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Antoine Caillon, Céline Grenier, Linda Grimaud, Emilie Vessieres, Anne-Laure Guihot, et al.. The angiotensin II type 2 receptor activates flow-mediated outward remodelling through T cells-dependent interleukin-17 production. *Cardiovascular Research*, 2016, 112 (1), pp.515 - 525. 10.1093/cvr/cvw172 . inserm-01382460

**HAL Id: inserm-01382460**

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Submitted on 17 Oct 2016

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# The angiotensin II type 2 receptor activates flow-mediated outward remodelling through T cells-dependent interleukin-17 production

Antoine Caillon<sup>1,2†</sup>, Céline Grenier<sup>1†</sup>, Linda Grimaud<sup>1</sup>, Emilie Vessieres<sup>1,3</sup>, Anne-Laure Guihot<sup>1</sup>, Simon Blanchard<sup>2,4</sup>, Eric Lelievre<sup>1</sup>, Marie Chabbert<sup>1</sup>, Etienne D. Foucher<sup>2</sup>, Pascale Jeannin<sup>2,4</sup>, Céline Beauvillain<sup>2,4</sup>, Pierre Abraham<sup>1,5</sup>, Laurent Loufrani<sup>1</sup>, Yves Delneste<sup>2,4</sup>, and Daniel Henrion<sup>1,3,5\*</sup>

<sup>1</sup>MITOVASC Institute, UMR CNRS 6214, INSERM U1083, Angers University, F-49045 Angers, France; <sup>2</sup>UMR CNRS 6299, UMR INSERM 892, Angers University, F-49045 Angers, France; <sup>3</sup>Cardiovascular Functions In Vitro (CARFI) Facility, Angers University, F-49045 Angers, France; <sup>4</sup>Department of Immunology and Allergology, University Hospital, F-49045 Angers, France; and <sup>5</sup>Department of Vascular Medicine, University Hospital, F-49045 Angers, France

Received 1 December 2015; revised 18 May 2016; accepted 9 June 2016; online publish-ahead-of-print 21 June 2016

Time for primary review: 38 days

## Aims

The angiotensin II type 1 receptor (AT1R) through the activation of immune cells plays a key role in arterial inward remodelling and reduced blood flow in cardiovascular disorders. On the other side, flow (shear stress)-mediated outward remodelling (FMR), involved in collateral arteries growth in ischaemic diseases, allows revascularization. We hypothesized that the type 2 receptor (AT2R), described as opposing the effects of AT1R, could be involved in FMR.

## Methods and results

We studied FMR using a model of ligation of feed arteries supplying collateral pathways in the mouse mesenteric arterial bed *in vivo*. Seven days after ligation, diameter increased by 30% in high flow (HF) arteries compared with normal flow vessels. FMR was absent in mice lacking AT2R. At Day 2, T lymphocytes expressing AT2R were present preferentially around HF arteries. FMR did not occur in athymic (nude) mice lacking T cells and in mice treated with anti-CD3ε antibodies. AT2R activation induced interleukin-17 production by memory T cells. Treatment of nude mice or AT2R-deficient mice with interleukin-17 restored diameter enlargement in HF arteries. Interleukin-17 increased NO-dependent relaxation and matrix metalloproteinases activity, both important in FMR. Remodelling of feeding arteries in the skin flap model of ischaemia was also absent in AT2R-deficient mice and in anti-interleukin-17-treated mice. Finally, remodelling, absent in 12-month-old mice, was restored by a treatment with the AT2R non-peptidic agonist C21.

## Conclusion

AT2R-dependent interleukin-17 production by T lymphocyte is necessary for collateral artery growth and could represent a new therapeutic target in ischaemic disorders.

## Keywords

Angiotensin II type 2 receptor • Interleukin-17 • Blood flow • Collateral artery growth

## 1. Introduction

Tissue perfusion following an ischaemic injury is increased by arteriogenesis, which results in the enlargement of native collateral arteries, or by *de novo* formation of arterioles.<sup>1</sup> Numerous clinical attempts to increase angiogenesis in patients with ischaemic disease by the administration of growth factors have failed,<sup>2</sup> leading to the increased recognition that strategies to improve tissue perfusion need to focus on increasing the extent of the collateral circulation and arteriogenesis.<sup>3</sup>

Flow (shear stress)-mediated outward remodelling of small arteries has a key role in collateral arteries growth.<sup>1</sup> Besides ischaemic disorders, it is also involved in somatic growth, in uterin arteries enlargement during pregnancy, in the vascular adaptation to exercising, and in tumour growth.<sup>1</sup> This remodelling requires perivascular macrophages accumulation during the early stage (first 2 days) following a chronic increase in blood flow in mesenteric resistance arteries,<sup>4</sup> and macrophage inactivation with clodronate prevents flow-mediated outward remodelling.<sup>5,6</sup> Hind limb revascularization following femoral artery

\* Corresponding author: BNMI, Faculté de Médecine, 49045 Angers, France. Tel: +33 2 41 73 58 45; fax: +33 2 41 73 58 95, E-mail: daniel.henrion@inserm.fr

† The first two authors contributed equally to the study.

ligation is strongly reduced in CD4<sup>-/-7</sup> or in CD8<sup>-/-</sup> mice,<sup>8</sup> whereas in natural killer cell-deficient mice,<sup>7</sup> regulatory T cell depletion increased post-ischaemic revascularization.<sup>9</sup> These studies suggest a role for T lymphocytes activation in collateral artery growth. Interleukin-17 (IL-17) has also been proposed to exert a protective effect in post-ischaemic revascularization.<sup>10</sup>

Angiotensin II is a pleiotropic growth hormone involved in most cardiovascular and metabolic disorders, with major effects on vascular hypertrophy, fibrosis, oxidative stress, endothelial dysfunction, and inflammation.<sup>11</sup> Interestingly, T cells possess a full renin–angiotensin system, and they express both angiotensin II type 1 and type 2 receptors (AT1R and AT2R, respectively).<sup>12</sup> Furthermore, endogenous angiotensin II increases T-cell activation through both AT1R and AT2R.<sup>13</sup>

Most of the vascular growth and inflammatory effects of angiotensin II are attributed to AT1R.<sup>14</sup> On the other hand, although largely unexpressed in adult tissues, AT2R appears at significant levels after vascular injury in stroke,<sup>15</sup> heart failure,<sup>16</sup> and heart attack,<sup>17</sup> and it is usually described as opposing the effects of AT1R.<sup>18</sup> Furthermore, following myocardial infarction in the rat, AT2R was detected in T cells in the peri-infarct area where it may provide myocardial protection.<sup>19</sup> Thus, we hypothesized that AT2R could play a role in flow-mediated outward remodelling of resistance arteries, involved in arteriogenesis and revascularization.

We investigated flow-mediated outward remodelling of resistance arteries using an *in vivo* model allowing to submit chronically 1 second-order mesenteric resistance artery to an increase in blood flow without systemic change in pressure or inflammation. Furthermore, other second-order arteries, not affected by the local change in blood flow, can be used as control vessels.<sup>4</sup>

## 2. Methods

An expanded Methods section is available in the Supplementary material online.

### 2.1 Animal protocols

Three-month-old mice (Charles River, L'Arbresles, France) were submitted to surgical procedures to increase locally blood flow in the mesenteric circulation or in the skin, as described below. Investigations were performed in agreement with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes (authorization of the laboratory # 00577). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC); Committee on the Ethics of Animal Experiments of 'Pays de la Loire' (CEEA, permits # CEEA PdL 2012.118 and 2012.141). Animals were anaesthetized with isoflurane (2.5%) and treated with buprenorphine (Temgesic<sup>®</sup>; 0.1 mg/kg, s.c.) before and after surgery.

### 2.2 Chronic increase in blood flow in mesenteric arteries

Three consecutive first-order arteries, equivalent in size, were used. Ligatures were applied to 4 second-order arterial branches. The artery located between the two ligated arteries was designed as high flow (HF) artery. Arteries located at distance from the ligated arteries were used as control [normal flow (NF)].<sup>20</sup>

### 2.3 Skin flap procedures

The skin ischaemia model consists of a U-shaped peninsular skin incision on the dorsal surface of the mouse. The vascular remodelling was evaluated to

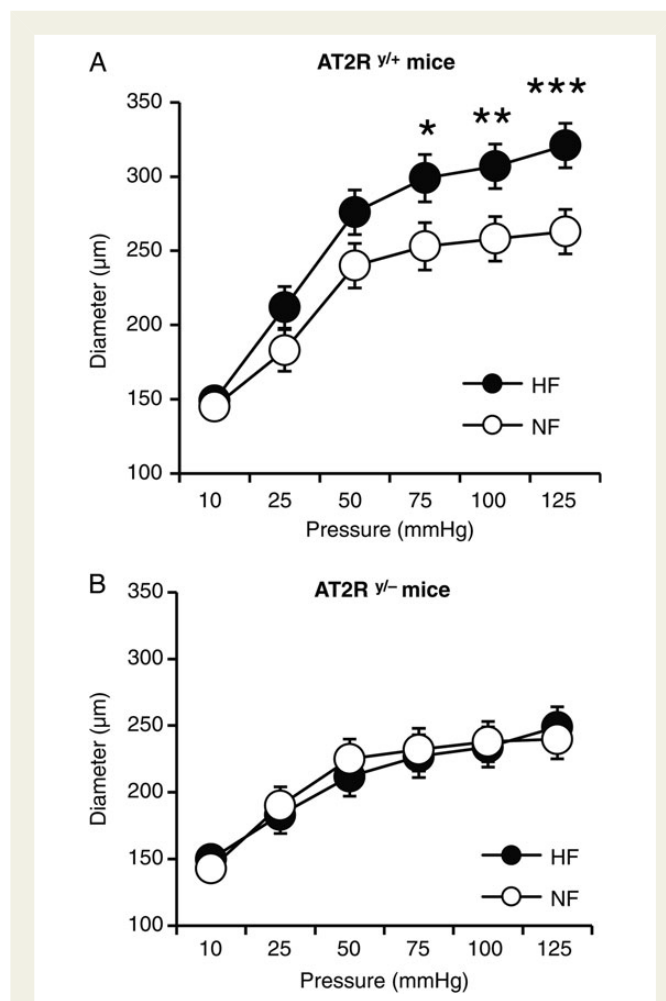
compare the right and left vascular pedicles arising from the lateral thoracic arteries at Day 0 and Day 21.<sup>21</sup>

### 2.4 Pressure–diameter relationship in mesenteric arteries *in vitro*

Arterial segments were cannulated at both ends and mounted in a video-monitored perfusion system (Living System, LSI, Burlington, VT) as previously described.<sup>22</sup>

### 2.5 Effect of IL-17 on arterial contractility and endothelium-dependent relaxation

Segments of mesenteric arteries (2 mm long each) were dissected and mounted in a wire myography (Danish Myo-technology, DMT, Copenhagen, DK).<sup>23</sup> Cumulative concentration–response curves to phenylephrine (0.001–10  $\mu\text{mol/L}$ ) and acetylcholine (0.01–10  $\mu\text{mol/L}$ ) were performed after 1 h incubation with IL-17A (10 ng/mL) or with the vehicle. Cumulative concentration–response curves to acetylcholine were obtained before and after incubation (20 min) with the NO-synthase inhibitor L-NAME (10  $\mu\text{mol/L}$ ). Acetylcholine-dependent relaxation was performed after the precontraction of the arteries with phenylephrine and serotonin to 70% of their maximal contractile response.<sup>24</sup>



**Figure 1** Flow-mediated outward remodelling of mesenteric arteries. Arterial diameter was measured 1 week after arterial ligation in HF and NF arteries isolated from WT (AT2R<sup>+/+</sup>) mice (A) and AT2R<sup>-/-</sup> mice (B). Mean  $\pm$  SEM is presented ( $n = 8$  mice per group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , HF vs. NF.

## 2.6 Quantitative real-time PCR

The levels of mRNA encoding selected proteins in HF mesenteric arteries from wild-type (WT) mice and AT2R<sup>-/-</sup> mice after 2 days of ligation were determined by real-time quantitative polymerase chain reactions (qPCRs), as previously described.<sup>6</sup>

## 2.7 Cell culture and activation of immune cells

Immune cells were isolated from lymph nodes and spleen of WT mice by grinding and washed by centrifugation. CD4<sup>+</sup> T cells were isolated by positive selection before purification of CD44<sup>high</sup>CD62<sup>low</sup> memory T cells and CD44<sup>low</sup>CD62L<sup>high</sup> naive T cells by fluorescence-activated cell sorting; purity was >99%, as assessed by flow cytometry.

Naive or memory CD4<sup>+</sup> T cells ( $1 \times 10^5$ ), cultured in X-VIVO 15 medium (Lonza, Basel, Switzerland), were stimulated with immobilized anti-CD3 mAb (145-2C11) and 1  $\mu$ g/mL anti-CD28 mAb (eBioscience). Cells were stimulated with angiotensin II, the non-peptidic AT2R agonist C21 (gift of Vicore Pharma, Sweden) or the peptidic AT2R agonist CGP42112 ( $10^{-8}$  M, Sigma-Aldrich). IL-6 plus TGF $\beta$  (respectively, 200 and 2 ng/mL) and IL-23 (200 ng/mL) were used as positive controls to induce IL-17A production by naive and memory T cells, respectively.

## 2.8 Flow cytometry analysis

Arteries with the surrounding perivascular tissue (including mainly fat tissue) were isolated and digested using 450 U/mL collagenase type I and 5 U/mL elastase for 1 h at 37°C, with intermittent agitation. Cell suspensions were then passed through a 100  $\mu$ m diameter filter before washing by centrifugation (800 g). Cells were fixed with 1% (v/v) paraformaldehyde. After washing, cells were stained for 25 min at 4°C with anti-CD3,

anti-CD45, anti-ROR $\gamma$ t, anti-T-bet, and anti-CD4 monoclonal antibodies (eBioscience) or isotype control monoclonal antibodies. Fluorescence was analysed on a LSR-II flow cytometer with DIVA software driving (Becton Dickinson Biosciences, Franklin Lakes, NJ). Data were analysed using the FlowJo software (Tree Star Inc., Ashland, OR).

## 2.9 Western-blot analysis and zymographic analysis of matrix metalloproteinases activity

See Material and Methods in the Supplementary material online.

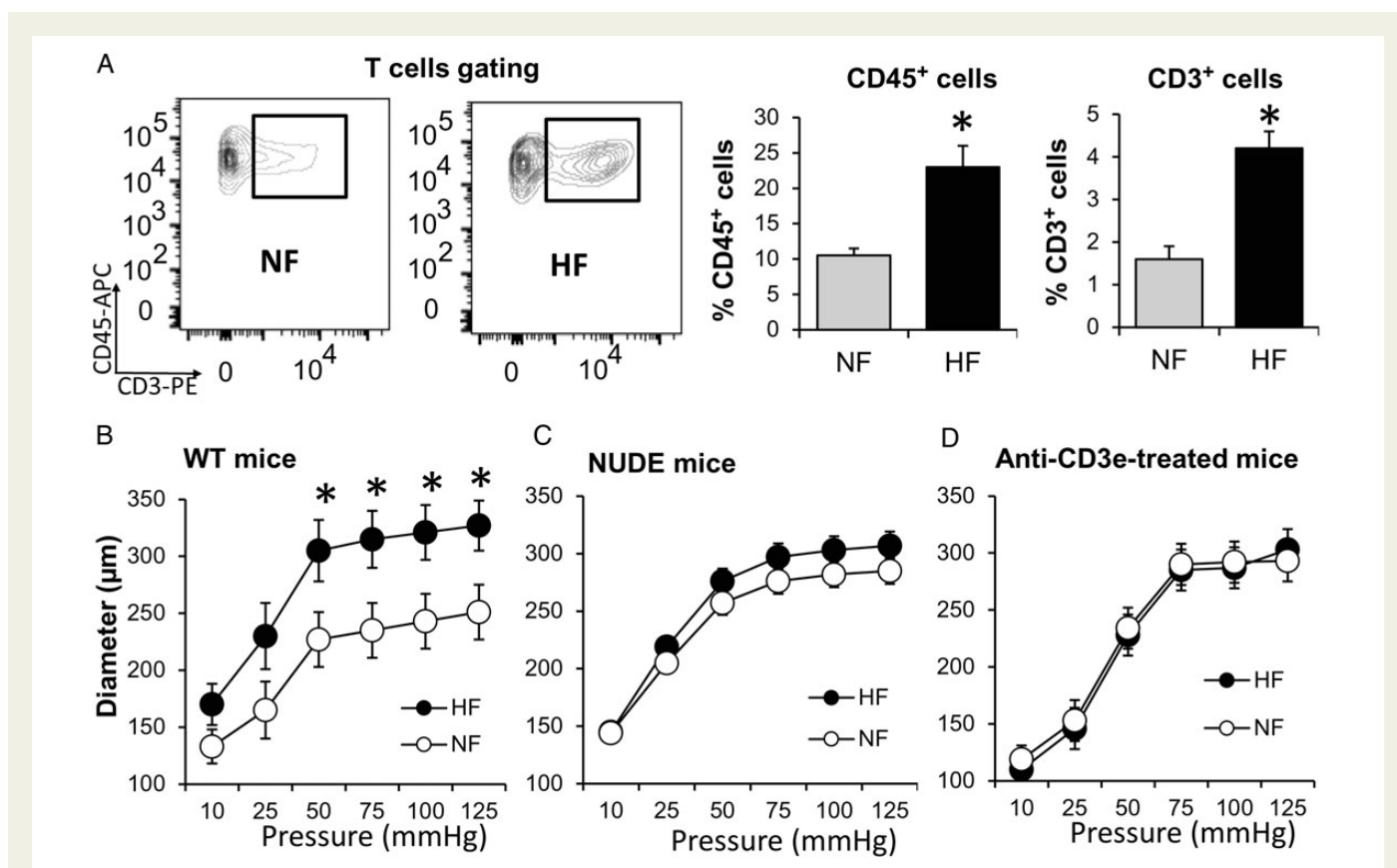
## 2.10 Statistical analysis

Results were expressed as means  $\pm$  SEM. Significance of the differences between groups was determined using two-way ANOVA for consecutive measurements (pressure–diameter curves and for concentration–response curves to acetylcholine and phenylephrine) followed by Bonferroni's test. When comparing two groups, Mann–Whitney test was used. For comparisons involving three or more groups, one-way ANOVA was performed followed by Bonferroni's test. Statistical analysis was performed under GraphPad<sup>®</sup>. Probability values of <0.05 were considered significant.

## 3. Results

### 3.1 Involvement of AT2R in flow-mediated diameter expansion

Arterial diameter in AT2R<sup>+/+</sup> mice was greater in HF arteries than that in NF vessels (Figure 1A), whereas no difference between HF and NF arteries was observed in AT2R<sup>-/-</sup> mice (Figure 1B). Similarly, we found in a



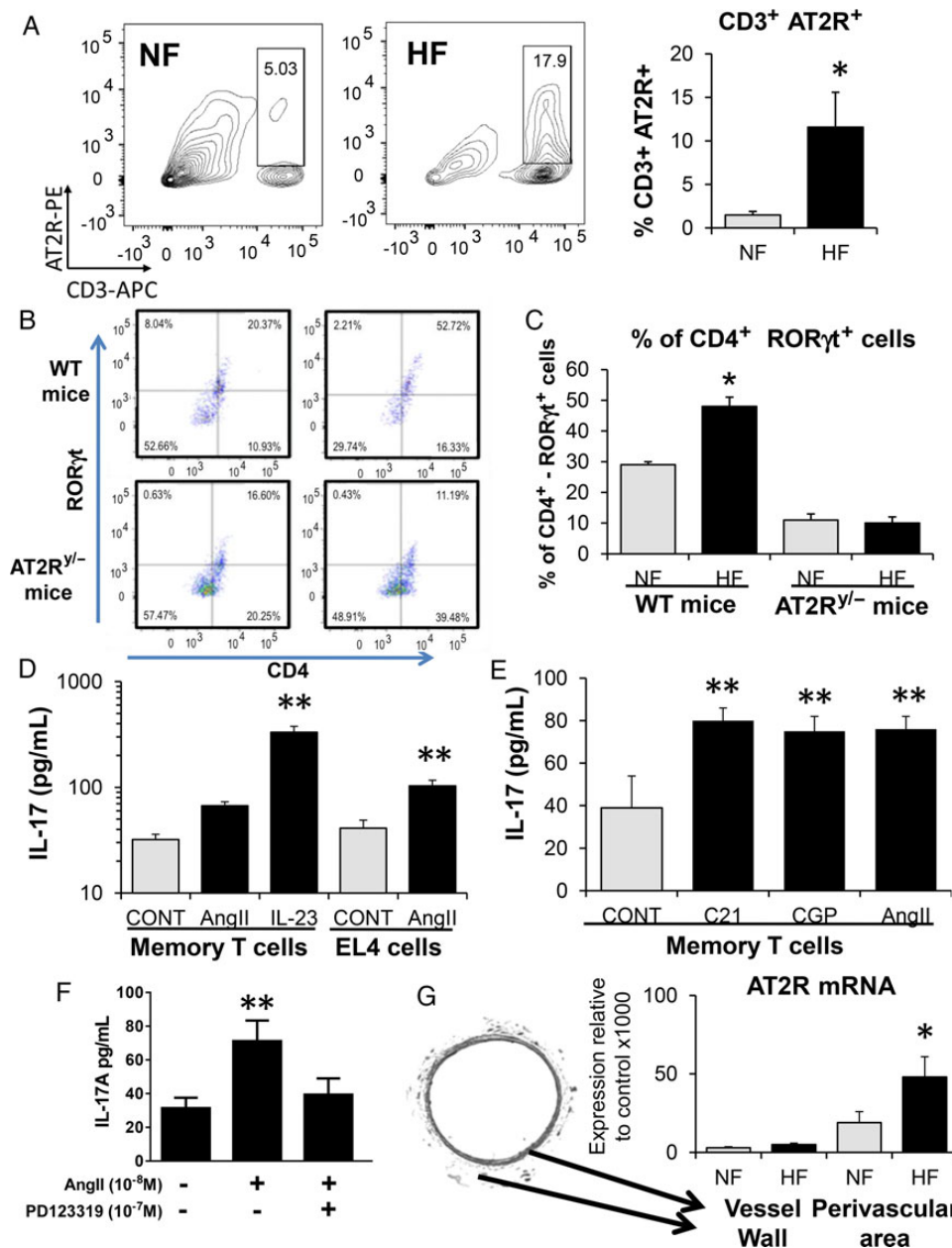
**Figure 2** Role of T cells in flow-mediated outward remodelling. Flow cytometry (A) of perivascular cells for CD45<sup>+</sup> and CD3<sup>+</sup> cells in NF and HF arteries 2 days after ligation. Arterial diameter measured in HF and NF arteries isolated from WT mice (B), nude mice (C), and mice treated with anti-CD3e antibodies (D). Mean  $\pm$  SEM is presented ( $n = 8$  mice per group). \* $P < 0.05$ , HF vs. NF arteries.

preliminary series of experiments that diameter enlargement did not occur in HF arteries isolated from rats treated with the AT2R blocker PD123319 (30 mg/kg per day) (Supplementary material online, Figure S1).

### 3.2 T lymphocytes in flow-mediated outward remodelling of resistance arteries

RT-qPCR analysis was represented on a heat map figure to regroup gene expression fold changes playing a role in HF remodelling

(Supplementary material online, Figure S2). The results revealed that the expression of MMP9 (*mmp9*) and TIMP-1 (*timp1*), essential for flow-mediated outward remodelling,<sup>25</sup> were clustered with CD11b (*itgam*) mRNA in HF vessels from WT mice and not in AT2R<sup>+/+</sup> mice, suggesting a role for immune cells in remodelling. Furthermore, the accumulation of leukocytes (CD45<sup>+</sup> cells), neutrophils (Ly6G<sup>+</sup> cells), and macrophages (F4/80<sup>+</sup> cells) was significantly larger around HF arteries than that around NF vessels (Supplementary material online,



**Figure 3** T cells expressing AT2R in HF arteries. Flow cytometry (A) of perivascular cells for CD45<sup>+</sup> and CD3<sup>+</sup> cells in NF and HF arteries 2 days after ligation showing AT2R expression in CD3<sup>+</sup> cells. (B and C) Flow cytometric analysis of the TH17 marker RORγt in CD4<sup>+</sup> cells isolated from WT and AT2R<sup>+/−</sup> mice. IL-17A (D) was quantified in the supernatants of memory CD4<sup>+</sup> T cells and EL4 cells treated for 7 days with anti-CD3 plus anti-CD28 mAb, in the absence or presence of angiotensin II (AngII, 10<sup>−8</sup> M) or IL-23 (200 ng/mL). IL-17A was also quantified after 7 days in the supernatants of memory CD4<sup>+</sup> T cells (E) treated without (control, CONT) or with the AT2R agonists C21 (10<sup>−8</sup> M) or CGP42112 (10<sup>−8</sup> M) or with angiotensin II (10<sup>−8</sup> M) in the presence of the AT1R blocker candesartan (Ang). Effect of candesartan (TCV, 10<sup>−7</sup> M) and PD123319 (10<sup>−7</sup> M) on AngII (10<sup>−8</sup> M)-induced IL-17 production (F). AT2R expression level in the perivascular area and in the wall of NF and HF arteries (G). Mean ± SEM is shown (n = 5–8 mice per group). \*P < 0.05, HF vs. NF arteries. \*\*P < 0.05, AngII, C21, CGP42112 or IL-23 vs. control (CONT).

Figure S3). This was confirmed by the analysis of T cells in the perivascular area showing a greater level of CD45<sup>+</sup> and CD3<sup>+</sup> cells around HF arteries (Figure 2A). Thus, we investigated flow-mediated remodelling in mice lacking T cells. Arterial diameter in WT mice was greater in HF arteries than that in NF arteries (Figure 2B), whereas diameter was equivalent in HF and NF arteries in (athymic) nude mice (Figure 2C) and in mice treated with a depleting anti-CD3 $\epsilon$  antibody (Figure 2D). These data suggest that T cells are most likely involved in outward arterial remodelling. In addition, AT2R was expressed by infiltrating CD3<sup>+</sup> cells surrounding HF arteries in contrast with NF vessels (Figure 3A).

### 3.3 Angiotensin II induces IL-17 production by mouse CD4<sup>+</sup> memory T cells via AT2R

AT2R was expressed only by infiltrating CD3<sup>+</sup> cells surrounding HF arteries in contrast with NF vessels (Figure 3A). CD4<sup>+</sup> cells surrounding HF arteries expressed more ROR $\gamma$ t, a marker of Th17, than NF arteries (Figure 3B). This was not observed in AT2R<sup>+/−</sup> mice (Figure 3C), suggesting a link between AT2R and Th17 lymphocytes in flow-mediated remodelling. As infiltrating CD3<sup>+</sup> and CD4<sup>+</sup> cells expressed AT2R, we evaluated the release of IL-17 by CD4<sup>+</sup> T lymphocytes stimulated by angiotensin II. Angiotensin II increased IL-17 production by anti-CD3, anti-CD28 mAb-stimulated memory T cells, and EL-4 cells, although at a lower level than IL-23 used as a positive control (Figure 3D). In contrast, angiotensin II did not induce IL-17 production by naive T cells (Supplementary material online, Figure S4). The AT2R agonists, C21 and CGP42112, as well as angiotensin II in the presence of the AT1R

blocker candesartan increased IL-17 production by memory T cells (Figure 3E). Blocking AT2R with PD123319 (10<sup>−7</sup> M) reduced IL-17 production by memory T cells by 30% (Figure 3F). Finally, AT2R mRNA level was mainly detected in the perivascular tissue, and its expression level was higher in HF than that in NF arteries (Figure 3G).

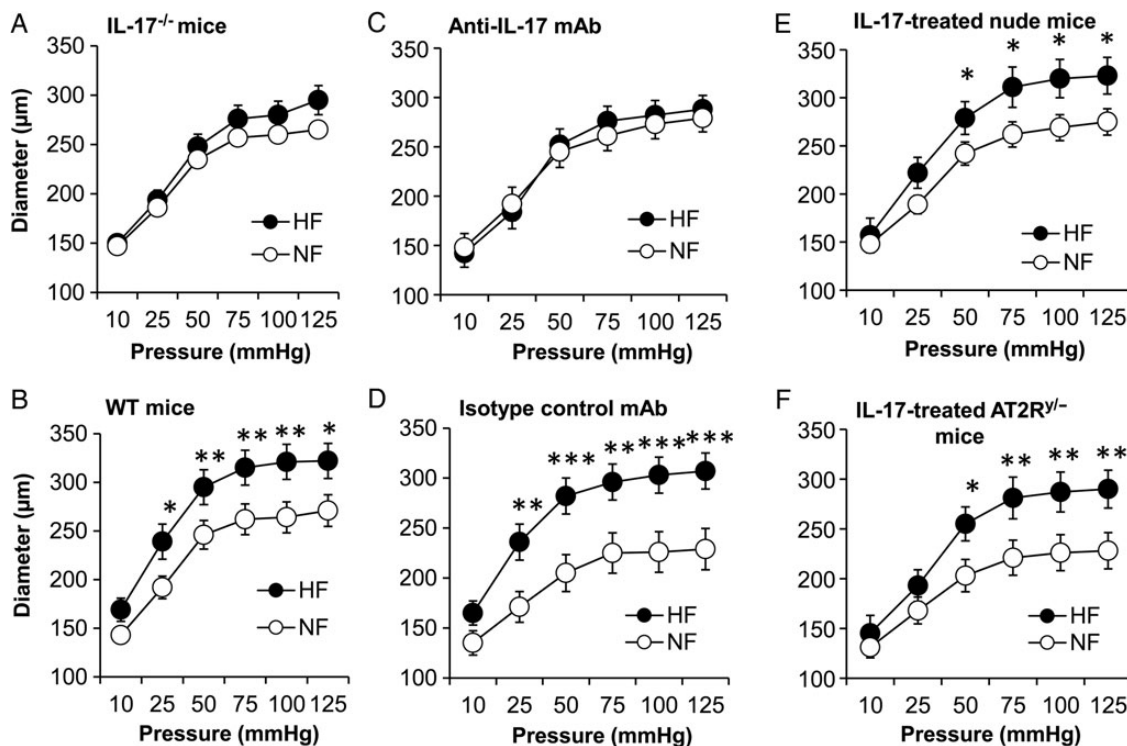
### 3.4 Involvement of IL-17 in flow-mediated diameter expansion

Diameter expansion did not occur in IL-17<sup>−/−</sup> mice compared with WT mice (Figure 4A and B). Furthermore, no diameter expansion occurred in mice treated with a neutralizing anti-IL-17 Ab (Figure 4C), compared with mice treated with the isotype control mAb (Figure 4D).

Notably, the lack of diameter expansion in AT2R<sup>+/−</sup> and in nude mice was corrected by a treatment with exogenous IL-17 (Figure 4E and F), suggesting that IL-17 facilitates outward remodelling downstream T cells activation by AT2R.

### 3.5 IL-17 amplifies endothelium (NO)-mediated relaxation

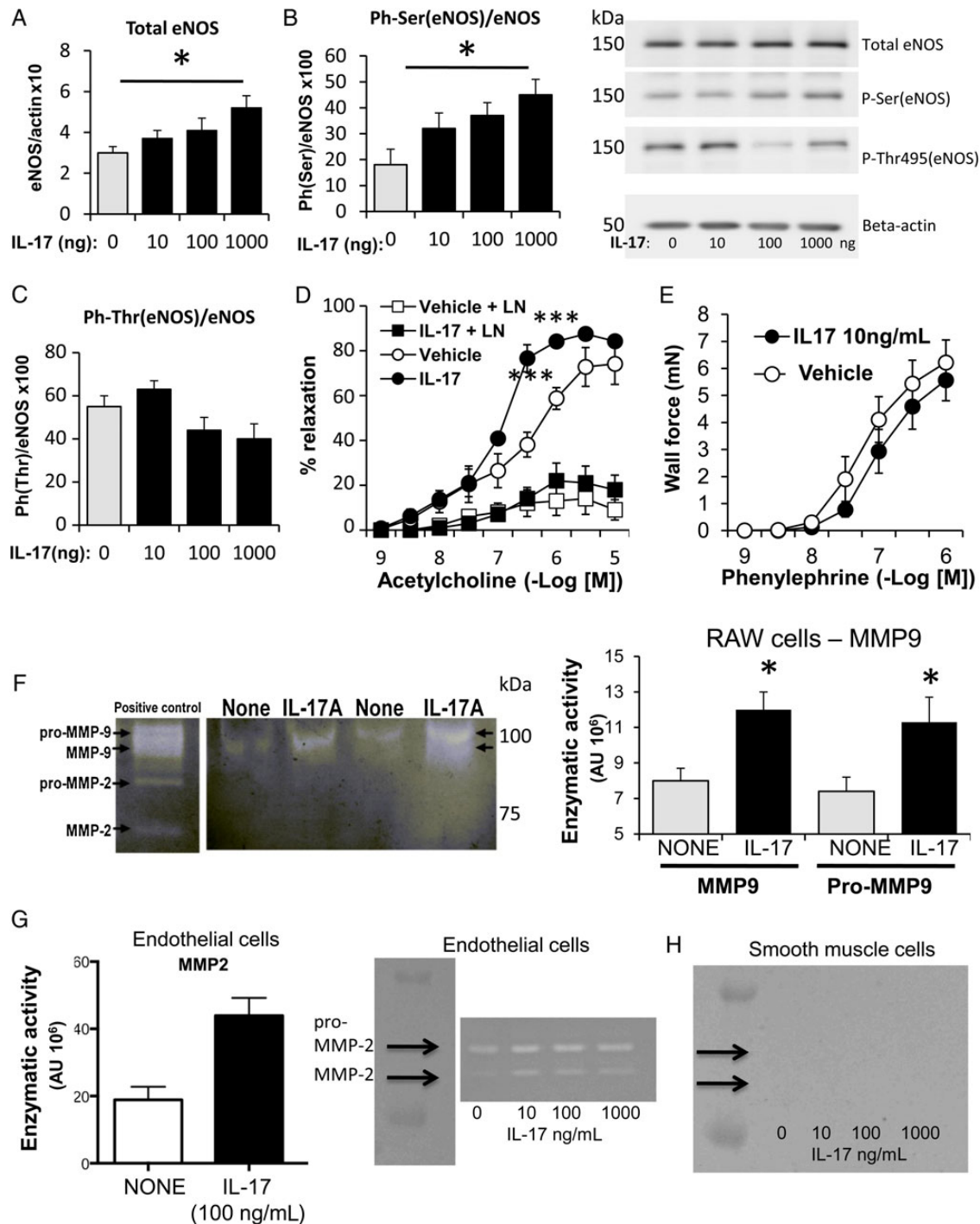
As IL-17 could be the the effector of AT2R-dependent diameter expansion, we investigated the effect of IL-17 on eNOS expression level, which is necessary in flow-mediated remodelling.<sup>25</sup> IL-17 increased eNOS expression levels in cultured mouse endothelial cells (Figure 5A). In addition, IL-17 increased the phosphorylation of eNOS on its activating site (Figure 5B) without significantly affecting phosphorylation on its inhibitory site (Figure 5C).



**Figure 4** Remodelling requires IL-17 production. Arterial diameter measured 1 week after ligation in NF and HF arteries isolated from IL-17<sup>−/−</sup> (A) and WT (B) mice as well as from mice treated with anti-IL-17 antibodies (C) or with isotype control antibodies (D). Arterial diameter was also measured in HF and NF arteries from nude (E) and AT2R<sup>+/−</sup> mice (F) treated with IL-17 (5 ng/kg per day). Mean  $\pm$  SEM is presented ( $n = 8$  mice per group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , HF vs. NF.

Thus, we tested the functional effect of IL-17 on endothelium (NO)-dependent relaxation. We found that IL-17 significantly increased acetylcholine-mediated relaxation of mesenteric resistance arteries *in vitro* (Figure 5D). Relaxation was reduced by the NO-

synthesis blocker L-NAME in arteries treated or not with IL-17, suggesting that the IL-17 was mainly acting on the production of NO. IL-17 did not change arterial contraction induced by phenylephrine (Figure 5E).



**Figure 5** IL-17 increased NO-dependent relaxation and MMP9 activity. Effect of IL-17 (0–1000 ng/mL) on eNOS (A), ph-Ser(eNOS) (B) and Ph-Thr(eNOS) (C) expression level in endothelial cells. Acetylcholine-mediated relaxation (D) measured before and after incubation with IL-17 (10 ng/mL) in the presence of IL-17 was measured before and after NO-synthesis blockade with L-NAME (LN, 100 mmol/L). Phenylephrine-mediated contraction (E) measured in NF and HF arteries. After incubation with IL-17 (10 ng/mL). Zymographic analysis of metalloproteinase activity in the supernatant of RAW264.7 (F), endothelial (G), and smooth muscle cells (H) was performed in the absence (None) or presence of IL-17 (100 ng/mL). Mean  $\pm$  SEM,  $n = 4$  mice per group. \* $P < 0.05$ , \*\*\* $P < 0.001$ , effect of IL-17.

### 3.6 IL-17 activated matrix metalloproteinases activity in macrophages and in endothelial cells

We also observed that the activity of MMP9 and pro-MMP9 (but not MMP2) was significantly increased in the supernatants of RAW264.7 cells stimulated with IL-17, compared with non-stimulated cells (Figure 5F). In contrast, the activity of pro-MMP2 and MMP2, not MMP9, was increased by IL-17 in the supernatant of endothelial cells (Figure 5G). The activity of MMP2 and MMP9 was not increased by IL-17 in smooth muscle cells (Figure 5H).

IL-17 also activated I $\kappa$ B $\beta$  and the ratio of phospho-ERK/ERK in endothelial cells with affecting significantly cJUN and phospho-p38/p38 (Supplementary material online, Figure S5). In smooth muscle cells, IL-17 activated phospho-NF- $\kappa$ B p65 without affecting significantly cJUN and phospho-p38/p38 and phospho-ERK/ERK (Supplementary material online, Figure S5).

### 3.7 Collateral arteries growth in a skin flap model of ischaemia

The pathophysiological implication of the present findings was assessed using the skin flap model of ischaemia, in which flow-mediated outward

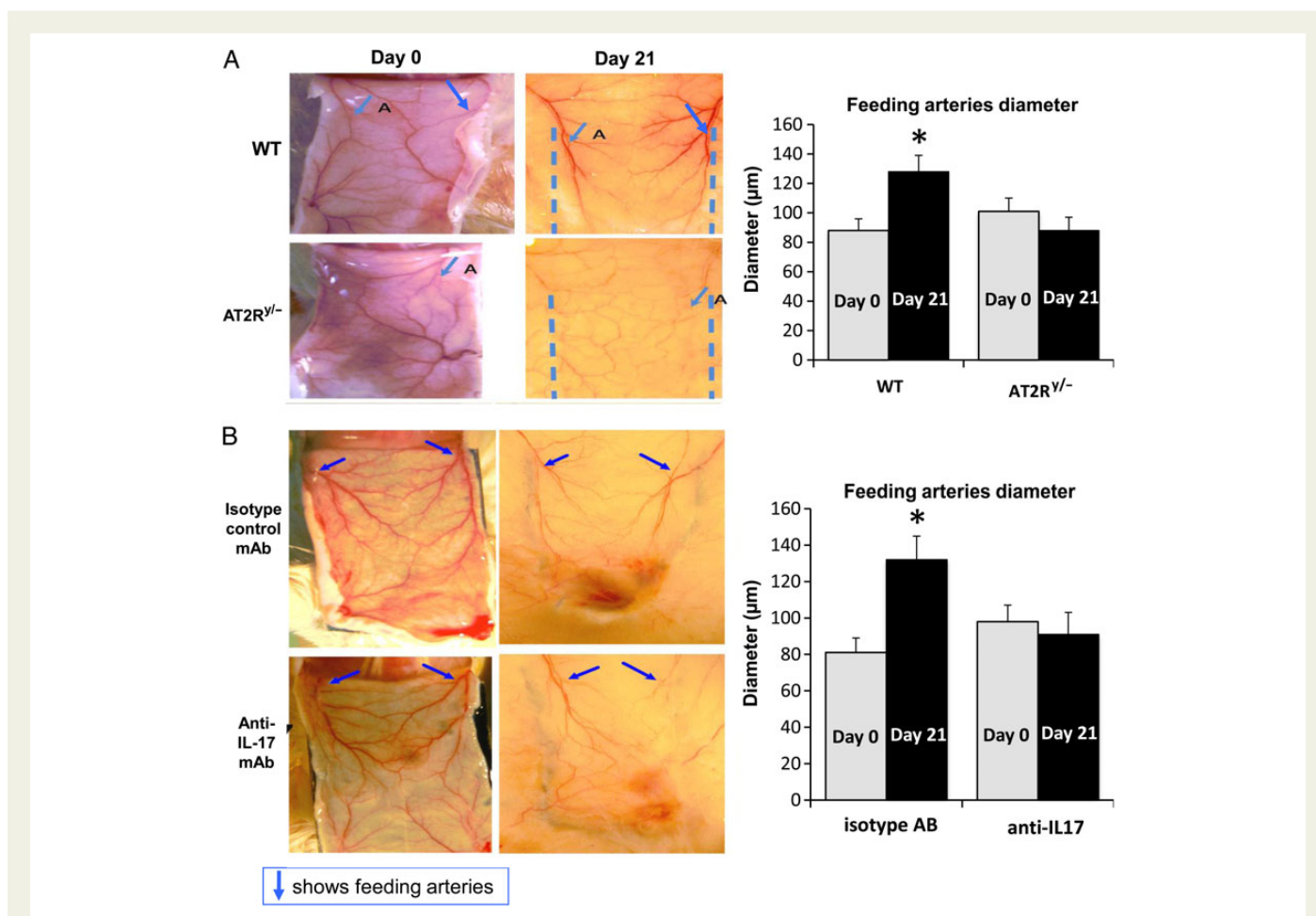
remodelling is essential for the revascularization of the ischaemic skin. In control mice (WT or isotype antibody-treated mice), the diameter of the feeding arteries increased significantly 21 days after surgery (Figure 6A and B), suggesting that outward remodelling occurred to revascularize the ischaemic skin. On the other hand, in AT2R<sup>-/-</sup> and in mice injected with a neutralizing anti-IL-17 mAb, outward remodelling of the lateral thoracic arteries was absent (Figure 6A and B).

### 3.8 AT2R stimulation restores HF-remodelling in old mice

As previously described,<sup>26,27</sup> outward remodelling of mesenteric resistance arteries did not occur in 12-month-old mice (Figure 7A and B). Nevertheless, remodelling occurred in 12-month-old mice treated with the AT2R agonist C21 (Figure 7C and D).

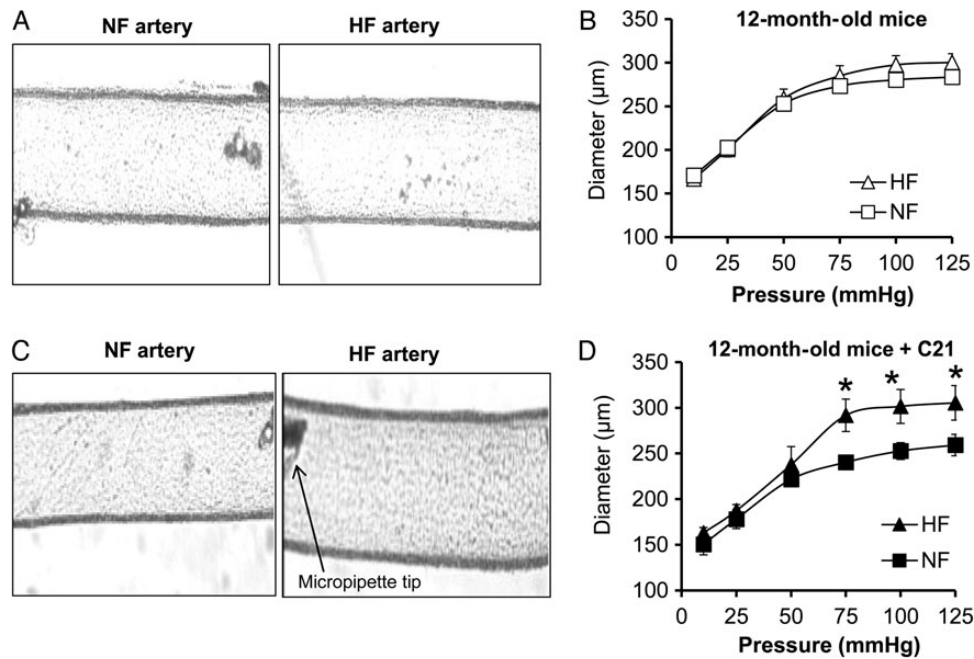
### 3.9 Animal characteristics

As different mouse strains were used, we measured arterial blood pressure in all the groups and did not find significant differences between them (Supplementary material online, Figure S6). Nevertheless, body weight was greater in AT2R<sup>-/-</sup> than that in AT2R<sup>+/+</sup> mice. Body weight



**Figure 6** AT2R is required for feeding arteries diameter expansion in the skin. Arterial diameter was measured at Day 0 and Day 21 in the lateral thoracic artery feeding the skin flap of WT and AT2R<sup>-/-</sup> mice (A) and in WT mice treated with an anti-IL-17 or a control (isotype) antibody (B). Blue arrows indicate lateral thoracic arteries. Arterial diameter measured at Day 0 and Day 21 is shown in the bar graphs. Mean  $\pm$  SEM,  $n = 5$  mice per group. \* $P < 0.05$ , Day 21 vs. Day 0.





**Figure 7** AT2R activation restores flow-mediated remodelling in old mice. Passive arterial diameter measured 1 week after ligation in NF and HF arteries isolated of 12-month-old mice treated with the vehicle (control, A and B) or with the AT2R agonist C21 (C and D). Mean  $\pm$  SEM,  $n = 5$  mice per group. \* $P < 0.05$ , HF vs. NF arteries.

was also larger in Swiss than that in AT2R<sup>+/+</sup> and IL-17<sup>+/+</sup> mice (Supplementary material online, Figure S6).

Consequently, we measured arterial remodelling in mice with different body weights (Supplementary material online, Figure S7) and found that remodelling occurred despite differences in NF arteries diameter. Nevertheless, no significant difference in arterial diameter in NF vessels was observed among the mouse groups used in the present study.

## 4. Discussion

The present work demonstrates the key role of AT2R in flow-mediated outward remodelling of resistance arteries, possibly through the activation of T cell-derived IL-17 production, as depicted in the scheme shown in Figure 8.

This interaction between vascular and immune systems is corroborated by the close phylogenetic relationship between angiotensin II and chemokine receptors<sup>31</sup> and might have roots in a putative evolutionary origin of the circulatory system from an ancestral lymphatic system.<sup>32</sup>

### 4.1 Role of T cells in flow-mediated remodelling of resistance arteries

We found that T cells have a central role in flow-mediated outward remodelling of mesenteric resistance arteries in physiological conditions. Indeed, T cell number increased in the first days after increasing blood flow. This observation is in agreement with previous studies showing limited but necessary macrophage accumulation around HF arteries on Days 1 and 2 following arterial ligation in the mesenteric vasculature.<sup>5</sup> In the present study, the functional role of T cells was demonstrated using nude mice and mice treated with anti-CD3 antibodies. Our observation is also in agreement with previous works showing a

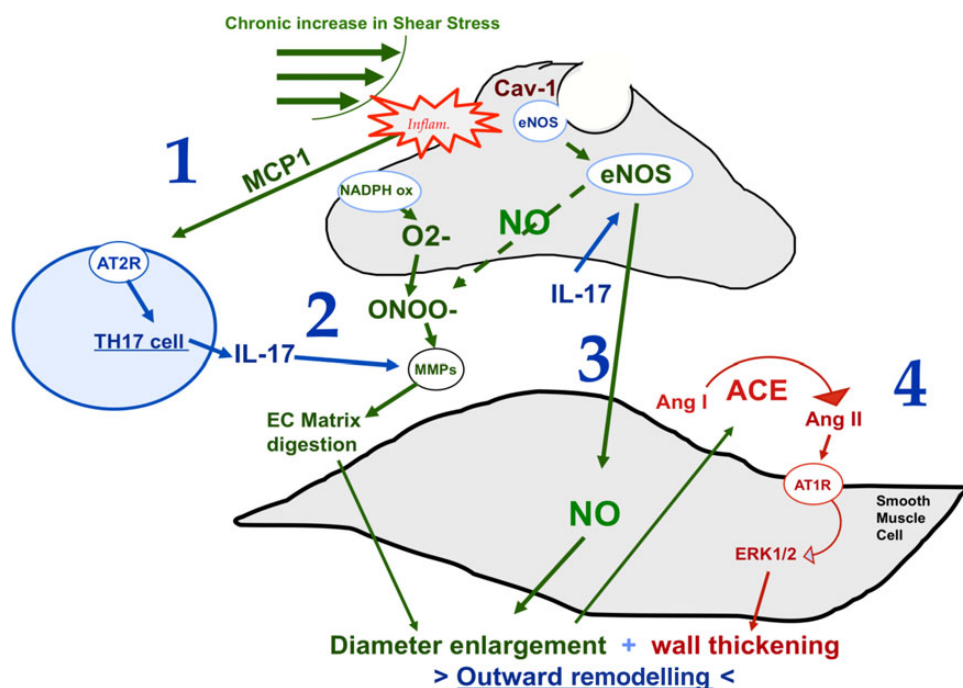
defect in hind limb revascularization following femoral artery ligation in CD4<sup>-/-</sup>,<sup>7,33</sup> CD8<sup>-/-</sup>,<sup>8</sup> or NK cell-deficient mice.<sup>7</sup>

### 4.2 Angiotensin II and memory T cell production of IL-17

We found that angiotensin II induced IL-17 production by memory T cells through AT2R activation. This finding is of major importance, as both angiotensin II and T lymphocytes have a key role in vascular homeostasis and are involved in remodelling in physiological conditions and in vascular disorders. We found that AT2R inhibition prevented IL-17 production, and that AT2R activation increased IL-17 production by T memory cells. This is a novel and important observation. So far, IL-17 has been suggested to have a dual effect with protective properties at low concentrations and deleterious properties at high concentration.<sup>34</sup> This latter study showed that IL-17 (ng/mL) induced eNOS phosphorylation in cultured human endothelial cells,<sup>34</sup> whereas IL-17 (μg/mL) inhibited eNOS activity in isolated rat arteries.<sup>35</sup> IL-17 has also been shown to be involved in post-ischæmic collateral growth.<sup>10</sup>

### 4.3 Role of AT2R in T cells-mediated outward remodelling

We found that AT2R-mediated IL-17 production was involved in the diameter expansion following a chronic increase in blood flow *in vivo*, as evidenced by the absence of diameter enlargement in mice lacking either AT2R or IL-17. Furthermore, it is likely that AT2R-mediated IL-17 production occurred following lymphocyte infiltration into the perivascular environment. In line with a role for IL-17 in the diameter expansion following AT2R activation, we found that IL-17 increased the vasodilator and NO-dependent activity of the endothelium, most probably through overexpression of eNOS in endothelial cells. The increase



**Figure 8** Scheme representing the pathway involved in the outward remodelling of the mesenteric artery in response to a chronic increase in blood flow. The first step (Step 1) involves the production of macrophage chemoattractant protein 1 (MCP1) within hours after the change in blood flow (shear stress). This is followed by an increase in the number of immune cells in the perivascular area surrounding the artery.<sup>5,28</sup> The chronic increase in flow also activates superoxide production ( $O_2^-$ ), which associate with NO produced by the endothelial NO synthase (eNOS), also activated by shear stress and form peroxynitrites ( $ONOO^-$ ).<sup>29</sup> Step 2: 2–3 first days following the chronic increase in flow. At this level, TH17 lymphocytes produce interleukine-17 (IL-17) after angiotensin II type 2 receptor (AT2R) activation (present study). IL-17 (present study) and  $ONOO^-$ <sup>29</sup> are then able to activate MMPs leading to the extracellular matrix digestion needed for diameter expansion<sup>25</sup> (Step 3). The diameter expansion is then driven by the vasodilatation due to NO production. This latter is being also activated by IL-17 (present study). Outward remodelling is stabilized when the diameter reaches a level allowing the normalization of shear stress. In this remodeled vessel wall, strain increases due to diameter expansion, and this leads to the activation of angiotensin II type 1 receptor and the MAP kinases ERK1/2 allowing a compensatory wall thickening<sup>30</sup> (Step 4).

in eNOS expression is essential for the diameter expansion following a chronic increase in blood flow *in vivo*.<sup>25</sup> We confirmed this assumption by showing that IL-17 induced MMP-9 activation in myeloid cells and MMP2 in endothelial cells, a key process in flow-mediated diameter expansion of both the carotid artery<sup>36</sup> and mesenteric resistance arteries.<sup>25</sup>

Thus, AT2R is certainly strongly involved in the sub-inflammatory state induced by the increase in blood flow leading to diameter expansion and shear stress normalization. This finding is in agreement with previous studies that have shown that a chronic increase in shear stress induces the production of MCP-1<sup>5,28</sup> followed by macrophage infiltration.<sup>5</sup>

#### 4.4 Role of AT2R and IL-17 in skin flap revascularization *in vivo*

As flow-mediated arterial diameter expansion is essential for collateral growth following ischaemia, we used the skin flap model of chronic hypoxia to extend our findings to pathological situations.<sup>21</sup> In this model, vertebral arteries undergo outward remodelling to revascularize the ischaemic part of the skin. As expected in view of our results obtained in the mesenteric artery, we found that the arteries originating from the lateral thoracic artery did not undergo diameter expansion in AT2R<sup>-/-</sup> mice in contrast with WT mice. Furthermore, the anti-IL-17 mAb also

prevented this diameter expansion. This finding further confirms the key role of AT2R and IL-17 in outward remodelling, which is essential for proper tissue revascularization under both physiological and pathological situations.

#### 4.5 Perspective

The involvement of AT2R in flow-mediated outward remodelling of resistance arteries through IL-17 is, to our knowledge, the most innovative finding of the present work. This remodelling, which is essential in somatic growth and pregnancy, has also a key role in post-ischaemic revascularization in a large number of diseases such as stroke, heart attack, and hind limb vascular insufficiency. Indeed, AT2R-dependent increase in collateral blood flow could explain in part the beneficial effect of the AT2R agonist C21 against ischaemic damages associated with stroke.<sup>37</sup> The possible protective effect of the AT2R in neurodegenerative diseases<sup>38</sup> could also involve, at least in part, the effect of AT2R on small arteries outward remodelling. The potential of a therapeutic use of AT2R stimulation is supported by our experiments showing that the non-peptidic AT2R agonist C21 could restore flow-mediated outward remodelling in 12-month-old mice. Previous studies have shown that flow-mediated outward remodelling is strongly reduced if not absent in old mice and rats. Indeed, in both 12-month-old mice<sup>27</sup> and in 24-month-old rats,<sup>26</sup> no diameter expansion occurs in

response to a chronic increase in blood flow *in vivo*. This finding is especially important as ageing is an independent and inevitable risk factor for peripheral ischaemic diseases.

In view of the findings of the present work, AT2R stimulation in resistance arteries could be a therapeutic alternative to the blockade of the renin–angiotensin system. Indeed, stimulating AT2R, instead of blocking either the production of angiotensin II or AT1R, is a novel approach that is especially promising as AT2R is usually minimally expressed, if not totally absent, in healthy tissues, whereas it is significantly expressed in diseased tissues.<sup>18</sup>

## 5. Conclusion

AT2R and IL-17 have a key role in the diameter expansion following a chronic increase in blood flow *in vivo*. Our results suggest a possible link between AT2R activation and IL-17 release by T cells. This finding provides a major breakthrough in the understanding of vascular remodeling and a potential therapeutic target as AT2R could be selectively stimulated to improve blood flow in vascular disorders.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

## Acknowledgements

Prof. L. Hein and Prof. U. Wenzel generated the AT2R<sup>+/−</sup> mice and provided them for experiments.<sup>39</sup> C21 was kindly provided by Vicore Pharma, Mölndal, Sweden.

**Conflict of interest:** none declared.

## Funding

We used the funding provided as a background funding to our laboratory by the INSERM (Institut National de la Santé et de la Recherche Médicale), CNRS (Centre National de la Recherche scientifique) and the university of Angers.

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## Corrigendum

doi:10.1093/cvr/cw197

**Corrigendum to:** Role of Hic-5 in the formation of microvilli-like structures and the monocyte–endothelial interaction that accelerates atherosclerosis [*Cardiovasc Res* 2015;**105**:361–371]

The authors wish to correct the following error: on page 362, in Section 2.8 the siRNA sequence was incorrectly reported in the paper as ‘siRNA (forward: 5’-GGA UCA UCU AUA CAG CAC-3’; reverse: 5’-CUC CUG CAA UAA ACC UAU A-3’ 10 nmol/L)’. The correct siRNA sequence is ‘siRNA (forward: 5’-GGA CCA GUC UGA AGA UAA G-3’; reverse: 5’-CUU AUC UUC AGA CUG GUC C-3’ 10 nmol/L)’.

The authors apologize for this error and confirm that it does not affect any of their analysis or conclusions.