of acute aortic dissection.

FIGURE 7 Flow Cytometry Analysis of Peripheral Blood CD45+ Mononuclear Cells



(A) Representative density plots and gating strategy for the cytometric analysis of peripheral blood mononuclear leukocytes, in control and P8RI-treated mice. **(B)** Quantitative analysis on CD45+ events (white blood cells, equal number of acquired events). Treatment with P8RI was associated with a significant reduction in circulating monocytes (CD11b+, myeloid origin), which was essentially due to a reduction in the main subpopulation (i.e., the pro-inflammatory [M1, Ly6C+/CX3CR1−] cells). Contrarily, the pro-reparative (M2, Ly6C+/CX3CR1+) monocyte population (representing <10% of peripheral blood monocytes) was increased in P8RI-treated mice. The experiment was repeated 4 times and yielded similar results each time. n = 10 mice/group; *p < 0.05.

when most of the monocytes/macrophages should have assumed the reparative phenotype.

Flow cytometric analysis of peripheral blood monocytes (CD45+CD11b+ mononuclear cells) revealed that the proportion of pro-reparative Ly6C+CX3CR1+ cells was significantly increased in the circulation of P8RI-treated mice (Figure 7). In contrast, the proinflammatory Ly6C+CX3CR1− cells (accounting for >90 peripheral blood monocytes) were significantly reduced in P8RI-treated mice, resulting in a decrease in the total circulating monocyte (CD11b+, mononuclear) number (Figure 7).

More importantly, at day 28, the vast majority of CD11b+ macrophages infiltrating the dissecting aneurysmal tissue expressed arginase I—which is predominantly expressed by M2 macrophages—in tissues derived from P8RI-treated mice, whereas the presence of iNOS+ M1 macrophages could only be detected in control mice (Figure 8).

Therefore, following the occurrence of an acute aortic dissection induced by Ang II infusion in Apo $E^{-/-}$ mice, the beneficial effects on aortic repair of the curative treatment with a CD31 agonist could be explained, at least in part, by the role of the agonist in the polarization of monocytes/macrophages from a proinflammatory to a pro-reparative phenotype.

DISCUSSION

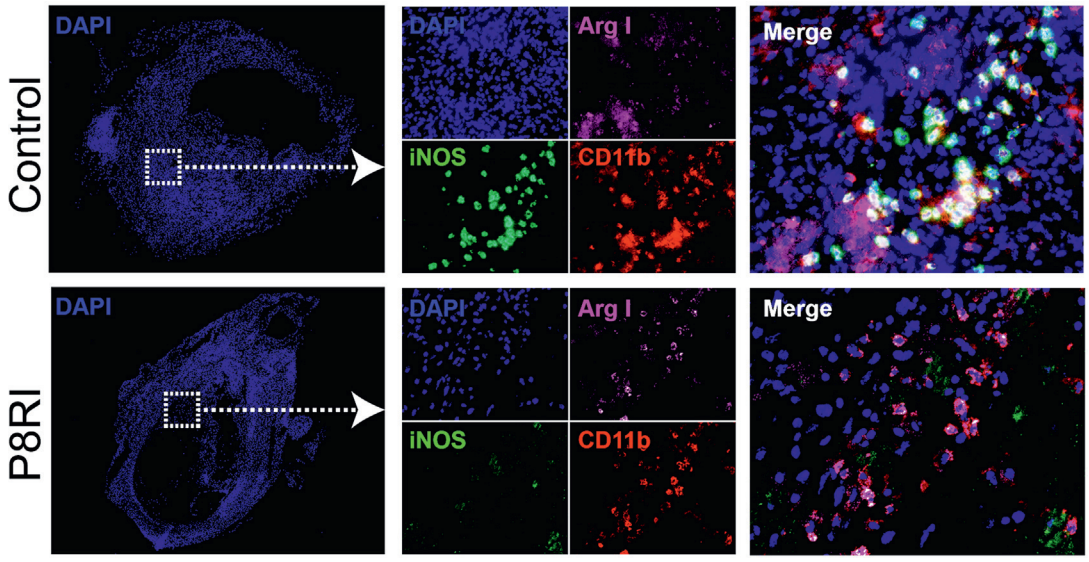
Appropriate arterial healing is crucial for the homeostasis of the circulatory system. Indeed, the arterial wall is continuously subjected to mechanical stress, which eventually causes focal injury such as intimal flaps that can give rise to intramural hematoma and/or arterial wall dissection. Although extensive work has been done in the field of skin and other soft tissue injury, little is known about the factors that can favor or hamper the healing of arterial wall injuries.

The analysis of ADIM samples from patients undergoing surgical aortic repair showed that the expression of CD31 is lost by the proinflammatory M1 macrophages that densely infiltrate the sites of acute arterial wall lesions, whereas CD31 re-expression accompanies the appearance of M2 and the disappearance of M1 macrophages at the sites of effective arterial wall healing, suggesting a role for CD31 in aortic tissue healing following ADIM (Central Illustration).

The chronic Ang II infusion model in Apo $E^{-/-}$ mice is widely used in preclinical studies to explore the therapeutic potential of novel treatments (12). The apparent luminal dilatation of the abdominal aorta due to the chronic Ang II infusion in aged male Apo $E^{-/-}$ mice (13) indeed reproduces the features of ADIM in humans, despite some specific differences (suprarenal instead of infrarenal injury because of the rupture of an aortic branch and the formation of intramural hematoma) (6).

Previously, we found that the administration of a CD31 agonist peptide could dramatically reduce the occurrence of dissection and intramural hematoma in this model (9). The administered peptide could bind to wound-associated leukocytes, including

FIGURE 8 Macrophage Phenotype Analysis by Immunofluorescence Microscopy in Experimental Dissecting Aneurysms



Representative immunofluorescence analysis of CD11b+ **(red)**, iNOS+ **(green)**, and arginase I+ **(pink)** leukocytes in dissecting aneurysms from control and P8RI-treated mice. Reparative, arginase I+ M2 macrophages were detectable in both groups, but at variance with the control mice, tissue-infiltrated macrophages (mononuclear CD11b+ cells) were virtually all negative for the M1 proinflammatory marker iNOS in P8RI-treated mice. The **magnified insets** were imaged within the site of elastin disruption in pseudoaneurysm cross sections **(white dashed boxes)**. The analysis was carried out on 4 sections from each pseudoaneurysm in 3 series of in vivo experiments.

macrophages. The role of macrophages in this model is dual, however, as macrophages are involved both in the initiation (10) and in the outcomes of events that lead to aneurysmal transformation (14). Thus, the prophylactic administration of the peptide might have prevented the initiation of the pathological processes rather that exerting an effect on the subsequent phases of wound healing in our previous work (9).

In the present study, the CD31 agonist was optimized for further clinical development and used as a curative treatment schedule with the treatment starting *after* the occurrence of the aortic complication, allowing us to specifically evaluate the therapeutic potential of CD31 agonists in arterial wound repair.

The protective effect of CD31 signaling in arterial wound healing most likely comprises multiple molecular and cellular targets involving several factors that affect the outcomes of tissue repair. We found that M1 macrophages consistently accumulate at the site of arterial injury both in patients' and in experimental mouse ADIM samples and that their persistence is associated with an unresolved intramural hematoma and defective collagen deposition at the wall of injured aortas. Whereas M1 macrophages are thought to be necessary for the clearance of neutrophils that first enter wounds in the early phases of healing (4), consistent evidence points at a crucial role for the switching of wound-associated macrophages from a proinflammatory M1 to a pro-reparative M2 phenotype to drive the resolution of the inflammatory phase and facilitate the wound healing process (4).

M2 macrophages are frequently found within human aneurysmal samples (15); however, to the best of our knowledge, the role of the M1 to M2 switch has never been studied in ADIM. Experimental studies show that the presence of blood, as observed in the case of intramural hematoma, drives an unrestrained proinflammatory M1 macrophage population that impairs wound healing (5). Moreover, in the context of experimental atherosclerosis, we previously found that the switch from an M1 to M2 phenotype is beneficial for the outcomes of vascular lesions (16).

Importantly, we found that CD31 and M2 marker expression at the surface of tissue-infiltrated macrophages increases with the healing stage following ADIM in patients. Furthermore, our in vitro data clearly show that CD31 signaling plays a direct and key role in the M1 to M2 phenotype switching of macrophages.

Beyond its role as an endothelial marker (17), CD31 is receiving increasing attention for its function as an immunoreceptor tyrosine-based inhibitory motif-bearing receptor (18), indicating its potential as a promising therapeutic target in inflammatory diseases (19). In the regulation of macrophage functions, CD31 signaling may play a crucial role in the resolution of inflammation, because, unlike other immunoreceptor tyrosine-based inhibitory motif-bearing receptors, it is constitutively expressed by both macrophages and neutrophils and because its trans-homophilic engagement is indispensable for the efficient engulfment of dead neutrophils (20), which is a prerequisite for favorable tissue repair irrespective of the injured organ.

STUDY LIMITATIONS. In its present form, the CD31 agonist that we have developed can be administered through an intravenous or subcutaneous injection. These administration routes are not suitable for prolonged treatment, but are compatible with in-hospital or short treatment regimens. Further studies in complementary experimental models are warranted from the perspective of using this therapeutic strategy in human ADIM, but the results of the present study strongly suggest that a treatment lasting 14 days can be sufficient to favor the repair and prevent subsequent aneurysmal transformation of an acute dissecting aortic wound.

CONCLUSIONS

We have identified a drug-suitable CD31 agonist peptide that favors the polarization of macrophages toward a reparative phenotype and promotes the healing of experimental ADIM.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Proinflammatory macrophages contribute to the adverse remodeling responsible for the expansion of dissecting aortic aneurysms that typically precedes rupture. In a mouse model of dissecting aortic aneurysm, a CD31 agonist peptide (P8RI) enhanced reparative macrophage function and reduced the rate of segmental aneurysm expansion.

TRANSLATIONAL OUTLOOK: Further investigation should focus on the regulatory mechanisms responsible for molecular switching of macrophage polarization in animal models and patients with acute arterial injury.

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KEY WORDS aortic dissection, CD31, drug discovery, intramural hematoma, macrophages, peptides

APPENDIX For an expanded Methods section as well as supplemental figures and tables, please see the online version of this paper.