



Matrix Metalloproteinase-12 Supports Pulmonary B Cell Follicle Formation and Local Antibody Responses During Asthma

To the Editor:

A protease–antiprotease imbalance is a feature of many chronic lung diseases, with concentrations and activity of several MMPs (matrix metalloproteinases) correlating with disease pathology. MMP-12 plays a prominent role in lung tissue remodeling owing to its capacity to degrade elastin and other extracellular matrix constituents but is also increasingly recognized for immunomodulatory functions central to the regulation of innate immunity. MMP-12 is elevated in patients with asthma and, in some instances, associated with more severe forms of disease (1, 2). Mouse models of allergic airway disease (AAD) have defined a central role for MMP-12 in driving airway remodeling and recruitment of innate immune cells (3, 4). However, the role of MMP-12 in regulating adaptive immunity in chronic lung diseases has been neglected.

In a mouse model of house dust mite (HDM)-induced AAD (Figure 1A) (5), C57BL/6 wild-type (WT) mice showed a strong induction of airway MMP-12, which was absent in *Mmp12*^{−/−} (KO) mice (Figure 1B). Although numbers of CD3⁺ T cells were comparable between WT and KO animals (Figure 1C), lung B cells were significantly reduced in HDM-treated KO animals (Figure 1D). Numbers of B cells in the lung draining mediastinal lymph nodes (Figure 1E) and peripheral blood (Figure 1F) were not significantly different in allergen-exposed WT and KO mice, indicating the reduced B cell response in KO animals was lung specific. Chronic HDM exposure induces the formation of tertiary lymphoid structures containing ectopic germinal centers in the lungs of mice. Significantly fewer germinal center B cells were present in the lungs of

HDM-exposed KO mice relative to WT control mice (Figure 1G), but T follicular helper cell numbers were comparable (Figure 1H).

Histological analysis revealed a reduction in the number and size of pulmonary B cell aggregates (Figures 1I–1K), and MMP-12 concentrations correlated with total PAX5⁺ area ($r = 0.5098$; $P = 0.0013$). Interrogation of precision-cut lung slices by confocal microscopy demonstrated the colocalization of germinal center B cells and CD4⁺ T cells (Figure 1L) and validated that the number of B cell aggregates (Figure 1M) and the individual aggregate volume (Figure 1N) were reduced in HDM-exposed KO mice.

In keeping with the diminished pulmonary B cell aggregates in HDM-KO mice, concentrations of airway total IgE (HDM-specific IgE below limits of detection) and HDM-specific IgA were also significantly reduced (Figures 1O and 1P). Systemic HDM-specific IgE was also reduced in HDM-treated KO mice (Figure 1Q), whereas IgG1 was comparable to WTs (Figure 1R). Airway MMP-12 positively correlated with IgE ($r = 0.612$; $P < 0.0001$) and IgA ($r = 0.482$; $P = 0.002$). Furthermore, mast cell protease-1, a surrogate for IgE-induced mast cell degranulation, was reduced in HDM-treated KO mice relative to WT control mice and showed a significant correlation with IgE ($r = 0.681$; $P < 0.0001$). These data show that MMP-12 is important for functional B cell aggregate formation and local antibody responses during AAD.

CXCL13 (chemokine (C-X-C motif) ligand 13) is a B cell chemokine demonstrated to drive aggregate formation in tertiary lymphoid structures in mouse models of AAD (6), and CXCL12 has been demonstrated to be important in distinct lung inflammatory models (7). MMP-12 can modulate the activity of CXC chemokines by proteolytic processing (8). Although lung CXCL13 (Figure 2A) and CXCL12 (Figure 2B) concentrations were comparable between HDM-treated WT and KO mice, MMP-12 proteolytically cleaves CXCL13, but not CXCL12, *in vitro* (Figure 2C). Mass spectrometry analysis demonstrated that recombinant CXCL13 (9,812 Da) yields a product of 8,239 Da upon exposure to MMP-12. Deconvolution of these data suggested that MMP-12 cleaves CXCL13 between Ser-94 (P1) and Leu-95 (P1') at the chemokine's C-terminal domain (Figure 2D). MMP-12 cleavage of CXCL13 was subsequently assessed *in vivo* in allergen-exposed mice by Western blot. Antibodies to CXCL13 frequently target its C-terminus and were thus found to bind MMP-12–cleaved CXCL13 with reduced affinity resulting in lower-intensity bands (Figures 2E and 2F). CXCL13 concentrations present in BAL fluid (BALF) and lungs of HDM-treated mice were below the limit of detection by Western blot (Figures 2E and 2F). When BALF of WT HDM-exposed mice was incubated *ex vivo* with CXCL13, it cleaved the chemokine in an analogous manner to MMP-12; however, no cleavage was observed with KO BALF (Figure 2E). CXCL13 immunoprecipitations of lung homogenate from HDM-treated WT and KO mice were performed to enrich the chemokine. Full-length CXCL13 was detectable in immunoprecipitations of MMP-12 KO mice, whereas the truncated form was detectable in WT immunoprecipitations (Figure 2F). Therefore, MMP-12 cleavage of CXCL13 occurs *in vivo* in HDM-exposed mice.

The excised fragment at the C-terminus of CXCL13 contains a binding site for the glycosaminoglycan heparan sulfate of the extracellular matrix (ECM) (9). Accordingly, MMP-12–cleaved CXCL13 demonstrated a reduced capacity to bind to biotinylated heparan sulfate (Figure 2G). Previous work demonstrated that cathepsin B operates within lymph nodes to cleave CXCL13 at a comparable site to MMP-12 and that ensuing cleavage-dependent

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Author Contributions: C.J.P., D.F.P., and R.J.S. designed and interpreted the experiments, performed statistical analysis, and prepared the manuscript. C.J.P., with the assistance of D.F.P., T.P., R.J., and R.J.S., performed the majority of the experiments. A.M.G., T.H., G.T., and A.S. were instrumental in the acquisition and processing of samples from the primary patient cohort. J.P., J.A.H., and C.M.L. provided key reagents and contributed discussions throughout the work.

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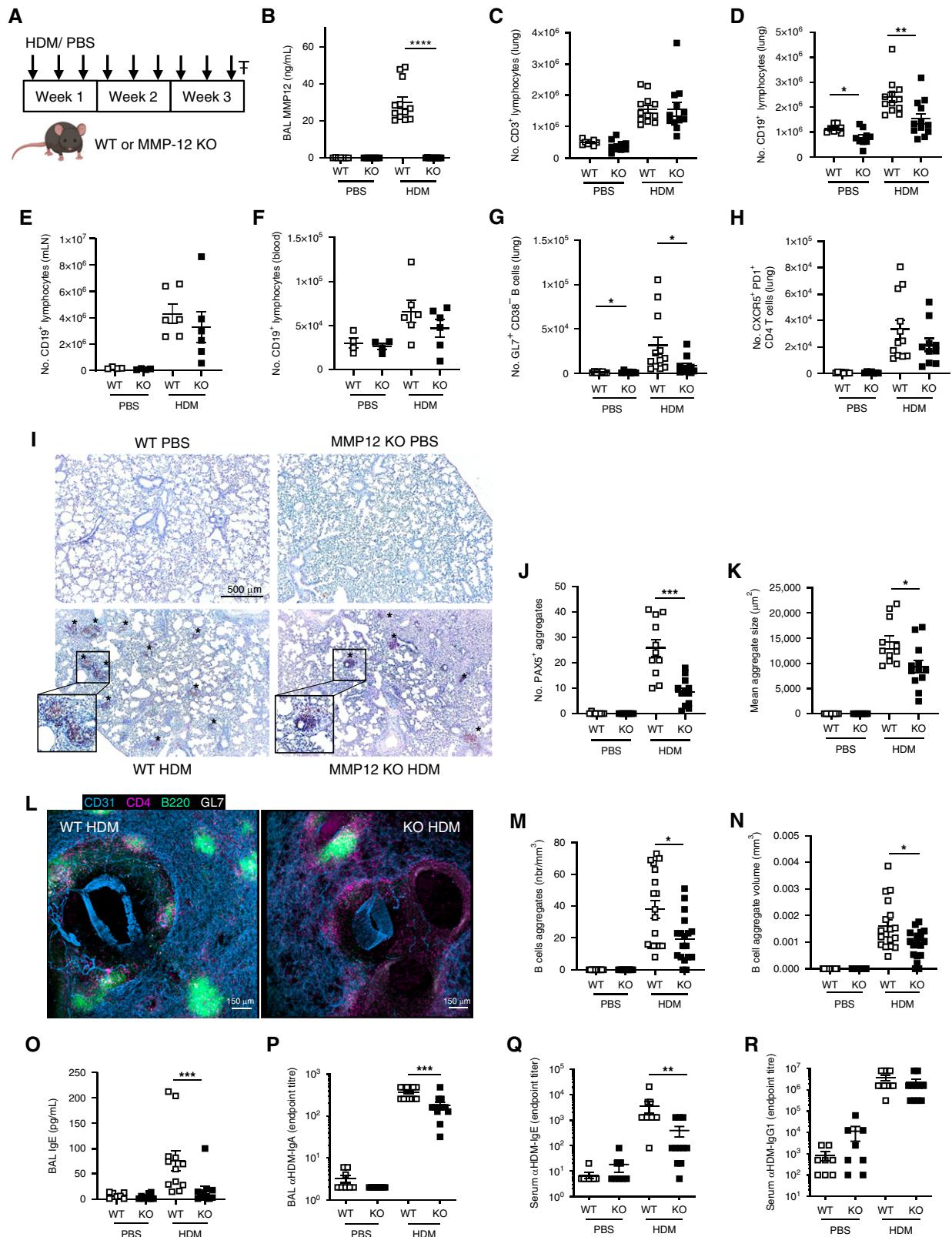


Figure 1. MMP-12 (matrix metalloproteinase-12) regulates formation of pulmonary B cell aggregates in a mouse model of allergic airway disease. (A) Male, 6- to 8-week-old C57BL/6 wild type (WT) and *Mmp12*^{-/-} (KO) mice (purchased from JAX and subsequently bred in-house) were administered 25 μ g house dust mite (HDM) extract (Citeq Biologics) or 50 μ l sterile phosphate-buffered saline (PBS) intranasally three times per week for 3 weeks. Mice were culled (T) 24 hours after the final dose of HDM or PBS (5). All animal procedures and care conformed strictly to

liberation of the chemokine from the ECM was critical for formation of B cell follicles (10). We suggest that MMP-12 cleavage of CXCL13 performs an analogous role within the lung, thus rationalizing the lung-specific deficiency observed in B cell follicle formation in HDM-treated KO mice.

The clinical significance of our findings was interrogated in a previously described (5) patient cohort of healthy volunteers and patients with moderate to severe asthma. Sputum MMP-12 was significantly elevated in patients with asthma relative to healthy control subjects (Figure 2H). Sputum IgE concentrations were generally very low and often undetectable (potentially attributable to sequestration by FcεR1-expressing cells) (Figure 2I). However, IgA was elevated in the airways of patients with asthma (Figure 2J) and significantly correlated with MMP-12 concentrations (Figure 2K), supportive of a role for this protease in local antibody responses in patients with asthma.

Although a protease imbalance is a hallmark of many chronic lung diseases, and MMP-12 has been associated with structural changes and modulation of innate immunity in this context, our studies also now suggest a prominent role for MMP-12 in defining B cell and local antibody responses in the lung in the context of asthma. We also highlight the capacity of MMP-12 to proteolytically modify the critical B cell chemokine CXCL13, reducing its capacity to bind to ECM heparan sulfate. Additional studies are needed to unequivocally demonstrate the significance of this MMP-12 processing of CXCL13 in defining pulmonary B cell and local airway antibody responses in mouse models of AAD and in patients with asthma.

Lung B cell aggregates have been previously reported in patients with asthma at a greater size and number than in healthy volunteers and correlating with eosinophilic infiltration, airway wall thickening, and disease severity (11), suggestive of a role in potentiating pulmonary pathology. Furthermore, allergen-specific IgE⁺ B cells have been described within these aggregates in the context of AAD in patients (12), again associating with pathological features of disease. Mucosal production of IgE and ensuing degranulation of mast cells is a major pathway of the inflammatory response underlying asthma. Secretory IgA responses to allergens in patients with asthma have been suggested to play a pathogenic role through eosinophil activation, with IgA shown to induce eosinophil degranulation *in vitro* and airway IgA

concentrations correlating with eosinophil cationic protein during late asthmatic responses (13). Moreover, it is plausible that our findings could also be of broader significance in the context of chronic inflammation or host immunity to infectious challenge. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

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Figure 1. (Continued). the UK Home Office Guidelines under the Animals (Scientific Procedures) Act 1986, and protocols were approved by the Home Office of Great Britain. (B) MMP-12 concentrations in BAL fluid were determined by ELISA (Abcam). (C and D) CD45⁺ (30-F11, BioLegend), CD3⁺ (145-2C11, eBioscience) T cells (C) and CD45⁺, CD3⁺, CD19⁺ (1D3, BioLegend) B cells (D) within lungs of mice were enumerated from single-cell suspensions by flow cytometry (5). (E and F) B cell numbers were enumerated in mediastinal lymph nodes (mLN; E) and peripheral blood (F) by flow cytometry. (G and H) Lung GL7⁺ (GL7, BioLegend), CD38⁺ (90, BioLegend) germinal center B cells (G) and CXCR5⁺ (L138D7, BioLegend), PD1⁺ (RMP1-30, BioLegend) T follicular helper cells (H) were enumerated by flow cytometry. (I–K) Representative images of formalin-fixed, wax-embedded lung sections (4 μm) (5) stained for PAX5 (30 ng/ml anti-PAX5 Antibody EPR3730[2]; Abcam). B cell aggregates (brown; *) were scored from four fields of view per section using a Leica DFC300FX microscope, and individual aggregate size was enumerated using Fiji software. (L) Representative images of agarose-inflated, paraformaldehyde-fixed precision-cut lung slices (PCLSs; 200 mm transverse sections) stained with B220 (RA3-6B2; Biolegend), CD4 (RM4-5; Biolegend), CD31 (polyclonal goat IgG; R&D Systems), and GL7 (GL7, Biolegend). PCLSs were imaged on an inverted laser scanning confocal SP5 (Leica Microsystems) using a 20× objective at a resolution of 512 × 512 pixels. (M and N) PCLS image analysis and rendering was performed using IMARIS software 8.1 version (Bitplane, Oxford Instruments) on six fields of view per PCLS. (O) Concentration of total IgE was determined in BAL fluid of mice by ELISA (eBioscience). (P) Concentration of HDM-specific IgA in BAL fluid was determined using an in-house ELISA, whereby serially diluted BAL fluid was incubated with plates precoated with HDM (50 μg/ml; Citeq), and bound IgA was subsequently detected using Clonotyping System-HRP (Southern Biotech). Data are expressed as the lowest dilution at which antibody is still detectable. (Q) Concentrations of HDM-specific IgE and (R) HDM-specific IgG1 were determined in the serum by ELISA using the same method outlined in P. Results are depicted as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 using Mann-Whitney statistical test.

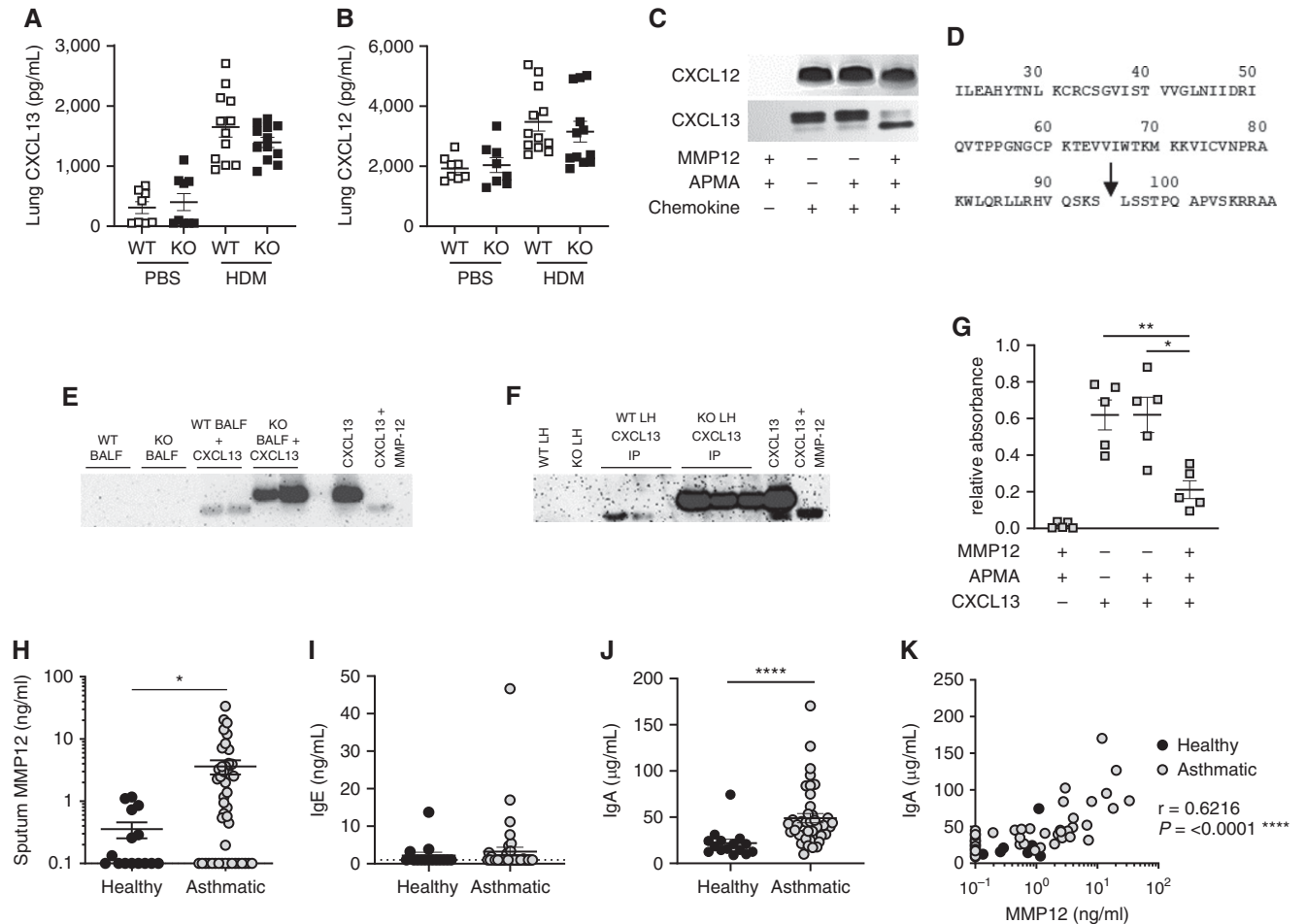


Figure 2. MMP-12 (matrix metalloproteinase-12) proteolytically processes CXCL13 to modulate glycosaminoglycan binding and correlates with airway antibody responses in patients with asthma. (A and B) The concentrations of CXCL13 (A) and CXCL12 (B) within lung homogenate (5) were measured by ELISA (R&D Systems). (C) Recombinant mouse CXCL13 and CXCL12 (both 100 μg/ml; Peprotech) were incubated with APMA (4-Aminophenylmercuric acetate)-activated (Sigma Aldrich) recombinant mouse MMP-12 (10 μg/ml; Abcam) for 8 hours at 37°C. The reaction products were separated by gel electrophoresis using NuPAGE 4–12% Bis-Tris Protein Gels (Thermo Fisher) and detected using Pierce Silver Stain kit. (D) Identification of murine MMP-12 cleavage products by an Applied Biosystems 4800 MALDI-TOF mass spectrometer in linear mode; MMP-12 cleavage site within murine CXCL13 denoted by arrow. (E) BAL fluid (BALF) from HDM-exposed wild-type (WT) or *Mmp12*^{-/-} (KO) mice was incubated with CXCL13 (50 ng; Peprotech) for 16 hours at 37°C. The reaction products were separated by gel electrophoresis using NuPAGE 4–12% Bis-Tris Protein Gels (Thermo Fisher). Comparable amounts of WT and MMP-12 KO BALF alone or recombinant CXCL13 alone (with or without MMP-12 cleavage as described above) were run as controls. Proteins were transferred to PVDF (polyvinylidene fluoride) membrane and probed with biotinylated anti-CXCL13 antibody (0.2 μg/ml; R&D Systems), followed by Streptavidin-HRP conjugate (R&D Systems). (F) Immunoprecipitations (IPs) were performed on pooled lung homogenate (LH) from HDM-exposed WT or MMP-12 KO mice using biotinylated anti-CXCL13 antibody (10 μg; R&D Systems) and Streptavidin Dynabeads (Invitrogen). Samples were interrogated by Western blot as detailed above. WT and MMP-12 KO LH or recombinant CXCL13 (with or without MMP-12 cleavage as defined above) were run as controls. (G) CXCL13–MMP-12 reaction products (10 pmol CXCL13/well) were assessed for their capacity to bind biotinylated heparan sulfate (Sigma). (H–K) Analysis of sputum samples from a cohort (5) of 15 healthy volunteers and 42 patients with moderate to severe asthma (defined by British Thoracic Society guideline criteria [disease steps 3–5]; Research Ethics Committee Reference 10/H1010/7). (H) Sputum MMP-12 measured by ELISA (Abcam). (I and J) Sputum IgE (I) and IgA (J) measured by ELISA (Invitrogen). (K) Correlation between sputum MMP-12 and IgA. Solid symbols represent healthy control subjects; open symbols represent patients with asthma. Results depicted as mean ± SEM. (A, B, G–J) **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001 using Mann-Whitney statistical test. Correlation analysis (K) was performed using Spearman rank test.

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Voltage-Gated Sodium Channel Na_v1.8 Dysregulates Na and Ca, Leading to Arrhythmias in Patients with Sleep-Disordered Breathing

To the Editor:

Sleep-disordered breathing (SDB) has evolved as a widespread disease with currently more than 1 billion affected patients worldwide (1, 2). Importantly, patients with SDB are at increased risk for cardiac arrhythmias such as atrial fibrillation, but drug treatment options are very limited (1, 3). Thus, new therapeutic approaches are urgently needed, for which detailed knowledge of cellular pathomechanisms is essential. Interestingly, the voltage-gated sodium channel isoform 1.8 (Na_v1.8) was demonstrated to be upregulated in ventricular hypertrophy and heart failure, favoring two proarrhythmic mechanisms, late sodium current and diastolic sarcoplasmic reticulum calcium leak (4). We conducted an observational study to test whether myocardial Na_v1.8 expression is specifically increased in patients with SDB independent of cardiac disease and if selective Na_v1.8 inhibition prevents proarrhythmic activity in SDB. Some of the following data have been previously reported in the form of an abstract (5).

Methods

This study was performed in compliance with the Declaration of Helsinki (2013 revision) and approved by the local ethics committee (University of Regensburg, Bavaria, Germany; 15-238-101). It is part of the prospective observational study CONSIDER-AF (Impact of Sleep-Disordered Breathing on Atrial Fibrillation and Perioperative Complications in Patients Undergoing Coronary Artery Bypass Grafting Surgery; NCT 02877745), analyzing right atrial appendage biopsies from 120 patients who underwent elective coronary artery bypass grafting and were tested for SDB by standard polygraphy during the preoperative night (6). An apnea–hypopnea index (AHI) ≥ 15 events/h defined SDB. Other inclusion criteria were age between 18 and 85 years, provision of written informed consent, and the availability of atrial biopsies. Exclusion criteria were existing home

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