

# The Inflammatory Pre-atherosclerotic Remodeling Induced by Intermittent Hypoxia is Attenuated by RANTES/CCL5 Inhibition

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**Support:** This study was supported by grants from AGIR@dom to M.D. and C.A., Fondation pour la Recherche Médicale (France) to C.A. and the Swiss National Science Foundation to F.M. (#3200BO-105896).

**Running Title:** Vascular remodeling due to intermittent hypoxia

**Subject Code List:** 8.18 (intermittent hypoxia)

**Word count:** 3056 (introduction: 478, methods: 452, results: 586, discussion: 1540)

## At a Glance Commentary

*Scientific Knowledge on the Subject:* Intermittent hypoxia in sleep apnea is a major risk factor for cardiovascular morbidity and mortality. Understanding the development of these outcomes may condition the management of apneic patients at risk for cardiovascular complications.

*What This Study Adds to the Field:* Intermittent hypoxia induced early systemic inflammation and inflammatory pre-atherosclerotic remodeling in mice, clearly involving the chemokine RANTES/CCL5. These results strengthen the need for early identification of apneic individuals at risk for cardiovascular complications, and suggest new therapeutic options for these patients.

This article has an online data supplement, which is accessible from this issue's table of content online at [www.atsjournals.org](http://www.atsjournals.org)

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Substantial contributions to conception and design: CA, MD

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Final approval of the version to be published: CA, PB, SL, JLP, CG, GP, FB, VB, CR, JPB, FM, PL, MD

## **ABSTRACT**

**Rationale:** The highly prevalent obstructive sleep apnea syndrome (OSA) with its main component intermittent hypoxia (IH) is a risk factor for cardiovascular mortality. The poor knowledge of its pathophysiology has limited the development of specific treatments, whereas the gold standard treatment, continuous positive airway pressure, may not fully reverse OSA chronic consequences and has limited acceptance in some patients.

**Objectives:** To examine the contribution of IH-induced inflammation to OSA cardiovascular complications.

**Methods:** We investigated systemic and vascular inflammatory changes in C57BL6 mice exposed to IH (21-5% FiO<sub>2</sub>, 60s cycle) or normoxia, 8h/day up to 14 days. Vascular alterations were reassessed in mice treated with a blocking antibody of RANTES/CCL5 signaling pathway, or with the IgG isotype control throughout the IH exposure.

**Measurement and Main Results:** IH induced systemic inflammation combining increased splenic lymphocyte proliferation and chemokine expression, with early and predominant RANTES/CCL5 alterations, and enhanced splenocyte migration toward RANTES/CCL5. IH also induced structural and inflammatory vascular alterations. Leukocyte-endothelium adhesive interactions were increased, attested by leukocyte rolling and ICAM-1 expression in mesenteric vessels. Aortas had increased intima-media thickness with elastic fiber alterations, mucoid depositions, NFkB-p50 and ICAM-1 overexpression, hypertrophy of smooth-muscle cells overexpressing RANTES/CCL5, and adventitial-periadventitial T-lymphocyte infiltration. RANTES/CCL5 neutralization prevented both intima-media thickening and inflammatory alterations, independently of the IH-associated proatherogenic dyslipidemia.

**Conclusions:** Inflammation is a determinant mechanism for IH-induced pre-atherosclerotic remodeling, involving RANTES/CCL5, a key chemokine in atherogenesis. Characterization of the inflammatory response could allow identifying at-risk patients for complications, and

its pharmacological manipulation may represent a potential complementary treatment of sleep apnea consequences.

**Word count:** 255

**Key words:** Sleep apnea; hypoxia; vascular remodeling; inflammation; RANTES/CCL5

## INTRODUCTION

Obstructive sleep apnea (OSA) is a worldwide public-health problem, affecting at least 5% of the general population and characterized by repetitive upper airway collapse during sleep leading to intermittent hypoxia (IH) (1). OSA is recognized as a cardiovascular risk factor leading to systemic arterial hypertension, coronary heart disease, arrhythmias, stroke and death (2,3). Recently, the American Heart Association and American College of Cardiology has jointly highlighted the cardiovascular risks of OSA, and acknowledged the lack of understanding that had limited the development of specific treatments (2,3). The current gold standard treatment, continuous positive airway pressure (CPAP) therapy, may not fully reverse OSA chronic cardiovascular consequences, e.g. hypertension (4,5). In addition, CPAP has poor effective long-term compliance in some individuals (6). OSA-cardiovascular morbidity could result mainly from IH-related inflammation (7), including elevated serum pro-thrombotic and pro-inflammatory factors that are major determinants for atherosclerosis development (8,9). OSA and common atherosclerosis share endothelial dysfunction, increases in leukocyte adhesion and platelet aggregation. Cytokines, chemokines and adhesion molecules expression are also elevated compared to controls (10). Circulating levels of inflammatory mediators parallel atherosclerotic plaque formation (9,11) and correlate with oxygen desaturation in OSA (12). Increased carotid intima-media thickness (IMT) is an early sign of atherosclerosis in OSA patients (13) and also correlates with serum inflammatory markers and oxygen desaturation (14,15). Together, these findings suggest an important promoting role for IH in systemic and vascular inflammation.

The specificity of OSA-related atherogenesis remains unknown whereas this knowledge could be useful for providing alternative treatment to CPAP in preventing OSA cardiovascular complications. At early stages, general mechanisms of atherogenesis include leukocyte

activation and recruitment into vascular tissue (8). These processes are partly under the control of chemokines and adhesion molecules, resulting in leukocyte rolling, endothelial adhesion, and transendothelial migration (16). Among leukocytes and chemokines, T-cells and the leukoattractant RANTES/CCL5 (regulated on activation, T-cell expressed, and secreted/chemokine ligand 5) appear to be critically and early involved in the atherogenesis (9,16), with serious prospects for future clinical treatments (17-19). RANTES/CCL5 mediates trafficking and homing of T-lymphocytes, monocytes, and other leukocytes, and has been implicated in arterial injury and atherosclerosis (9), circulating levels being predictive of cardiac events and mortality (20,21). Several studies have provided evidence for T-lymphocyte involvement in the atherosclerosis of OSA-patients (22), however the role of RANTES/CCL5 is still unknown. It is of interest to characterize the role of chemokines in the IH-related inflammation, as they represent potential targets for future pharmacological manipulations.

The aim of the present study was to identify the biological events leading to pre-atherosclerotic remodeling through systemic and vascular inflammatory responses to IH in a murine model of sleep apnea. On the basis of our results, we secondarily focused our investigations on the specific role of the chemokine RANTES/CCL5 in the IH-related vascular remodeling. Some of the results of this study have been previously reported in the form of an abstract (23).

## **METHODS**

Details are provided in the online supplement.

### **Animals**

Male C57BL/6J mice (8 weeks old) were randomized to 5 and 14 days of IH (21-5% FiO<sub>2</sub>, 60s cycle, 8h/d) or air (N, normoxic), as previously described (24). Blood was collected under anesthesia for hematocrit, chemokine and cholesterol determinations. Tissues were harvested and frozen until analysis. All efforts were made to reduce the number of animals used to an unavoidable minimum.

### **Assessment of systemic inflammation**

***Proliferation assay.*** Splenocytes were isolated, cultured at a concentration of  $5 \times 10^6$  cells.ml<sup>-1</sup>, and stimulated in triplicates with graded concentrations of concanavalin-A (Sigma). Cell proliferation was determined 48h later using a cell proliferation kit (CellTiter 96® AQueous non-radioactive cell proliferation assay, Promega).

***Splenocyte chemokine mRNA expression.*** Total mRNA was extracted from spleen, and chemokine expression was assessed by qRT-PCR and normalized with the 18S ribosomal-RNA level.

***Ex vivo transmigration assay.*** Interferon-gamma (IFN- $\gamma$ ) stimulated and unstimulated splenocytes were assessed for transwell migration toward RANTES/CCL5 (R&D Systems).

### **Assessment of vascular inflammation and remodeling**

***Leukocyte rolling.*** Intravital microscopy was performed on exteriorized mesenteric microvasculature since the technique requires thin transparent tissue. Rolling of rhodamine 6G-stained leukocytes was assessed by two blinded observers.

**ICAM-1, alpha-smooth muscle actin ( $\alpha$ SMA) and NF $\kappa$ B immunoblotting.** Expressions of mesenteric and aortic ICAM-1, aortic  $\alpha$ SMA and NF $\kappa$ B were assessed by Western blot. Tissues were homogenized and proteins were separated on 8% SDS-PAGE, immunoblotted for ICAM-1 (R&D systems) or  $\alpha$ SMA (Abcam), visualized using enhanced chemoluminescence detection system and quantified by densitometric analysis using Image J® software. For NF $\kappa$ B, nuclear proteins were separated on 10% SDS/PAGE and immunoblotted for NF $\kappa$ B-p50 (Abcam).

**Aortic IFN $\gamma$  mRNA expression** Total mRNA was extracted from aortas, and IFN $\gamma$  expression was assessed by qRT-PCR and normalized using ubiquitin mRNA levels.

**Immunohistochemistry.** OCT-embedded aorta sections (10- $\mu$ m) (Tissue tek®-OCTTM, Sakura) were stained with hematoxylin-eosin, Verhoeff, alcian blue, Oil-red-O and sirius red to assess global tissue morphology, elastic fibers, mucoid degeneration, lipid deposition and collagen content, respectively. Additional sections were immunostained for CD3 (DakoCytomation) and RANTES/CCL5 (R&D systems). Morphometric analysis (up to 30 measurements on 3 non-contiguous midthoracic aorta sections per animal, n=7-10 mice per group) was performed with a light microscope (Nikon Eclipse 80i) and LUCIA-G software V5.0.

**RANTES/CCL5 neutralization.** To confirm the role of RANTES/CCL5 in IH-induced vascular remodeling, additional mice were treated as previously described (25), using a rat anti-mouse RANTES/CCL5 monoclonal antibody (R&D systems, intraperitoneal injection, 250  $\mu$ g, 3 times per week) or its rat IgG isotype control (ATCC) throughout the 14-day exposure to IH or air. Mice were assessed for vascular remodeling as described above.

### **Statistical analysis**

Results were expressed as means  $\pm$  SEM and analyzed using either ANOVA and t-test or Mann-Whitney U-test, according to normality and variance homogeneity. Statistical significance was set at  $p < 0.05$ .

## RESULTS

### SYSTEMIC INFLAMMATION

We assessed three indices reflecting systemic T-lymphocyte activation. Spleen-isolated T-cells were analyzed for their proliferative response to concanavalin A, and their capacity to express chemokines and migrate upon a chemokine gradient.

**1) Splenocyte proliferation:** No significant difference in the baseline proliferation rate emerged between N and IH. The dose-response relationship resulted in bell-shaped curves, the hypoxic mice showing a significantly higher peak proliferation with 0.5 µg/ml of Con-A at day 5 and 0.5-1 µg/ml at day 14 (Figures 1A and 1B).

**2) Splenocyte expression of leukostimulating and leukoattractant chemokines:** IH induced a predominant elevation in RANTES/CCL5 after only 5 days of IH that was still present at 14 days. Expressions of MIP1α/CCL3 and MIP1β/CCL4 increased more progressively, whereas modifications of MCP1/CCL2 did not reach significance (Figures 1C to 1F).

**3) Splenocyte migration:** Unstimulated and IFN-γ stimulated splenocytes from IH-mice showed higher migration capacity towards the leukoattractant RANTES/CCL5 (Figure 1H), again confirming that IH induced splenocyte activation.

### VASCULAR INFLAMMATION

#### **IH increases leukocyte rolling and ICAM-1 expression**

To investigate the effect of IH on circulating leukocyte recruitment, we used intravital microscopy on the mesenteric microcirculation. We found an increased number of rolling leukocytes in hypoxic mice (Figures 2A and 2B). Furthermore, whereas leukocyte arrest was not significantly altered, mesenteric ICAM-1 protein was overexpressed after 14 days of IH (Figures 2C and 2D).

## **IH induces structural and inflammatory aorta remodeling**

**1) Structural remodeling:** While the internal perimeter was unaffected (N vs IH,  $1942 \pm 54$  vs  $1993 \pm 71$   $\mu\text{m}$ ), IMT was increased after 14 days of IH (Figures 3A and 3B), suggesting an expansive remodeling. The enlarged wall involved the tunica media, in which elastic lamellae were thicker (N vs IH,  $1.7 \pm 0.1$  vs  $2.6 \pm 0.2$   $\mu\text{m}$ ,  $p < 0.01$ ) with a less harmonious organization and some discontinuities of the elastic network (Figure 3C). Distance between the elastic lamellae was increased (Figure 3D) due to smooth-muscle cell (SMC) hypertrophy, without an alteration in the number of SMC nuclei in the media (Figure 3E). Alcian blue staining showed some mucoid accumulations between sub-intimal elastic fibers (Figure 3F), that could result from elastoid degeneration and/or mucoid degenerating SMC. In contrast, no lipid deposition or collagen alteration was observed (data not shown).

**2) Aorta inflammation:** 14 days of IH induced a slight but significant increase in T-cell (CD3 positive cells) infiltration in the aortic wall, almost exclusively located in the adventitia-periadventitia (Figures 4A and 4B). RANTES/CCL5 expression was increased in IH aortas, as shown by immunostaining, either underlining the elastic fibers of the media, or covering the cytoplasm of SMCs. For the latter, there was a characteristic spindle-shaped staining, especially for the hypertrophic SMCs between the two most internal layers of elastic fibers (Figure 4C). IH-aortas also exhibited increased expression of nuclear NF $\kappa$ B-p50 and cytoplasmic ICAM-1 (Figures 4D and 4E).

### **3) RANTES/CCL5 neutralization prevents IH-induced aorta remodeling**

The antibody selectively binds to the chemokine RANTES/CCL5 and inhibits the interaction of RANTES/CCL5 with its receptors, thereby neutralizing RANTES/CCL5 activity. RANTES/CCL5 inhibition prevented both structural and inflammatory alterations of the

aortic wall due to IH. Indeed, anti-RANTES treatment attenuated the increases in IMT,  $\alpha$ SMA, T-cell infiltration and RANTES/CCL5 expression (Figures 5A, 5B, 5D and 5E). This was associated with a global anti-inflammatory effect, as RANTES/CCL5 neutralization also prevented the IH-induced NFkB-p50 and Th1 cytokine IFN $\gamma$  overexpression (Figures 5C, 5F and 5G). In contrast, RANTES/CCL5 neutralization did not prevent hematocrit (normoxic IgG and anti-RANTES/CCL5 treated mice:  $43.5\pm 0.5$  vs  $42.3\pm 0.6\%$ ; hypoxic IgG and anti-RANTES/CCL5 treated mice:  $46.6\pm 0.4$  vs  $46.8\pm 0.6\%$ ), cholesterol and weight alterations associated with IH (online Figures E1B to E1D).

## **DISCUSSION**

The specific pathophysiology of OSA-induced atherogenesis remains poorly understood. In the current study, we demonstrated in mice that IH, the key hallmark of OSA, induced systemic and vascular inflammation, with pre-atherosclerotic remodeling. Both inflammatory and structural vascular alterations were attenuated with RANTES/CCL5 neutralization. This provides a strong rationale to consider new treatment strategies combined to CPAP when addressing OSA cardiovascular consequences.

### **Methodological considerations**

**Mouse strain.** As previously published (26), we used C57BL6 mice exposed to 14 days of IH to study pre-atherosclerotic remodeling due to IH, i.e. early stages of vascular changes before any fibrolipid lesions. C57BL6 mice can develop atherosclerosis but after a long IH exposure (12 weeks) with a concomitant atherogenic diet resulting in dyslipidemia (27). Atherosensitive mice such as apolipoprotein-E deficient mice could be potentially interesting, but they spontaneously develop very advanced atherosclerotic lesions with systemic inflammation and severe dyslipidemia, even without IH. Therefore the use of C57BL6 mice without confounders (e.g. atherogenic diet, spontaneous inflammation and dyslipidemia), as performed in this study, allowed assessing the effects of IH per se on the vasculature at early stages.

**IH stimulus.** The hypoxic stimulus used in this study has been extensively used by our group and others (24). With a nadir arterial oxygen saturation around 60% (26), our experimental model mimics severe sleep apnea, representing less than 10% of patients. In the absence of confounding factors (i.e. obesity...) or genetic susceptibility for complications, IH needs to be severe enough to induce detrimental effects (24), but also leads to weight loss as observed in

our study. However despite this limitation, the IH model proved to be useful during the last two decades for investigating the various consequences elicited by sleep apnea (24).

### **IH induces systemic inflammation**

During the inflammatory response, lymphocytes are activated into lymphoid organs such as the spleen where they proliferate. Then, lymphocytes are recruited into non-lymphoid inflamed tissues under the control of chemokines and adhesion molecules, and release inducible chemokines (9). In the present study, we observed splenocyte activation in response to IH, including enhanced proliferative and migratory capacities, as well as increased chemokine expression. Our data are consistent with the literature reporting specific activation of lymphocytes in OSA patients (22). Thus from our experimental data, we can hypothesize that the hypoxic component of OSA plays a major role in lymphocyte activation. Splenic-derived T lymphocytes may be directly activated by hypoxia through HIF-1 $\alpha$  and NF $\kappa$ B activation, with complex interactions between these two hypoxia-sensitive transcription factors (28,29). Additionally, lymphocytes may be indirectly activated through the numerous IH-induced changes including oxidative stress (30), sympathetic activation (31) and hormonal alterations including leptin (32). We further specifically assessed four chemokines that are known to be determinant in atherosclerosis (9), and we found an early and predominant RANTES/CCL5 overexpression. This splenic overexpression of chemokines confirmed the IH-induced splenocyte activation. We also found that activated splenocytes had enhanced migration toward RANTES/CCL5 gradient, supporting an important role for this specific chemokine in the IH response.

### **IH increases leukocyte-endothelium interactions**

Leukocyte recruitment is an early stage linking systemic and tissue inflammation, with leukocyte rolling being one of the first step. In the present study, we showed increased adhesive interactions between leukocytes and microvessels in the IH animals. Similar microcirculation alterations, with increased leukocyte rolling and adhesion, have been recently shown after only three hours of experimentally induced obstructive apneas (33). These early alterations may rely on biomechanical activation (34), such as the oscillatory shear stress due to blood pressure fluctuations (26). This mechanism could be particularly relevant to the obstructive apnea condition that also includes respiratory efforts and related hemodynamic changes (33). Whereas leukocyte arrest was not significant in our IH model, we evidenced a mesenteric upregulation of the ICAM1 protein, which is crucial in the adhesion and transmigration steps of leukocyte recruitment (35) and could precede adhesion of leukocytes. This result is in agreement with clinical studies in OSA patients showing elevated circulating adhesion molecules (12).

### **IH induces structural remodeling of large arteries**

IH-mice exhibited an enlarged IMT without vascular dilatation, suggesting an expansive remodeling (34,36). While collagen alterations and lipid depositions were absent, elastic fibers were thicker, disorganized and separated by a larger distance due to SMC hypertrophy. These alterations suggest mechanical adaptations of the arterial wall. Indeed, we previously reported at a similar time point that IH induced blood pressure elevation and enhanced vasoconstriction to norepinephrine due to sympathetic hyperactivity (26). In response to elevated blood pressure, early functional and structural vascular plasticity occurs, including SMC hypertrophy and hyperplasia, elastic fiber thickening and abnormal extracellular matrix turnover (37). In addition to hemodynamic strains, hypoxia itself and the numerous alterations

generated by IH can contribute to SMC hypertrophy, through sympatho-adrenergic hyperactivity, endocrine and paracrine factors, as well as oxidative, inflammatory and growth factors (36). We also observed some mucoid depositions in the media which may originate from elastoid degeneration and/or mucoid degenerating SMC.

### **IH-induced inflammation of large arteries involves RANTES/CCL5**

To investigate whether the IH-induced vascular remodeling exhibited an inflammatory component, we studied the nuclear NFkB protein expression and some of its downstream targets. We found a higher NFkB-p50 expression in hypoxic mice, which is in accordance with recent studies showing NFkB activation in cardiovascular tissue from IH-mice (38) and in cells cultured under IH (7). This consistent IH-induced NFkB activation could underlie the inflammatory alterations in apneic patients, including increased adhesion molecules and chemokines (12,39) that play an important role in vascular remodelling (40). Among downstream NFkB targets, we uncovered RANTES/CCL5 overexpression in the media. This RANTES/CCL5 expression seemed to stem essentially from SMC, as suggested by the spindle-shaped staining, and not from T-cells as the latter were located in the adventitia-periadventitia. Another possible source of RANTES/CCL5 could be activated platelets, depositing RANTES/CCL5 on endothelial cells (40).

Chemokines and their receptors have a great impact on initiating and progressing vascular alterations, controlling each step of the remodeling process (40). Therefore, by expressing this leukoattractant chemokine, SMC could have participated in recruiting leukocytes into aortas during IH (41), as suggested by the enhanced *ex-vivo* splenocyte migration toward RANTES/CCL5. However, other chemokines, cytokines and adhesion molecules, downstream NFkB and produced by vascular cells, may also contribute to inflammatory remodeling (9). Indeed, ICAM-1 overexpression in IH-aortas may have also contributed to T-

lymphocyte infiltration, as ICAM-1 is critical in leukocyte recruitment into inflamed tissues (35). The slight aortic T-cell infiltration did not enlarge IMT, as lymphocytes were few and peripherally located. This rather indicates tissue inflammation, and the lymphocyte localization is in line with the recent concept of the "outside-to-inside" signaling (42). Indeed, atherosclerosis has been usually looked at as a vascular process starting at the intima level and progressing to media and adventitia (i.e. inside-out process). In contrast in our study, periadventitial T-cells suggest a role of the periadventitial fat in IH-related vascular inflammation (i.e. outside-in process). The perivascular fat is a specific subset of the white adipose tissue that secretes proatherogenic cytokines and chemokines, such as RANTES/CCL5; this may contribute to the early steps of atherogenesis by attracting inflammatory cells via the periadventitial vasa vasorum rather than from the systemic circulation at the intima level (42)."

The important role of RANTES/CCL5 has already been described in early vascular remodeling (43), as well as in advanced atherosclerotic process (19). Since we found predominant alterations of RANTES/CCL5 in splenocytes from IH mice, we hypothesized that RANTES/CCL5 inhibition could exert a beneficial effect on IH-induced vasculature remodeling. RANTES/CCL5 neutralization attenuated both intima-media thickening and vascular inflammation, without affecting the IH-induced proatherogenic dyslipidemia and HIF-related hematocrit elevation. These results demonstrate the significant contribution of inflammation to the vascular damages due to IH, and support RANTES/CCL5 involvement. Although RANTES/CCL5 expression can result from NF $\kappa$ B activation, RANTES/CCL5 neutralization also reduced IH-induced NF $\kappa$ B activation, likely due to reciprocal interactions (44) (figure 5G). It may result from a global anti-inflammatory effect, as similarly observed when using RANTES/CCL5 antagonist (45), despite HIF activation attested by hematocrit elevation. Indeed, besides preventing T-cell recruitment, RANTES/CCL5 neutralization

prevented activation of T-cells and possibly of SMC as suggested by the abolition of IH-induced Th1 cytokine IFN $\gamma$  expression.

## **Conclusion**

Atherosclerosis is considered as an immuno-inflammatory disease (8), suggesting that targeting inflammation could provide clinical benefit. In view of our results and of the literature, OSA should be considered as an immuno-inflammatory disorder as well. In the present study, we found moderate inflammatory changes, suggesting low-grade inflammation following IH exposure. Besides hemodynamic (26) and metabolic (46) alterations, we showed that the inflammatory response due to severe IH is an early and determinant factor that could contribute to OSA-associated pre-atherosclerotic remodeling.

Emerging therapies aiming at decreasing leukocyte activity and recruitment have led to promising results in atherosclerosis research, either using anti-CD3 antibody (18) or blocking chemokine pathways (19). Orally-administered small molecules to neutralize chemokines are under development and will have clear benefits over antibody treatment in terms of cost and ease of use, but their safety especially for chronic use, will need to be determined.

In this study, we evidenced RANTES/CCL5 involvement in the vascular remodeling initiated by severe IH, with an early involvement in both systemic and vascular inflammatory responses, implicating various cell-types (leukocytes, SMC). This preclinical study suggests that the inflammatory response should be considered in apneic patients, as a potential target for treatment of this disease.

**Acknowledgements:** The authors thank S. Cachot, N. Miguet and S. Jacquard for technical help.

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## FIGURE LEGENDS

### **Figure 1. Intermittent hypoxia induces splenocyte activation**

Splenocyte proliferation in response to concanavalin-A after 5 (A) and 14 (B) days of intermittent hypoxia (IH) or air (N); \* $p < 0.05$  vs N (n=6-12 per group). Splenocyte mRNA expression of RANTES/CCL5 (C), MIP-1 $\alpha$ /CCL3 (D), MIP-1 $\beta$ /CCL4 (E) and MCP-1/CCL2 (F). Measurements were normalized to the eukaryotic 18S ribosomal-RNA (G) and expressed as fold induction of their baseline values; \* $p < 0.05$  vs N (n=6 per group). Splenocyte migration toward RANTES/CCL5 after 14 days of IH or air (H). Splenocytes were tested without stimulation or after 50 ng.ml<sup>-1</sup> interferon-gamma (IFN- $\gamma$ ) stimulation;  $p < 0.05$  vs unstimulated\* or stimulated $\dagger$  splenocytes from N-mice (n=4).

### **Figure 2. Intermittent hypoxia increases leukocyte-endothelium adhesive interactions**

Leukocyte-microvessel interplay was assessed by leukocyte rolling and ICAM-1 expression in mesenteric vessels from mice exposed to intermittent hypoxia (IH) or air (N) for 14 days. (A) Representative photographs showing leukocyte rolling (arrow). (B) Rolling quantification (n=4-5 each). Representative immunoblotting (C) and quantitative analysis (D) of ICAM-1 expression (n=4 each).

### **Figure 3. Intermittent hypoxia induces structural aorta remodeling**

Vascular remodeling was assessed in mice exposed to intermittent hypoxia (IH) or air (N) for 14 days. (A) Hematoxylin-eosin stainings (10x10 magnification and digitally magnified insets). (B) Histomorphometric analysis of intima-media thickness, n=7 each. (C) Verhoeff coloration showing thicker elastic fibers in IH-mice (10x40 magnification). Elastic fiber separation (D) and smooth-muscle cell (SMC) nuclei (E) in the media (n=7 each). (F) Alcian

blue coloration showing mucoid depositions between the elastic fibers in IH-mice (arrows, 10x40 magnification).

#### **Figure 4. Intermittent hypoxia induces aorta inflammation**

Inflammation was assessed in mice exposed to intermittent hypoxia (IH) or air (N) for 14 days. (A) CD3 immunostaining with arrows showing T-cells (10x20 magnification). (B) Quantitative analysis of CD3-positive cell infiltration according to the various tunica of the aortic wall. Note that T-cells predominated in the adventitia-periadventitia tunica (n=9-10 per group). (C) Representative RANTES/CCL5 immunostaining (10x20 magnification). Immunoblottings and quantifications of nuclear NFkB-p50 (D) and cytosolic ICAM-1 (E) (n=4 each).

#### **Figure 5. RANTES/CCL5 neutralization attenuates IH-induced aorta remodeling**

Mice were exposed to intermittent hypoxia (IH) or air (N) for 14 days, and treated either with the anti-RANTES/CCL5 monoclonal antibody or with the control IgG throughout the exposure. (A) Intima-media thickness (n=7-8 each). (B) Cytosolic  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression with representative immunoblotting and quantitative analysis (n=5-7 each). (C) Nuclear NFkB-p50 expression with representative immunoblotting and quantitative analysis (n=4-7 each). (D) Quantification of T-cell infiltration in the aortic wall (n=7-8 each). (E) Representative RANTES/CCL5 immunostaining (10x20 magnification). (F) IFN $\gamma$  mRNA expression normalized to ubiquitin (n=4-9 each). \*p<0.05 vs N-IgG; †p<0.05 vs anti-RANTES treated IH-mice. (G) Regulation pathway of NFkB activation and subsequent leukocyte recruitment and activation (adapted from Ye et al, (44)).