

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Tight junction, mucin, and inflammasome-related molecules are differentially expressed in eosinophilic, mixed, and neutrophilic experimental asthma in mice

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Abstract

Background: Asthma is a chronic respiratory disease with marked clinical and pathophysiological heterogeneity. Specific pathways are thought to be involved in the pathomechanisms of different inflammatory phenotypes of asthma; however, direct in vivo comparison has not been performed.

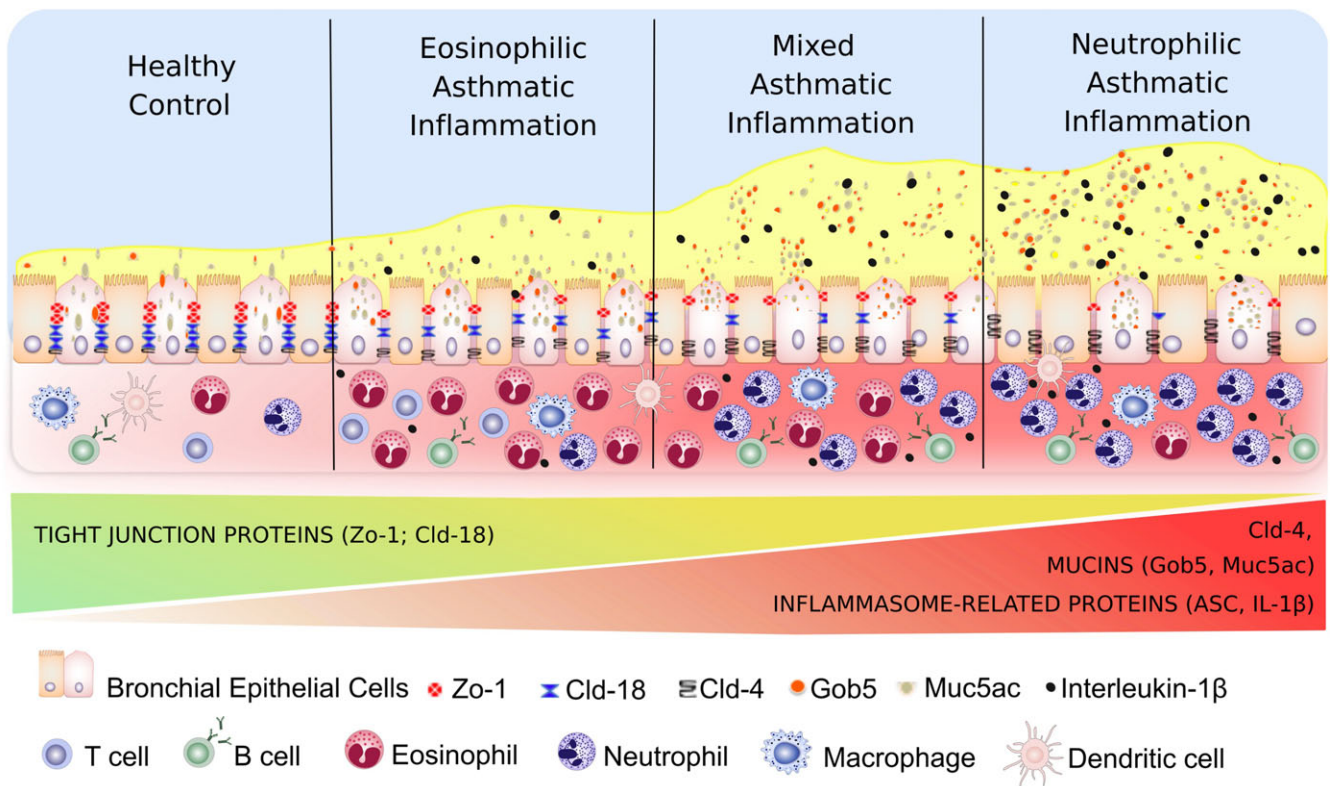
Methods: We developed mouse models representing three different phenotypes of allergic airway inflammation—eosinophilic, mixed, and neutrophilic asthma via different methods of house dust mite sensitization and challenge. Transcriptomic analysis of the lungs, followed by the RT-PCR, western blot, and confocal microscopy, was performed. Primary human bronchial epithelial cells cultured in air-liquid interface were used to study the mechanisms revealed in the in vivo models.

Results: By whole-genome transcriptome profiling of the lung, we found that airway tight junction (TJ), mucin, and inflammasome-related genes are differentially expressed in these distinct phenotypes. Further analysis of proteins from these families revealed that Zo-1 and Cldn18 were downregulated in all phenotypes, while increased Cldn4 expression was characteristic for neutrophilic airway inflammation. Mucins Clca1 (Gob5) and Muc5ac were upregulated in eosinophilic and even more in neutrophilic phenotype. Increased expression of inflammasome-related molecules such as Nlrp3, Nlrp4, Casp-1, and IL-1 β was characteristic for neutrophilic asthma. In addition, we showed that inflammasome/Th17/neutrophilic axis cytokine—IL-1 β —may transiently impair epithelial barrier function, while IL-1 β and IL-17 increase mucin expressions in primary human bronchial epithelial cells.

Conclusion: Our findings suggest that differential expression of TJ, mucin, and inflammasome-related molecules in distinct inflammatory phenotypes of asthma may be linked to pathophysiology and might reflect the differences observed in the clinic.

KEYWORDS

endotype, epithelial barrier, house dust mite, phenotype, precision medicine



GRAPHICAL ABSTRACT

Tight junction, mucin, and inflammasome-related molecules are differentially expressed in the lungs in distinct inflammatory phenotypes of allergic airway inflammation (AAI): eosinophilic, mixed, and neutrophilic AAI. Zo-1 and Claudin-18 are downregulated, whereas Clca1 (Gob5) and Muc5ac are upregulated in all phenotypes. Claudin-4, Nlrp3, Nlrp4, Casp-1, and IL-1 β are upregulated in neutrophilic AAI. IL-1 β impairs epithelial barrier function and increases MUC5AC expression in differentiated primary human bronchial epithelial cells (HBECS). IL-17 also increases MUC5AC expression in HBECS.

1 | INTRODUCTION

Asthma is a chronic respiratory disease with complex clinical and pathophysiological features. Extensive research on clusters of clinical and molecular features of asthma improved our understanding of different asthma phenotypes and their underlying molecular processes, named endotypes.¹⁻³ Distinct inflammatory phenotypes of eosinophilic, mixed, neutrophilic, and paucigranulocytic asthma are identified, while many endotypes are yet to be confirmed.² Generally, there are two main complex asthma endotypes: (a) the type 2 asthma, observed in early-onset allergic asthma, late-onset eosinophilic asthma, and probably in exercise-induced asthma phenotypes; and (b) the non-type 2 asthma, observed as obesity-related asthma, neutrophilic asthma, and paucigranulocytic asthma.² Type 2 asthma involves Th2 cells, type 2 innate lymphoid cells (ILC2s), type 2 B cells, eosinophils, mast cells, and their major cytokines—IL-4, IL-5, and IL-13. In contrast, non-type 2 asthma is characterized by infiltration of Th1/Th17 cells, neutrophils, the presence of type I interferons, and IL-17.⁴ Pathophysiology behind subgroups of non-type 2 asthma remains poorly understood. Patients with neutrophilic and mixed asthma respond poorly to corticosteroid therapy,⁵ and increased numbers of

neutrophils with persistent eosinophilia are seen in severe asthma and sudden-onset fatal asthma.^{6,7} In both types of complex phenotypes, other factors such as genetics,^{8,9} epithelial barrier dysfunction,¹⁰ innate immune response,^{11,12} environmental exposures,^{13,14} viral infections,¹⁵ and comorbidities¹⁶ may further modulate inflammation, bringing the stability of dominant physiopathological mechanisms to question.¹⁷ Therefore, better understanding of the characteristics of each inflammatory subgroup is crucial for extensive development of personalized/precision medicine.^{18,19} Several animal models have been developed to mimic and study in vivo asthma-like airway inflammation, but most of them are focused on acute eosinophilic inflammation as a hallmark of the type 2 asthma. So far, the detailed unbiased in vivo studies on molecular responses among different kinds of inflammation in asthma models are lacking.²⁰

Airway epithelial cells and mucus are the first physical barriers protecting against inhaled allergens and pathogens, and mounting an innate immune response against these environmental exposures.²¹ Recently, we and others have shown that epithelial tight junction (TJ) molecules' expression and barrier function are impaired in asthma patients.^{10,22} In addition, several important mucus genes such as MUC5AC and CLCA1 (GOB5) are upregulated in asthmatic airways.^{23,24} It is, however, poorly

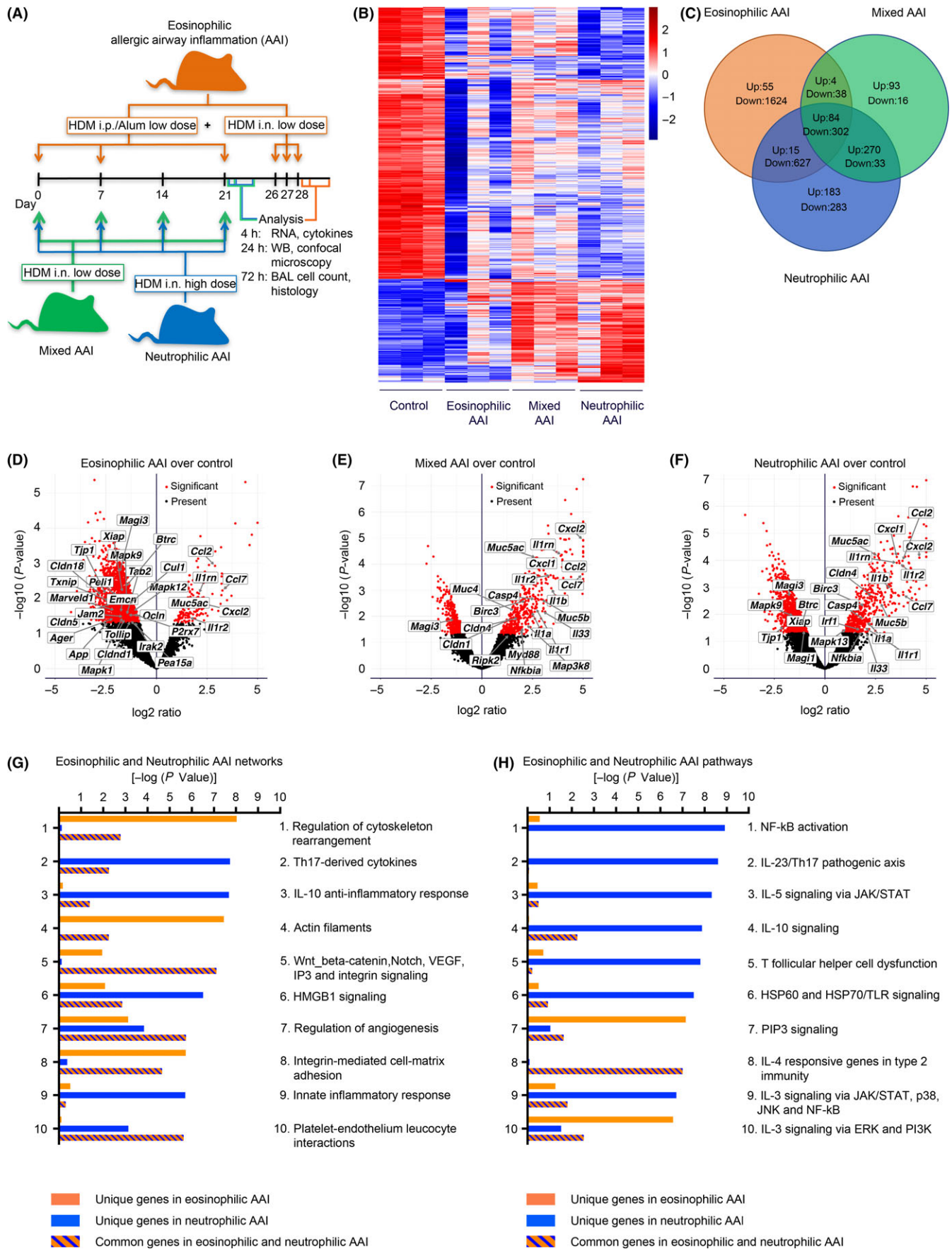


FIGURE 1 Distinctive lung transcriptomic profiles in eosinophilic, mixed, and neutrophilic asthma-like allergic airway inflammation in mice (AAI). A, In vivo models of eosinophilic, mixed, and neutrophilic AAI, performed in the study. HDM low dose refers to 0.75 DU Der f and 0.75 DU Der p in HDM extract. HDM high dose refers to 7.5 DU Der f/7.5 DU Der p in HDM extract. Mice were killed at 4 h for RNA analysis by microarray and RT-PCR, at 24 h for protein analysis by Western blot and confocal microscopy and at 72 h for BAL cell count and histology after the last challenge in each model. B, All differentially regulated genes ($P < 0.01$) in the whole lung tissue between all phenotypes and control at 4 h after last allergen exposure ($n = 3$ mice/group). C, Venn diagram of differentially regulated genes in eosinophilic AAI (orange, $n = 2749$, $P < 0.05$), mixed AAI (green, $n = 840$, $P < 0.05$), and neutrophilic AAI (blue, $n = 1797$, $P < 0.05$) as compared to control group in the whole lung tissue. D, Volcano plot of eosinophilic AAI over control ($P < 0.05 = -\log_{10}(p) > 1.3$). E, Volcano plot of mixed AAI over control ($P < 0.05 = -\log_{10}(p) > 1.3$). F, Volcano plot of neutrophilic AAI over control ($P < 0.05 = -\log_{10}(p) > 1.3$); x-axis depicts $\log_2$ fold change with upregulated genes on the right and downregulated genes on the left, y-axis depicts level of significance. Designated genes are from tight junction, mucin, and inflammasome-related gene groups. G, Top ten (most significantly enriched) general networks within differentially expressed genes (defined as in c) unique for eosinophilic (orange) or neutrophilic (blue) AAI or common for both phenotypes (orange-blue stripes). H, Top ten (most significantly enriched) pathways within differentially expressed genes (defined as in c) unique for eosinophilic (orange) or neutrophilic (blue) AAI or common for both endotypes (orange-blue hatched). Networks and pathways with $P < 0.05$ ($-\log_{10}(p) > 1.3$) are considered statistically significant. i.p., intraperitoneal; i.n., intranasal; HDM, house dust mite

understood, whether the expression of TJs and mucins is linked to specific asthma phenotypes or endotypes.

Recent data suggest that neutrophilic inflammation in asthma is based on the axis of inflammasome activation and concomitant IL-1 β /IL-17-induced neutrophil activation.^{25,26} Inhibition of NLRP3 inflammasome activation in mouse model of severe, neutrophilic asthma prevented the disease,^{26,27} while it was entirely ineffective in the allergic eosinophilic asthmatic inflammation in mice.²⁸ Therefore, the direct comparison of the expression of inflammasome complexes in different types of asthmatic phenotypes is essential for further understanding of these phenomena.

Hence, we sought to perform an in vivo unbiased investigation of the molecular pathomechanisms of different phenotypes of allergic airway inflammation (AAI), namely eosinophilic, mixed, and neutrophilic. Based on the differentially expressed gene families, we studied in detail the mRNA, protein expression, and localization of TJ, mucin, and inflammasome-related molecules in order to define signatures of each phenotype. To translate our findings into humans, we analyzed the role of IL-1 β and IL-17 on the barrier function and mucins in human primary differentiated bronchial epithelial cells.

2 | METHODS

Full methods are provided in the Data S1.

2.1 | Mouse models of different inflammatory phenotypes in AAI

Three different inflammatory acute asthma phenotypes—eosinophilic, mixed, and neutrophilic, were induced in Balb/c mice via different ways of house dust mite sensitization (Figure 1A). All animal procedures were performed with approval from the University of Marburg local authorities, that is, the Regierungspräsidium Giessen (approval number: MR 20/13 Nr. 63/2013).

2.2 | Human bronchial epithelial cell culture, cytokine stimulations, transepithelial resistance (TER), and paracellular flux

Human bronchial epithelial cells (HBECs) (Lonza, Basel, Switzerland) were cultured in air-liquid interface (ALI) with IL-1 β (R&D Systems, Minneapolis, MN, USA), IL-17A (PeproTech), or medium alone. Transepithelial resistance (TER) was measured by a Millicell ERS Volt-Ohm Meter (Millipore, Billerica, MA, USA). Epithelial permeability was measured by adding fluorescein isothiocyanate (FITC)-dextran (2 mg/mL; Sigma-Aldrich, St. Louis, Missouri, United States).

2.3 | mRNA isolation and RT-PCR

mRNA was isolated using the RNeasy Mini and Micro kit (Qiagen, Hilden, Germany). Reverse transcription was performed with RevertAid RT kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). cDNA was amplified using SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, United States) in QuantStudio 7 Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Primer sequences can be found in the Table S1.

2.4 | Gene array analysis

Total RNA was processed for GeneChip analysis using the GeneChip WT Plus Reagent Kit and analyzed using GeneChipTM Mouse Gene 2.0 ST microarray chips (Affymetrix, Santa Clara, CA, USA). The entire data set has been submitted to the NCBI GEO: accession number GSE108417. Networks and pathway maps were generated using the MetaCore version 6.30 software and STRING version 10.5.²⁹

2.5 | Western blotting

Twenty micrograms of lysate protein was loaded onto 4%–20% Mini-PROTEAN TGX polyacrylamide gels (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to nitrocellulose

membranes, blocked, and then incubated with primary antibodies (1:500 for Zo-1, 1:250 for Claudin-18, 1:200 for Claudin-4, 1:10 000 for Clca1, 1:2000 for Muc5ac, and 1:1000 for IL-1 β [R&D Systems]). The blots were incubated with the HRP-conjugated secondary antibodies (1:10 000).

2.6 | Immunofluorescence staining

Permeabilization and blocking were done before incubation with polyclonal rabbit anti-Zo-1 (Invitrogen, Camarillo, CA, USA), monoclonal rabbit anti-Claudin-18 (Invitrogen), polyclonal rabbit anti-Claudin-4 (Abcam, Cambridge, UK), or monoclonal rabbit anti-Clca1 (Gob5) (Abcam) at 1:500; or polyclonal rabbit anti-ASC or monoclonal mouse anti-Mucin 5AC (Abcam) at 1:100 in 1% BSA in PBS, followed by incubation with Alexa Fluor 546-labeled goat anti-rabbit antibody.

2.7 | Statistics

Data analysis was performed with GraphPad Prism Version 7 software (GraphPad Software, La Jolla, CA, USA). Differences between groups were analyzed by using *t* test, Mann-Whitney *U* test, one-way ANOVA, or Kruskal-Wallis test with post hoc analysis, as appropriate. The significance level α was set to 0.05.

3 | RESULTS

3.1 | Development of eosinophilic, mixed, and neutrophilic models of AAI via different sensitization schemes

To study the characteristic features of eosinophilic, mixed, and neutrophilic asthma, we used different mouse models of AAI using house dust mite (HDM) extract via different routes, doses, and timing of sensitization (Figure 1A). The eosinophilic model was induced by injecting low-dose HDM i.p., together with alum preceding subsequent low-dose intranasal (i.n.) allergen challenges. Majority of cells in BAL were eosinophils, with nearly a complete absence of neutrophils. In contrast, by giving high doses of HDM intranasally (i.n.) to the mice, we observed high neutrophil counts in the BAL, with limited numbers of eosinophils. Mixed airway inflammation with both eosinophils and neutrophils in the BAL was induced by using low doses of i.n. HDM (Table S2). The analysis of cytokine levels in the BAL and in lung homogenates in mice with eosinophilic infiltration showed an abundance of IL-5, little amount of IFN- γ , and practically no IL-17A, confirming the type 2 endotype. In the neutrophilic inflammation, high abundance of IL-17A and IFN- γ with low IL-5 suggests predominant type 1/17 endotype with only some type 2-like background. In the mixed inflammation, there was more IL-5 with relatively less amounts of IL-17A and IFN- γ , implying indeed mixed type 2 and type 1/17 endotype (Table S2).

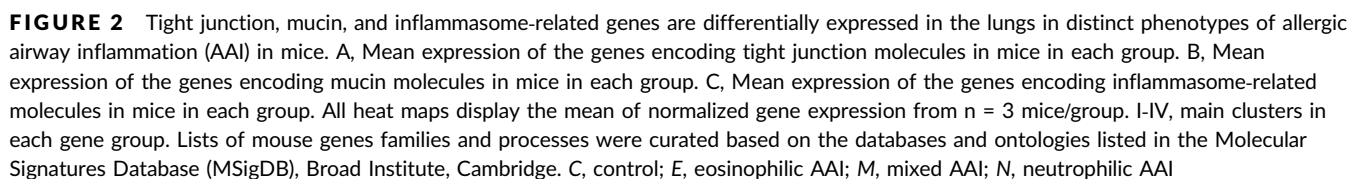
3.2 | Airway barrier molecules, mucins, and inflammasome-related genes are differentially expressed in distinct phenotypes of AAI

Molecular characteristics of three different types of AAI in mice were first profiled by transcriptome analysis of the whole lungs. It revealed a striking difference between control and diseased mice (Figure 1B-F) with the evident changes also between different phenotypes (Figures 1C, S1A-D). A total of 1679 transcripts were uniquely regulated in eosinophilic inflammation, 466 in neutrophilic inflammation, and only 109 in mixed phenotype (Figure 1C). Eosinophilic AAI was most distinct, while mixed AAI and neutrophilic AAI were resembling each other (Figures 1D-F, S2A-C). Therefore, we further compared eosinophilic and neutrophilic inflammation for pathways and networks. Several most significantly changed genes in eosinophilic AAI belonged to cytoskeleton, cell adhesion, and junction process networks and pathways; and to innate immunity and Th17-related networks and pathways in neutrophilic AAI (Figures 1G,H, S2A-C). Intriguingly, IL-5 signaling via JAK/STAT pathway appeared to be uniquely enriched in the neutrophilic AAI (Figure 1H). More detailed analysis of the genes in this category revealed though, that the same genes were changing in similar direction in the eosinophilic AAI vs control, yet not reaching statistical significance. Apparently, several genes belonging to this category depend not only on IL-5 signaling, but also on other upregulated pathways in neutrophilic AAI, such as NF- κ B activation, Th17-pathogenic axis, and IL-3 signaling (Figure 1G,H). Thus, their stronger enrichment in neutrophilic inflammation might result from the complex regulation of JAK/STAT pathway. Furthermore, genes which were uniformly enriched in both inflammatory phenotypes belonged to the pathway of IL-4-responsive genes in type 2 immunity (Figure 1H) pointing at a common underlying process.

Further in-depth analysis of these enriched networks and pathways revealed that they all contained genes from the three distinct families: tight junction (TJ), mucin, and inflammasome-related genes (Figures 2A-C, S2A-C). Therefore, we proceeded to study mRNA and protein expression together with lung tissue localization of the several molecules from these gene families, in order to define signatures of eosinophilic, mixed, and neutrophilic AAI.

3.3 | Zo-1 and Claudin-18 are downregulated in all phenotypes, while Claudin-4 upregulation is characteristic for neutrophilic AAI

Transcriptomic analysis of genes from the TJs family showed three main clusters: (i) genes upregulated in eosinophilic AAI, but not in other phenotypes, consisting mostly of Claudins (cluster I, Figure 2A); (ii) genes downregulated in all phenotypes of AAI including *Tjp1* (Zo-1), *Cld18*, and *Ocln* (cluster II, Figure 2A); and (iii) genes upregulated in neutrophilic and mixed AAI including *Cldn1*, *Cldn4*, and *Cldn7* (cluster III, Figure 2A). Confirmatory RT-PCR experiments



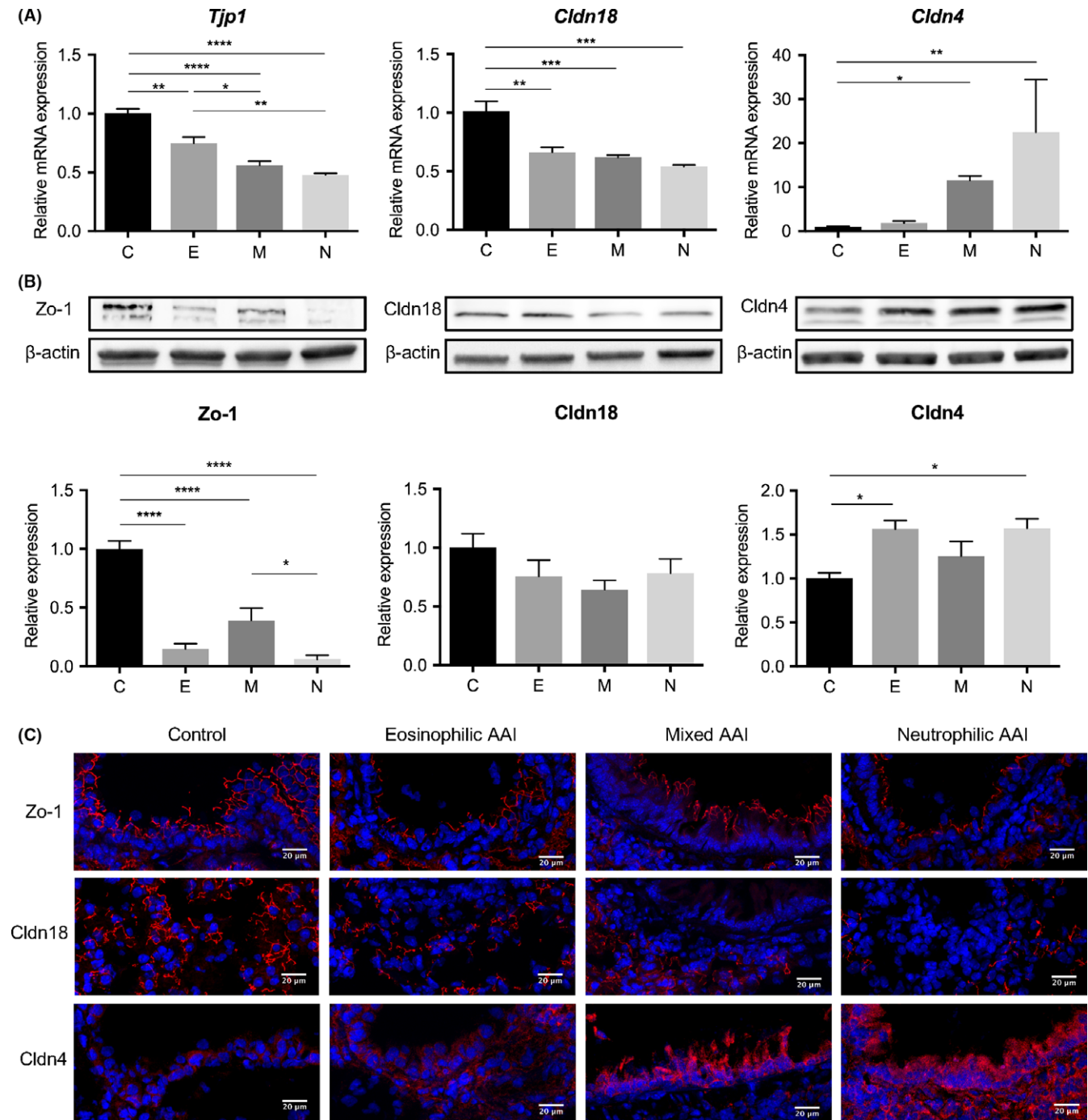


FIGURE 3 Zo-1 and Claudin-18 are reduced in all phenotypes of AAI, while Claudin-4 is upregulated in neutrophilic AAI in mice. A, mRNA expression of *Tjp1* (Zo-1), *Cldn18*, and *Cldn4* analyzed by real-time PCR in lung lysates 4 h after last house dust mite (HDM) or saline treatment. Gene expression was assessed using RT-PCR, normalized to housekeeping *Eef2* gene, and represented as a relative quantification ($RQ = 2^{-\Delta\Delta CT}$) compared to control mice. Data represent mean and SEM from three mice/group, each performed in duplicate. B, Western blot (WB) gel images and quantitative analysis of Zo-1, Cldn4, and Cldn18 protein in lung lysates 24 h after last HDM or saline treatment. The WBs are representative of three independent experiments from three mice. The bar graphs show the mean and SEM of densitometry results, presented as the ratio of total protein to β -actin. C, Confocal immunofluorescence images of the tight junction proteins Zo-1, Cldn4, and Cldn18 24 h after last HDM or saline treatment. Images are representative of three independent experiments from three mice, each showing similar results; scale bars: 20 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by the Kruskal-Wallis test with Dunn post hoc analysis. C, control; E, eosinophilic AAI; M, mixed AAI; N, neutrophilic AAI

supported these transcriptome findings (Figures 3A, S3). *Tjp1* (Zo-1) and *Cldn18* were significantly decreased in all inflammatory phenotypes compared to control, with the lowest expression in the

neutrophilic type (Figure 3A). Other genes with similar expression pattern included *Ocln*, *Magi3*, *Marved1*, *Cldn8*, *Magix*, *Cldn15*, and *Cldn17* (Figure S3). In contrast, *Cldn4* mRNA expression was

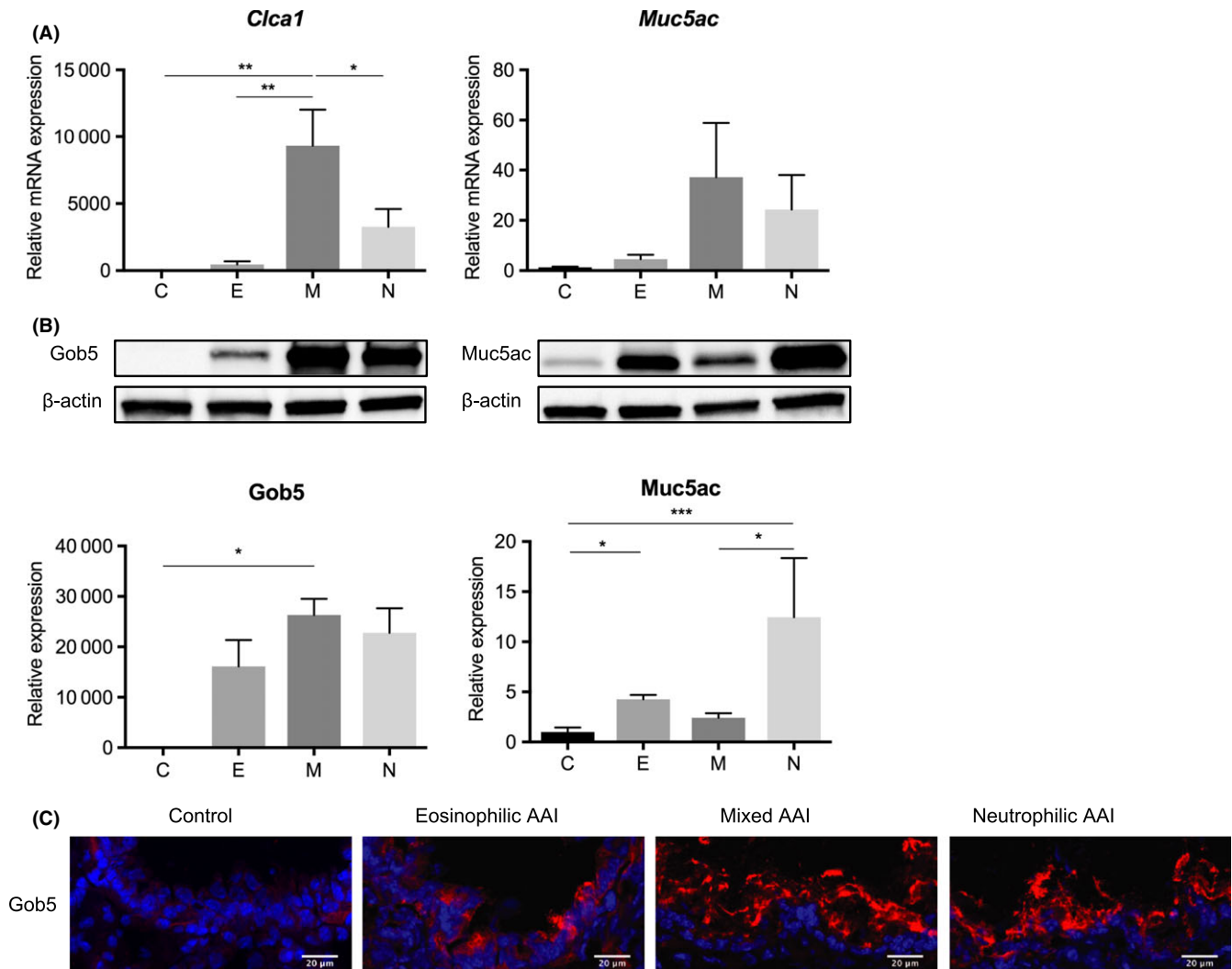


FIGURE 4 *Clca1* (*Gob5*) and *Muc5ac* are upregulated in eosinophilic, mixed, and neutrophilic AAI in mice. A, mRNA expression of *Clca1* (*Gob5*) and *Muc5ac* in lung lysates 4 h after last house dust mite (HDM) or saline treatment. Gene expression was assessed using RT-PCR, normalized to housekeeping *Eef2* gene, and represented as a relative quantification ($RQ = 2^{-\Delta\Delta CT}$) compared to control mice. Data represent mean and SEM from three mice/group, each performed in duplicate. B, Western blot (WB) gel image and quantitative analysis of *Gob5* and *Muc5ac* protein in lung lysates 24 h after last HDM or saline treatment. The WBs are representative of three independent experiments from three mice. The bar graphs show the mean and SEM of densitometry results, presented as the ratio of total protein to β -actin. C, Confocal immunofluorescence images of *Gob5* 24 h after last HDM or saline treatment. Images are representative of three independent experiments from three mice, each showing similar results; scale bars: 20 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by the Kruskal-Wallis test with Dunn post hoc analysis. C, control; E, eosinophilic AAI; M, mixed AAI; N, neutrophilic AAI

significantly increased in mixed and neutrophilic AAI (Figure 3A). Similarly, *Cldn1*, *Cldn25*, *Cldn7*, *Cldn27*, and *Tmem235* were mostly upregulated in neutrophilic AAI (Figure S3). Western blot analysis (Figure 3B) and confocal microscopy (Figure 3C) showed the same trends for the protein expression of Zo-1, *Cldn18*, and *Cldn4*. Notably, Zo-1 and *Cldn4* were mostly expressed in the bronchial epithelium, while *Cldn18* was only found in alveolar epithelial type cells. Overall, our findings suggest that even though majority of TJ molecules seem to be downregulated in all AAI phenotypes, there are few molecules, such as *Cldn4*, which are uniquely upregulated in neutrophilic AAI, demonstrating phenotypical differences in epithelial barrier.

3.4 | Increased expression of mucins *Clca1* (*Gob5*) and *Muc5ac* in eosinophilic and neutrophilic AAI

For the mucin genes, transcriptomic analysis revealed two main clusters: (i) genes upregulated in any phenotype, with several mucin genes significantly upregulated in eosinophilic phenotype including *Clca1* (*Gob5*), while others such as *Muc5ac* and *Muc5b* were mostly upregulated in neutrophilic phenotype (cluster I, Figure 2B); (ii) genes downregulated/with mixed regulation in different AAI phenotypes, for example, *Muc1* and *Muc20* (cluster II, Figure 2B). *Clca1* (*Gob5*) was indeed highly upregulated at the mRNA level (Figure 4A) and also at the protein level in the whole lungs

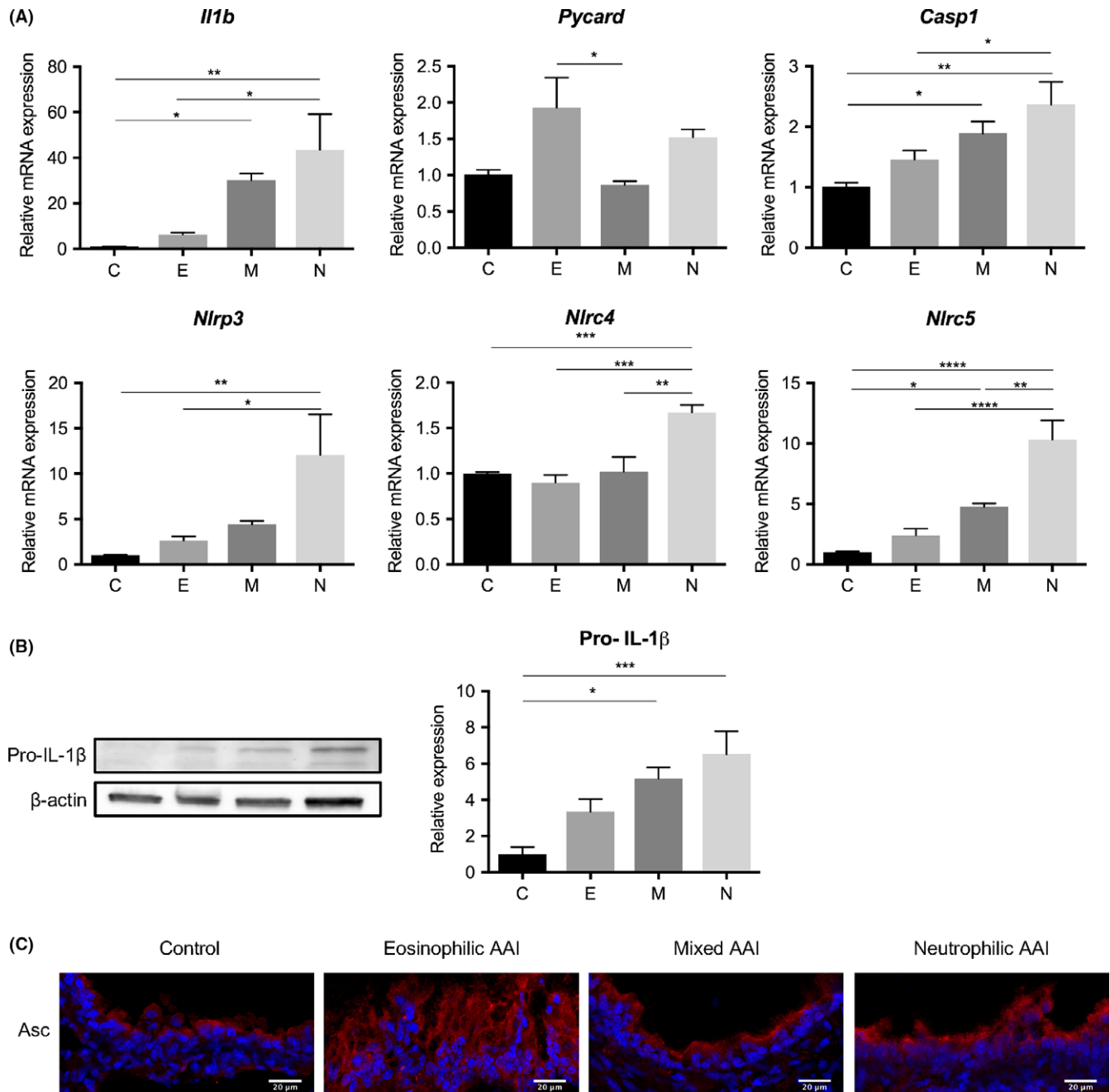


FIGURE 5 Upregulation of inflammasome-related molecules in mixed and neutrophilic AAI in mice. A, mRNA expression of *Il1b*, *Pycard* (*Asc*), *Casp1*, *Nlrp3*, *Nlr4*, and *Nlr5* in lung lysates 4 h after last house dust mite (HDM) or saline treatment. Gene expression was assessed using RT-PCR, normalized to housekeeping *Eef2* gene, and represented as a relative quantification ($RQ = 2^{-\Delta\Delta CT}$) compared to control mice. Data represent mean and SEM from three mice/group, each performed in duplicate. B, Western blot (WB) gel image and quantitative analysis of pro-IL1 β protein in lung lysates 24 h after last HDM or saline treatment. The WBs are representative of three independent experiments from three mice. The bar graphs show the mean and SEM of densitometry results, presented as the ratio of total protein to β -actin. C, Confocal immunofluorescence images of Asc 24 h after last HDM or saline treatment. Images are representative of three independent experiments from three mice, each showing similar results; scale bars: 20 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by the Kruskal-Wallis test with Dunn post hoc analysis. C, control; E, eosinophilic AAI; M, mixed AAI; N, neutrophilic AAI

(Figure 4B), as well as in bronchial epithelium (Figure 4C) in eosinophilic and much stronger in the mixed and neutrophilic AAI compared to controls. In the control conditions, Gob5 expression was barely detectable (Figure 4B). Interestingly, Muc5ac protein was strongly increased in all inflammatory phenotypes, but the

highest expression was observed in neutrophilic AAI (Figure 4B). We found comparable expression profiles for *Muc4* and *Muc5b* (Figure S4). In summary, our findings suggest that while several mucins are upregulated by the type 2 airway inflammation (eosinophilic), their expression might be also initiated and/or further

enhanced by ongoing type 1/17 inflammatory response (neutrophilic).

3.5 | Inflammasome-related molecules' expression profile is characteristic for neutrophilic AAI

For inflammasome-related genes, we observed four main clusters from transcriptomic analysis: (i) genes highly expressed in eosinophilic AAI, for example, *Pycard* (Asc); (ii) genes of lower expression compared to control, evident mainly in eosinophilic and neutrophilic AAI, for example, *Aim2* and *Il18*; (iii) biggest cluster of genes highly expressed in neutrophilic and mixed and only some of them in eosinophilic AAI, for example, *Nlrp3*, *Nlrp5*, *Myd88*, and *Il1b*; and (iv) genes downregulated in the eosinophilic phenotype

only (Figure 2C). We next observed that the majority of further tested genes and proteins displayed cluster III expression pattern showing increasing expression from eosinophilic to neutrophilic inflammation (Figures 2C, 5A, S5). Among genes investigated in detail, *Il1b* mRNA whose mature product is a central output of inflammasome activation showed increasing expression of mRNA across phenotypes, with neutrophilic AAI having the highest expression (Figure 5A). *Casp1*, *Nlrp3*, *Nlrp4*, *Nlrp5* mRNA (Figure 5A), as well as other inflammasome-related genes, such as *Casp4*, *Casp8*, *Il1a*, *Aim2*, *Ddx58* (*Rig1*), and *Ifih1*(*Mda5*), showed highest expression in neutrophilic inflammation with a similar pattern (Figure S5). Western blot analysis of pro-IL-1 β protein (Figure 5B) was in agreement with gene expression results. Of note, we did not detect the mature, cleaved form of IL-1 β , which

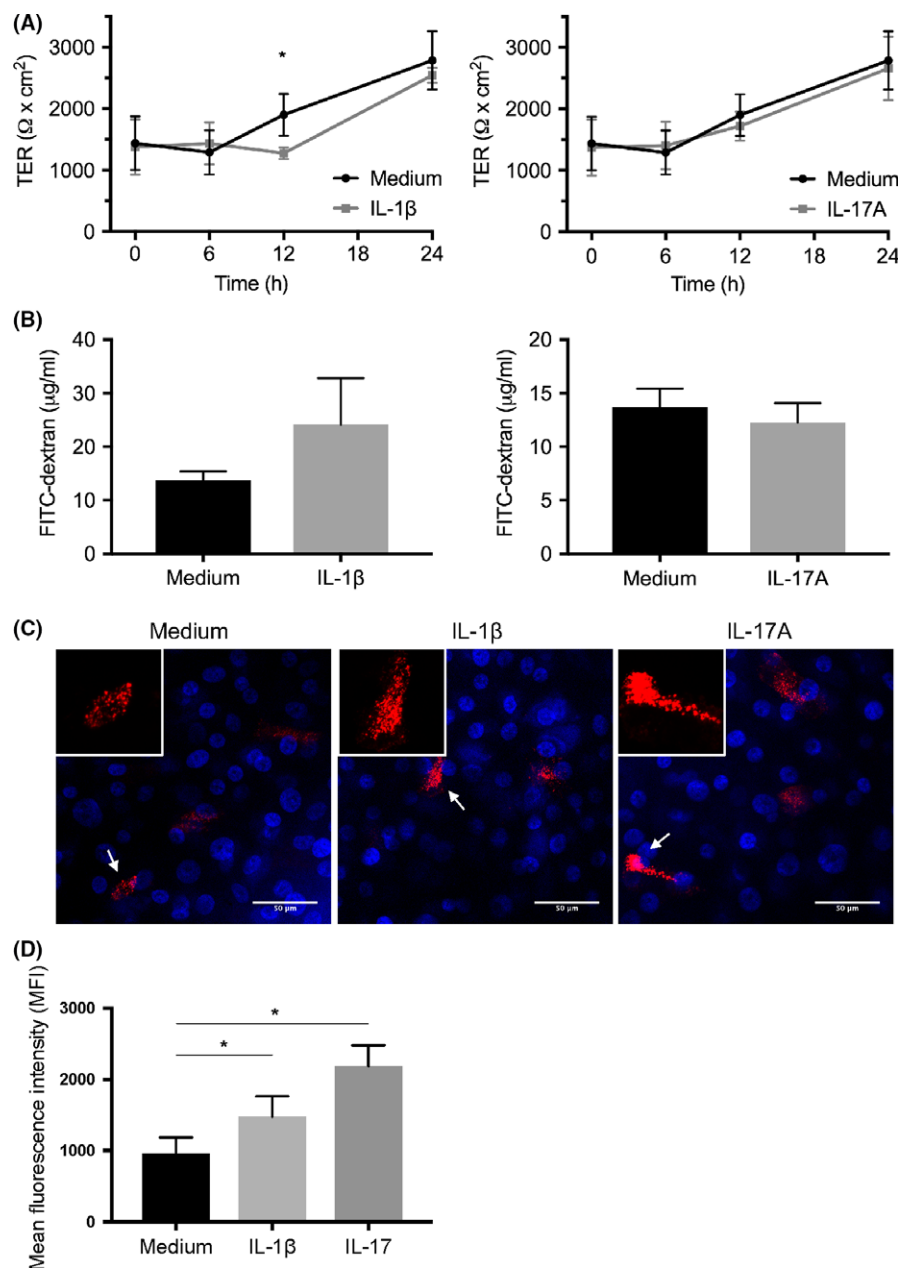


FIGURE 6 IL-1 β slightly disturbs epithelial barrier integrity, while both IL-1 β and IL-17A increase MUC5AC expression in human primary bronchial airway epithelial cells. A, Transepithelial resistance (TER) of human primary bronchial epithelial cells (HBECs), grown in air-liquid interface (ALI) conditions, treated in the basal compartment with IL-1 β and IL-17A or medium control for 6, 12, and 24 h. Data represent mean and SEM from five independent experiments from five different donors, performed in duplicate. B, FITC-dextran diffusion of HBECs, grown in ALI conditions, treated in the basal compartment with IL-1 β and IL-17A or medium control for 12 h. Data represent mean and SEM from five independent experiments from five different donors, performed in duplicate. C, Representative confocal immunofluorescence images or (D) quantification of MUC5AC expression in ALI-HBECs at 24 h after stimulation with IL-1 β , IL-17A, or medium. Scale bars: 50 μm . Data represent mean and SEM from five independent experiments from five different donors. * $P < 0.05$, by the t test, Mann-Whitney U test or Kruskal-Wallis test with Dunn post hoc analysis

suggests that inflammasome molecules, while generally upregulated in neutrophilic AAI, have not yet formed fully activated inflammasome complexes.

We confirmed that *Pycard* (Asc) mRNA was more expressed in eosinophilic and to a lesser extent also in neutrophilic AAI in the analysis of cluster 1 (Figure 5A). Asc, which is an integral adaptor molecule for the majority of inflammasome complexes upon their activation, was also increased in eosinophilic and neutrophilic AAI in the whole lungs and in the bronchial epithelium (Figure 5A,C). Interestingly, from cluster II, we confirmed that *Il18* mRNA expression was lower than in controls, especially in eosinophilic inflammation (Figure S5), in contrast to *Aim2*, which showed increased expression in neutrophilic AAI (Figure S5). We did not observe any genes with the specific expression pattern from cluster IV. In summary, these data demonstrate that especially in neutrophilic AAI, there is an increased expression of inflammasome sensor molecules (*Nlrp3*, *Nlrc4*, *Nlrc5*, *Ddx58* [Rig-I]), adaptor molecules (Asc), caspase-1, and the final product to be cleaved by inflammasome activation pro-IL-1 β .

3.6 | IL-1 β slightly influences epithelial barrier function, while both IL-1 β and IL-17A increase mucin expression in human primary airway epithelial cells

To further investigate the mechanistic link between inflammasome-related IL-1 β and Th17-type of inflammation with TJs and mucin gene expression, and to translate our findings to humans, we stimulated primary differentiated human bronchial epithelial cells cultured in the air-liquid interface with IL-1 β and/or IL-17A. We found a decrease in TER, the global measurement of the TJs functionality, at 12 hours after stimulation with IL-1 β (Figure 6A). There was also a trend of increased permeability of ALI cultures upon IL-1 β treatment, measured by FITC-dextran flux (Figure 6B). In contrast, stimulation with IL-17A did not have any significant effect on TER and paracellular flux at any investigated time point (Figure 6A-B). IL-1 β and IL-17A did not influence *ZO-1*, *CLDN1*, or *CLDN4* mRNA expression (Figure S6). Next, we observed in confocal microscopy that 24 hours of stimulation with IL-1 β or IL-17A significantly increased the protein expression of MUC5AC (Figure 6C,D). In summary, these results suggest that IL-1 β in humans may transiently impair global epithelial barrier function at the early time points, while IL-1 β and IL-17 may increase the expression of mucins.

4 | DISCUSSION

Our findings that most significantly dysregulated genes in eosinophilic inflammation belonged to cytoskeleton, cell adhesion, and barrier and junction processes, while neutrophilic inflammation involved innate immunity, inflammasome, and Th17-related processes is in agreement with previous findings,^{4,10,30} with an added value of the unbiased—omics comparative approach. It suggests that our experimental models phenotypically and transcriptionally, effectively reflect

heterogeneity of asthma in humans. Nevertheless, since all three models represent acute airway inflammation in response to different HDM exposure conditions, they might not reflect different stages in the disease development, observed in some patients during disease progression.

Impaired barrier function caused by disruption of epithelial TJs leads to aberrant communication with the underlying mesenchyme, remodeling, and a more chronic inflammatory phenotype.³¹ Assembly of epithelial barrier requires coordinated activity of multiple domains of the tight junction protein ZO-1.³² Our finding of significantly downregulated Zo-1 in all inflammatory phenotypes of AAI is in agreement with previous findings of reduced ZO-1 in asthmatic patients,²² through pathomechanisms involving Th2 cells and ILC2 via IL-4, IL-13, and activity of histone deacetylases.^{10,33} Also, IFN- γ has been found to reduce epithelial barrier in patients with chronic rhinosinusitis.³⁴ Claudin-4 which has sealing function³⁵ is upregulated during early stages of acute lung injury.³⁶ Therefore, the highest upregulation seen here in neutrophilic AAI may reflect the compensatory effect in relation to the disruption of other TJs and may indicate the more severe form of inflammation in neutrophilic AAI. Recent studies demonstrated that Claudin-18 deficiency impairs alveolar epithelial barrier function in vivo and in vitro^{37,38} and is associated with type 2 inflammation in asthma.³⁹ However, no study has previously linked Claudin-18 expression with non-type 2 endotype, while we observed here that Claudin-18 is further reduced in neutrophilic inflammation. These findings suggest that personalized targeting of the barrier function proteins for therapeutic intervention could be useful both for type 2 and non-type 2 endotypes.

Airway mucus contributes to the resistance of the lungs to environmental injury; however, when there is impaired clearance or increased production, it contributes to the pathogenesis of airway diseases. *Clca1* (Gob5) is one of the most highly induced genes in epithelial cells in asthma.⁴⁰ Upregulation of mucins in eosinophilic AAI is expected as mucus hypersecretion and goblet cell hyperplasia are regulated through IL-13- and IL-9-dependent pathways.⁴¹ An even higher expression of *Clca1*, *Muc5ac*, *Muc4*, and *Muc5b* observed here in neutrophilic AAI has not been clearly documented before. This further enhanced expression of mucins might be induced by the ongoing Th17 and inflammasome-mediated inflammation.^{24,42} It has been shown that IL-17 and IL-1 β could stimulate MUC5AC and MUC5B by a JAK2-dependent autocrine/paracrine loop²⁴ and/or via NF- κ B-based mechanism,^{43,44} the mechanisms important in chronic obstructive pulmonary disease.⁴⁵ We confirmed these observations both in our in vivo murine model and in vitro in HBECs. Therefore, although the mucus overproduction in asthma is typically associated with type 2 immune response, increasing evidences suggest the association also with non-type 2 immune mechanisms.

The relationship between asthma phenotypes and inflammasomes has been lately linked with neutrophilic asthma and severe, steroid-resistant asthma.²⁵⁻²⁷ Increased mRNA expression of NLRP1, NLRP3, and NLRC4 inflammasome sensors and increased IL-1 β

protein levels are observed in the sputum of patients with neutrophilic asthma.²⁵⁻²⁷ Accordingly, NLRP3 inflammasome inhibition in HDM-induced or Chlamydia-induced and Haemophilus-induced neutrophilic asthma model prevents the disease,^{26,27} while it does not contribute to the pathogenesis of the “classical” eosinophilic OVA-mediated allergic asthma in mice.²⁸ Our current findings on the strong transcriptomic enrichment and increasing expression of inflammasome sensor molecules—*Nlrp3*, *Nlr4*, *Nlr5*, *Ddx58* (*Rig-I*), adaptor molecules—*Asc*, as well as caspase-1 and pro-IL-1 β , from eosinophilic to neutrophilic AAI, are in agreement with these findings and further support the link between Th17 pathway and inflammasome activation.⁴⁶ Enrichment of inflammasome pathway in mouse models with neutrophil infiltration and also in patients with neutrophilic asthma may indicate its strong association with mechanisms initially leading to that type of inflammatory response. In addition, several factors responsible for temporary neutrophilic inflammation, such as frequent virus-induced exacerbation of asthma, which lead to inflammasome activation and IL-1 β release,^{47,48} may contribute subsequently to the change in the phenotype and progression of the disease. Consequently, inhibition of inflammasome axis^{49,50} might constitute a novel opportunity of targeting chronic neutrophilic inflammation, especially in the situation of resistance to steroids.

Our in vitro experiments showing that IL-1 β alone may transiently influence epithelial barrier function, while both IL-1 β and IL-17A may increase mucin expression, suggest a mechanistic link between increased inflammasome-Th17-related axis with an impaired barrier function in humans. IL-1 β can induce development of IL-17-producing T cells in cooperation with IL-23.⁵¹ IL-17, in agreement to our observations, was found to stimulate mucin genes, *MUC5B* and *MUC5AC*,²⁴ in airway epithelium. In addition, inflammasome is also a potential regulator of goblet cell secretion.⁵² Together, these data suggest that complex barrier dysfunction in neutrophilic asthma may be the consequence of the axis of inflammasome activation—IL-1 β /IL-17—neutrophilic infiltration.

In conclusion, we demonstrated here the differences in the expression of TJ, mucin, and inflammasome-related molecules in different inflammatory phenotypes of AAI. These data suggest an association of cellular phenotypes with molecular endotypes and open a new window for more precision approaches to asthma patients.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

H-T.T.T., S.H., F.R., and U.R. contributed to the acquisition, analysis, and interpretation of data. G.T., C.A., A.E., M.M., and M.A. contributed to the analysis and interpretation of data. C.A.A. and H.G. contributed to the conception and design of study and interpretation of data. M.S. contributed to the conception and design of study, acquisition, analysis, and interpretation of data. H-T.T.T. and M.S. wrote the manuscript, and all authors revised and approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary Material

Tight junction, mucin and inflammasome-related molecules are differentially expressed in eosinophilic, mixed and neutrophilic experimental asthma in mice

Short title: Inflammatory phenotypes of experimental asthma differ in tight junction, mucin and inflammasome-related molecules

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Supplementary Methods

Mouse model of different inflammatory phenotypes in allergic airway inflammation (AAI)

Three different AAI phenotypes- eosinophilic, mixed, and neutrophilic, were induced in Balb/c mice via different ways of house dust mite (HDM) sensitisation (Figure 1a). *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* represent the most common HDM species to which allergic/asthmatic responses are elicited (1-3). Quite often, simultaneous sensitization to both allergen families (Der p and Der f) is observed (4-6). Allergic inflammation was induced in mice by exposure to a mixture of standardised Der p (*Dermatophagoides pteronyssinus*) and Der f (*Dermatophagoides farinae*) allergen extracts (ALK-Abelló A/S, Horsholm, Denmark). The extracts were standardised against an in-house reference and amounts are expressed in development units (DU) as described previously (7).

For eosinophilic AAI, mice were sensitised by three intraperitoneal (i.p.) injections of HDM extract containing 0.75 DU Der f and 0.75 DU Der p (ALK-Abelló A/S) adsorbed to 1.5 mg Al(OH)₃ (Pierce, Rockford, IL, USA) in 200 µl PBS on days 0, 7 and 21. On days 26 to 28, mice were challenged intranasally (i.n.) with the similar dose of HDM extract dissolved in 50 µl PBS. For induction of the mixed or neutrophilic phenotype, mice were i.n. sensitised /challenged with the HDM extract (0.75 DU Der f /0.75 DU Der p or 7.5 DU Der f/7.5 DU Der p, respectively) in 50 µl PBS once a week a total of four times. Intranasal application of only PBS served as a control.

Depending on the intended main read-out mice were sacrificed at different time points following last challenge in each model according to results of preceding optimization experiments. In detail, mice were sacrificed at 4 h to harvest lungs and BAL for RNA extraction and cytokines analyses, respectively. For analysis of lung proteins by WB and confocal microscopy, mice were sacrificed at 24 h after the last challenge. Finally, for the measurements of BAL cellular content and performing histology of the lungs, mice were sacrificed at 72 h.

All animal procedures were performed with approval from the University of Marburg local authorities, i.e. the Regierungspräsidium Giessen (Approval number: MR 20/13 Nr. 63/2013).

Periodic acid–Schiff (PAS) staining, inflammation scores, bronchoalveolar lavage (BAL) cell counts, and cytokines in BAL and lung homogenates were measured as described previously (8) (n=5-6 mice/group). IL-5, IL-17A and IFN γ in BAL and in lung homogenates were measured by Cytometric Bead Array (*Biotechne, Wiesbaden, Germany*) using FACSArray. Lung samples were also snap frozen in liquid nitrogen for transcriptome analysis (n=3 mice/group) as well as for further analysis by immunofluorescence staining, Western blotting and mRNA expression (n=3 mice/group).

Human bronchial epithelial cell culture

Human bronchial epithelial cells (HBECs) from five different donors (Lonza, Basel, Switzerland) were cultured in T75 T flasks with bronchial epithelium growth medium including the Single Quot Bullet Kit (Lonza). Medium was changed after 24 hours and every second day from then on. Cells were harvested at a confluence of 90% by trypsinisation (5 min at 37°C, 5% CO₂; Trypsin EDTA 0.05%, Gibco). Trypsin was neutralized with trypsin neutralizer solution (TNS) (Gibco).

Air-liquid interface (ALI) cultures, cytokine stimulations, and TER

HBECs were seeded onto 6.5-mm-diameter polyester membranes with a pore size of 0.4 μ m (Oxyphen, Wetzikon, Switzerland) at a density of 150,000 cells per well. Dulbecco modified Eagle medium (Gibco-BRL, Invitrogen) with fresh retinoic acid was mixed 1:1 with bronchial epithelium basal medium (BEBM, Lonza) supplemented with the Single Quot kit except for triiodothyronine and retinoic acid (Lonza) and used as the medium in ALI cultures. Experiments were conducted with cells from five different donors in a minimum of experimental duplicates, and average numbers were used for analysis. Medium was changed every second day. At complete cell confluence, the apical compartment was freed of any medium to allow further cell differentiation at the ALI to take place. Stimulation experiments were initiated 21 days after seeding, when cells were fully differentiated. Opti-MEM Reduced Serum Medium (Gibco, Paisley, Scotland, UK) was used from then on. ALI cultures were stimulated by adding different cytokines to the basolateral compartment: IL-1 β , 10 ng/mL (R&D Systems, Minneapolis, MN, USA) and IL-17A, 10 ng/mL (Peprotech). TER was measured in $\Omega \times \text{cm}^2$ by using a Millicell ERS Volt-Ohm Meter (Millipore,

Billerica, MA, USA). Measurements were done at 0, 6, 12, and 24 hours after stimulation. For the use of ALI cultures in immunofluorescence, cells were fixed with paraformaldehyde 4% (Sigma-Aldrich, St. Louis, MO, USA). For mRNA isolation, cells were stored in RNeasy lysis buffer (RLT; Qiagen, Basel, Switzerland) plus β -mercaptoethanol (Sigma-Aldrich).

Paracellular flux measurements

Epithelial permeability as a surrogate marker of layer integrity was measured by using fluorescein isothiocyanate (FITC)–dextran (Sigma-Aldrich) flux measurements. FITC-dextran (2 mg/mL) was added apically at 12 hours after stimulation. Three and 24 hours after addition, the FITC intensity of basolateral fluids was assessed with an ELISA reader (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) at 480 nm excitation.

mRNA isolation and RT-PCR

Snap frozen lung samples were placed in RNeasy lysis buffer (Qiagen) with β -mercaptoethanol (Sigma-Aldrich) using gentleMACS Dissociator (Miltenyi Biotec) according to manufacturer's instructions. mRNA was isolated using the RNeasy Mini and Micro kit (Qiagen) respectively for mouse lung samples and human ALI cultures, according to the manufacturer's protocol. Reverse transcription of mRNA samples was performed with RevertAid RT kit (Thermo Fisher) with random hexamers according to the manufacturer's protocol. The prepared cDNAs were amplified using SYBR green PCR master mix (Thermo Fisher Scientific) according to the manufacturer's recommendations in QuantStudio 7 Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Primer sequences can be found in the Supplementary table S1 below.

PCR amplification of the endogenous control gene encoding eukaryotic translation elongation factor 2, *Eef2* (for mouse samples) or elongation factor (*EF*)-1 α (for human samples) was performed in each plate to allow normalisation between samples. Relative quantification and calculation of the range of confidence was performed using the comparative $\Delta\Delta CT$ method. Arbitrary units show the $2^{-(\Delta\Delta CT)}$ values relative to control group.

Gene arrays analysis

Total RNA (300 ng) was processed for GeneChip analysis using the Gene Chip WT Plus Reagent Kit and analysed using GeneChipTM Mouse Gene 2.0 ST microarray chips (Affymetrix, Santa Clara, CA) following manufacturer's directions. The entire data set has been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO; <http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi>), accession number GSE108417. Gene expression data was preprocessed by the Robust Multichip Analysis (RMA) algorithm using Affymetrix Expression Console. One way ANOVA and post-hoc tests were performed for comparisons between groups. Significance level of $p < 0.05$ was applied for the Venn diagrams and functional pathways and network analysis, in order to find out the most complete enrichment in several pathways. Venn diagrams and volcano plots were generated using Shiny apps by R-Studio (written by the Functional Genomic Center Zurich). Hierarchical cluster analysis and heat maps generation was done using R software (R Core Team, Vienna, Austria; <http://www.R-project.org/>). Networks and pathway maps were generated using the MetaCore version 6.30 software (MetaCore, Thomson Reuters). In detail, filtered lists of differentially expressed genes in each phenotype as compared to control group were uploaded to the software. Next, comparative enrichment analysis in pathway maps, process networks, GO processes, biomarkers and diseases were run for eosinophilic and neutrophilic AAI. Metacore-generated pathways and process networks were rank ordered according to their significance score. Significance scores of those pathways are indicated by the $-\log(P\text{-value})$ which represents significant results when greater than 1.3. Comparative enrichment analysis also revealed genes which were uniquely enriched in eosinophilic or neutrophilic AAI or were commonly enriched in both phenotypes. All most significant pathways and processes are shown in the Supplementary Figures. Interaction networks were also generated by STRING version 10.5 (<https://string-db.org/>).

Western blotting

Snap frozen lung samples were placed in chilled T-PER tissue protein extraction reagent (Thermo Fisher Scientific) with protease and phosphatase inhibitors (Roche) in gentleMACS M tubes for homogenisation using gentleMACS Dissociator

(Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. The Western blot procedure was the same for lung and ALI cultures. Cell lysates were centrifuged, and equal amounts of protein lysate (20 µg) were loaded onto 4–20% Mini-PROTEAN TGX polyacrylamide gels (Bio-Rad, Hercules, Calif). After electrophoresis, the proteins were transferred to nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in PBS with 0.1% Tween-20 (PBST; Sigma-Aldrich). The blots were then incubated with primary antibodies (1:500 for ZO-1 (rabbit polyclonal IgG, Invitrogen, catalog number 40-2200), 1:250 for Claudin-18 (rabbit monoclonal IgG, clone: 34H14L15, Invitrogen, catalog number 700178), 1:200 for Claudin-4 (rabbit polyclonal IgG, Abcam, catalog number ab15104), 1:10000 for Clca1 (rabbit monoclonal IgG, clone: EPR12254-88, Abcam, catalog number ab180851), 1:2000 for Muc5ac (mouse monoclonal IgGκ1, clone: 45M1, Abcam, catalog number ab3649), 1:1000 for IL-1β [polyclonal goat IgG, R&D Systems, catalog number AF-401-NA]) overnight at 4 °C and washed with PBST. The blots were incubated with HRP-conjugated secondary antibodies (1:10000 dilution of Peroxidase AffiniPure Goat anti-Rabbit IgG for ZO-1, Claudin-18, Claudin-4, Clca1 visualization, Jackson Laboratories, catalog number: 111-035-003; 1:10000 dilution of Peroxidase AffiniPure Goat anti-Mouse IgG for MUC5AC visualization, Jackson Laboratories, catalog number: 115-035-146; 1:10000 dilution of Peroxidase AffiniPure Donkey anti-Goat IgG for IL-1β visualization, Jackson Laboratories, catalog number: 705-035-003) for 1 hour. After washing, the blots were developed with SuperSignal West Femto Maximum Sensitivity ECL Substrate (Thermo Fisher Scientific) and the signals were captured with LAS-1000 gel documentation system (Fujifilm, Tokyo, Japan).

Immunofluorescence staining

Snap frozen lung specimens from mice were embedded in OCT compound Tissue Tek (Sakura Finetek, Alphen aan den Rijn, The Netherlands). Sections were cut to a thickness of 7 µm on a cryomicrotome (MICROM, Waldorf, Germany) at -25°C and fixed with 4% paraformaldehyde (Sigma-Aldrich). The staining procedure was the same for cryosections and ALI cultures. Permeabilisation and blocking were accomplished by means of incubation with a mixture of 10% goat serum

(DakoCytomation, Glostrup, Denmark), PBS containing 0.1% Triton X (Acros Organics, Geel, Belgium), 1% BSA (Sigma-Aldrich) in PBS and 0.02% SDS. Specimens were then incubated with polyclonal rabbit IgG anti-Zo-1 (Invitrogen, catalog number: 40-2200), monoclonal rabbit IgG anti-Claudin-18 (clone: 34H14L15, Invitrogen, catalog number: 700178), polyclonal rabbit IgG anti-Claudin-4 (Abcam, catalog number, ab15104) or monoclonal rabbit IgG anti-Clca1 (Gob5) (clone EPR12254, Abcam, catalog number ab180851) at 1:500; or polyclonal rabbit IgG anti-ASC (Santa Cruz Biotechnology, catalog number: sc-22514-R) or monoclonal mouse IgG1 κ anti-Mucin 5AC (clone 45M1, Abcam, catalog number ab3649) at 1:100 in 1% BSA in PBS, followed by incubation with Alexa Fluor 546-labeled secondary antibodies (1:2000 dilution of AF546 goat anti-rabbit polyclonal IgG for ZO-1, Claudin-18, Claudin-4, Clca1 and ASC visualization, Molecular Probes, catalog number: A11010; 1:2000 dilution of AF546 goat anti-mouse IgG for MUC5AC visualization, Molecular Probes, catalog number: A11003). Slides were mounted with ProLong-Gold mounting medium (Prolong-Gold DAPI, Invitrogen) and analysed with a Zeiss LSM 780 confocal microscope (Carl Zeiss, Jena, Germany). Images were then processed in Image J version 2.0.0-rc-43/1.51h.

Statistics

Data analysis was performed with GraphPad Prism Version 7 software (GraphPad Software, La Jolla, Calif). Differences between groups were analysed by using t-test, Mann-Whitney U test, one-way ANOVA or Kruskal Wallis test with post-hoc analysis, as appropriate. The significance level α was set to 0.05.

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260

261 **Table S1:** PCR primers for gene expression assays

Gene	Protein	Forward primer sequence (5'→ 3')	Reverse primer sequence (5'→ 3')
MOUSE			
Tight junction			
<i>Cldn1</i>	Claudin-1	CCACCATTGGCATGAAGTGC	ACTAATGTCGCCAGACCTGA
<i>Cldn4</i>	Claudin-4	GGGAATCTCCTTGGCAGTCC	CGATGTTGCTGCCGATGAAG
<i>Cldn5</i>	Claudin-5	GTGCCGGTGTACAGAAAGTA	TTGACCGGGAAGCTGAACTC
<i>Cldn6</i>	Claudin-6	CGGCAACAGCATCGTCGTG	TCTTGGTGGGATATTCGGAGG
<i>Cldn7</i>	Claudin-7	AGCATGTTCTTGGATTGGTC	CTCCCCAGCTCACACGTATT
<i>Cldn8</i>	Claudin-8	GCAACCTACGCTCTTCAAATGG	TGGTGCGATGGGATGGTAC
<i>Cldn13</i>	Claudin-13	GCATCTCTACAGCTGGACCC	GCTGATGGCCTCTTGTTTGC
<i>Cldn15</i>	Claudin-15	GCAACGTCATCACCACCAAC	TGGACATACCCAGAGAGGGC
<i>Cldn17</i>	Claudin-17	CGAGAACTTGGAGGAGCACTCT	GCCCGCTCCAATGAAG
<i>Cldn18</i>	Claudin-18	ACCGTTCAGACCAGGTACAC	GTCATCTGGTGTACAGGCCA
<i>Cldnd1</i>	Claudin domain containing 1; Claudin-25	GAGAGTAGGGTGCTGTGGTC	TCACCTGCATACGCCCATC
<i>Tmem235</i>	Transmembrane protein 235; Claudin-27	CACCTGTGAAGGGCAGAACA	TGTGCGATGCAGGGAGA
<i>Patj</i>	InaD-like protein (Inadl)	CTGCCAGAAACGGTGTGTTG	GCCAGTCCTCCAGGAACAAT
<i>Jam2</i>	Junction adhesion molecule B	CCTGATGCTGCTGCTGCTAC	AGCCAAAATAGCCTCTTGGAAC
<i>Jam3</i>	Junction adhesion molecule C	TGTGCAAGTGAAGCCAGTGACCC	AGCCCTCGCTCTCTTGGAAC
<i>Magi1</i>	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1	GCACAACCTTCGGTTCGGAT	GCCAAGATCCGGTCTCCTAC
<i>Magi3</i>	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 3	TGAGCTCATTCAGGCTGGTG	AAGGAGCCAAACCGTGGTC
<i>Magix</i>	PDZ domain-containing protein MAGIX	CCACACGGAGGGTGTAGTG	GTTACTGGAAGTGGCTCCGT
<i>Marveld1</i>	MARVEL domain-containing protein 1	GGTGCCACCTCTAAGGTTTCC	GACTGCCAAACCTTCTGCTG
<i>Marveld2</i>	MARVEL domain-containing protein 2	CTCGGAGACATCGGGAGTTC	CCTGATCCCTCTGTGATCACT
<i>Ocln</i>	Occludin	CTTATCTTGGGAGCCTGGACAT	GATTGGGTTTGAATTCATCAGG TCT
<i>Tjp1</i>	Tight junction protein ZO-1; Zonula occludens protein 1	GATGTTTATGCGGACGGTGG	CATTGCTGTGCTCTTAGCGG
Mucin			
<i>Clca1</i>	calcium-activated chloride channel regulator 1, Gob-5	AACGGAGCAGGTGCCGATGC	AGTGACTCCTCCCAGAGCCCA
<i>Muc4</i>	Mucin-4	CAGGACCAGATGGCTCTGAA	GCATTGGCCTCCATTGTGAC
<i>Muc5ac</i>	Mucin-5AC	TGTGCCTGCCTGTACAATGG	CCAGAACATGTGTTGGTGCAG
<i>Muc5b</i>	Mucin-5B	ATGGGCAGCAGAACTGGAG	ATGGAGTCACTATACACTCTCT GAC

Inflammasomes			
<i>Aim2</i>	Interferon-inducible protein AIM2	TGGGAACAAGACAGTACAGAGG	GAAAACTCCCTGACGCCACC
<i>Casp1</i>	Caspase-1	TGTAATGAAGACTGCTACCTGGC	CCTTGTTTCTGAGGATGAAGGA TGT
<i>Casp4</i>	Caspase-4	CATTCCCAGATGCCCACCAT	GTTGCTTTGTTCTCAGTTGCC
<i>Casp8</i>	Caspase-8	AGCACAGAGAGAAGAATGAGCC	GAGTCACACAGTTCCGCCAT
<i>Ddx58</i>	ATP-dependent RNA helicase DDX58; RIG-I	TGTGCCCCTACTGGTTGT	AGTTGCCTGCTGCTCATAG
<i>Il18</i>	Interleukin-18	ATGGAGACCTGGAATCAGACAA	TGGGGTTCCTGCGCACTTTG
<i>Il1a</i>	Interleukin-1 alpha	CGCTTGAGTCGGCAAAGAAATC	GAGAGAGATGGTCAATGGCAG A
<i>Il1b</i>	interleukin-1 beta	GCCACCTTTTGACAGTGATGAG	CAAAGGTTTGGAAGCAGCCC
<i>Ifih1</i>	Interferon-induced helicase C domain-containing protein 1; MDA5	GTCTTCTGCCTGCTGTCGAT	ACTTGTGTCTGATTCTGTGGTC
<i>Nlr4</i>	NLR family, CARD domain-containing 4	TCACCACGGATGACGAACAG	GTCAATCAGACCACCTGGCA
<i>Nlr5</i>	NLR family, CARD domain containing 5	AGCTGACCATGCTCCTGAAC	CAGGGCTCTTCCAACCTGAG
<i>Nlrp3</i>	NACHT, LRR and PYD domains-containing protein 3	ACCAGCCAGAGTGGAATGAC	ACAAATGGAGATGCGGGAGAG
<i>Pycard</i>	PYD and CARD domain-containing protein; Asc	AGCCAGAACAGGACACTTTGT	AAGCATCCAGCACTCCGTC
Endogenous control			
<i>Eef2</i>	Elongation factor 2	CATGTGGACCACGGCAAGTC	GGAGATGGCGGTGGATTTGAT
HUMAN			
<i>CLDN1</i>	Claudin 1	CAGTCAATGCCAGGTACGAATTT	AAGTAGGGCACCTCCCAGAAG
<i>CLDN4</i>	Claudin 4	TGTACCAACTGCCTGGAGGAT	GACACCGGCACTATCACCATAA
<i>TJPI</i>	tight junction protein 1; ZO-1	ACAGTGCCTAAAGCTATTCCTGT GA	TCGGGAATGGCTCCTTGAG
<i>EEF1A1</i>	Eukaryotic translation elongation factor 1 alpha 1; EF-1α	TGAAGTCTGGTGATGCTGCC	CAAAGCGACCCAAAGGTGGA

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263 **Table S2:** Scores, cell counts and cytokine levels in each phenotype (n=5-6)

	Control	Eosinophilic	Mixed	Neutrophilic
Inflammation score	0.35 ± 0.13	5.93 ± 0.51*	4.61 ± 0.57*	7.02 ± 0.52*
In BAL:				
Total cells [x 10 ⁵ /BAL]	0.57 ± 0.09	3.73 ± 1.48*	2.61 ± 0.53*	1.72 ± 0.29*
Eosinophils [x 10 ⁵ /BAL]	0.00 ± 0.00	2.65 ± 1.16*	1.22 ± 0.33*	0.07 ± 0.01*#
Neutrophils [x 10 ⁵ /BAL]	0.01 ± 0.01	0.01 ± 0.01	0.38 ± 0.07*	0.26 ± 0.08*#
Lymphocytes [x 10 ⁵ /BAL]	0.00 ± 0.00	0.50 ± 0.08*	0.21 ± 0.05*	0.17 ± 0.04*
Macrophages [x 10 ⁵ /BAL]	0.55 ± 0.09	0.79 ± 0.22	0.85 ± 0.15	1.21 ± 0.19*
IL-5 [pg/ml]	0.00 ± 0.00	39.28 ± 6.71*	11.60 ± 6.05	2.80 ± 0.86#
IL-17A [pg/ml]	0.00 ± 0.00	0.00 ± 0.00	2.00 ± 2.00	143 ± 39.44*#
IFN-γ [pg/ml]	n.d.	n.d.	n.d.	n.d.
In lung homogenates:				
IL-5 [pg/ml]	1.62 ± 1.35	40.16 ± 20.87	45.92 ± 7.73	26.98 ± 9.74
IL-17A [pg/ml]	0.25 ± 0.16	0.22 ± 0.15	10.78 ± 2.84	147.56 ± 44.82*#
IFN-γ [pg/ml]	30.19 ± 10.14	27.54 ± 8.02	31.25 ± 9.49	56.33 ± 19.91

264 Values presented as mean ± SEM; n.d., not detectable. * p < 0.05 compared to control; #
265 p < 0.05 compared to eosinophilic allergic airway inflammation (one way ANOVA with post-
266 hoc analysis).

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Supplementary Figure Legends

Supplementary Figure 1. Distinctive lung transcriptomic profiles in eosinophilic, mixed and neutrophilic allergic airway inflammation in mice. (A) Volcano plot of eosinophilic over neutrophilic AAI ($p < 0.05 = -\log_{10}(p) > 1.3$). (B) Volcano plot of eosinophilic over mixed AAI ($p < 0.05 = -\log_{10}(p) > 1.3$). (C) Volcano plot of neutrophilic over mixed AAI ($p < 0.05 = -\log_{10}(p) > 1.3$). x-axis depicts $\log_2$ fold change with upregulated genes on the right and down-regulated genes on the left, y-axis depicts level of significance. Designated genes are from tight junction, mucin and inflammasome-related gene groups. (D) 50 most significantly regulated ($p < 0.05$) genes in the whole lung tissue between all AAI phenotypes and control at 4 h after last allergen exposure ($n=3$ mice/group).

Supplementary Figure 2. Distinctive gene interaction networks in the lungs during eosinophilic, mixed and neutrophilic allergic airway inflammation in mice. (A) Interaction network in eosinophilic AAI. (B) Interaction network in mixed AAI. (C) Interaction network in neutrophilic AAI. Network nodes represent differentially expressed genes (DEGs) in each phenotype as compared to control (n input=500 most significant DEGs; genes not interacting with each other are not shown). Edges represent gene-gene associations, with the line thickness indicating the strength of data support. Networks are clustered using kmeans to eight clusters of different colours, based on the distance matrix obtained from the global interaction scores. Colours of the clusters are random for each phenotype. Indicated clusters of importance to the paper.

Supplementary Figure 3. Tight junctions' genes are differentially regulated in eosinophilic, mixed and neutrophilic AAI in mice. mRNA expression of *Ocln*, *Magi3*, *Marveld1*, *Cldn8*, *Magix*, *Cldn15*, *Cldn17*, *Cldn1*, *Cldnd1*(*Cldn25*), *Cldn7*, *Tmem235* (*Cldn27*), *Cldn5*, *Cldn6*, *Cldn13*, *Patj* (*Inadl*), *Jam2*, *Jam3*, *Magi1* and *Marveld2* in lung lysates 4 h after last HDM or saline treatment. Gene expression was assessed using RT-PCR, normalised to housekeeping *Eef2* gene and represented as a relative quantification ($RQ = 2^{-\Delta\Delta CT}$) compared to control mice. Data represent mean

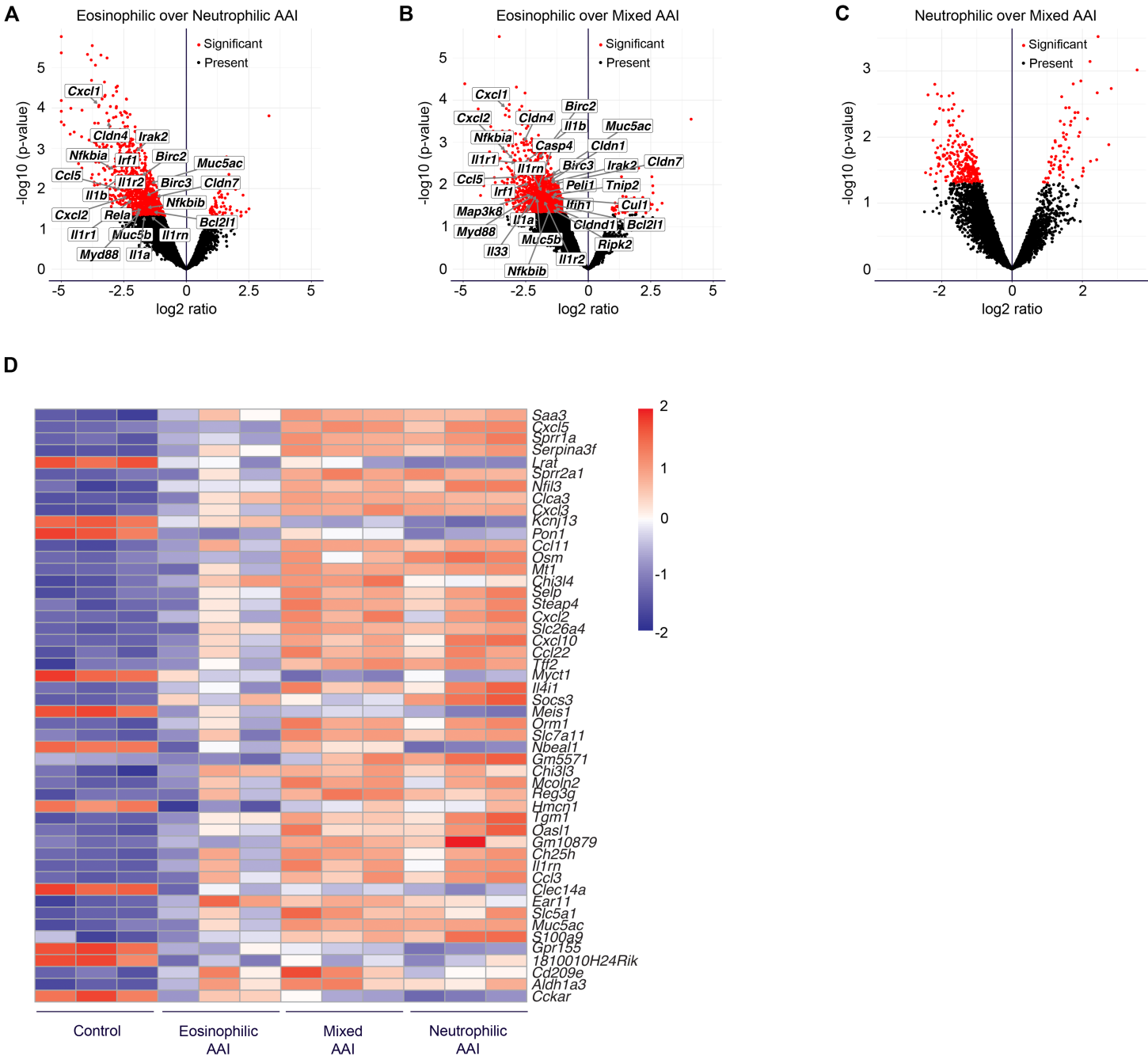
and SEM from three mice/group, each performed in duplicate. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by the Kruskal-Wallis test with Dunn post-hoc analysis. *C*, control; *E*, eosinophilic AAI; *M*, mixed AAI; *N*, neutrophilic AAI.

Supplementary Figure 4. *Muc4* and *Muc5b* are upregulated in mixed and neutrophilic AAI in mice. mRNA expression of *Muc4* and *Muc5b* in lung lysates 4 h after last HDM or saline treatment. Gene expression was assessed using RT-PCR, normalised to housekeeping *Eef2* gene and represented as a relative quantification ($RQ = 2^{-\Delta\Delta CT}$) compared to control mice. Data represent mean and SEM from three mice/group, each performed in duplicate. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by the Kruskal-Wallis test with Dunn post-hoc analysis. *C*, control; *E*, eosinophilic AAI; *M*, mixed AAI; *N*, neutrophilic AAI.

Supplementary Figure 5. Upregulation of inflammasome-related molecules in mixed and neutrophilic AAI in mice. mRNA expression of *Casp4*, *Casp8*, *Il1a*, *Aim2*, *Ddx58 (Rig1)*, *Il18* and *Ifih1 (Mda5)* in lung lysates 4 h after last HDM or saline treatment. Gene expression was assessed using RT-PCR, normalised to housekeeping *Eef2* gene and represented as a relative quantification ($RQ = 2^{-\Delta\Delta CT}$) compared to control mice. Data represent mean and SEM from three mice/group, each performed in duplicate. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by the Kruskal-Wallis test with Dunn post-hoc analysis. *C*, control; *E*, eosinophilic AAI; *M*, mixed AAI; *N*, neutrophilic AAI.

Supplementary Figure 6. mRNA expression of *TJPI (ZO-1)*, *CLDN1*, and *CLDN4* in human bronchial epithelial cells after 24 h stimulation with IL-1 β , IL-17A or medium. Gene expression was assessed using RT-PCR, normalised to housekeeping *EEF1A1* gene and represented as a relative quantification ($RQ = 2^{-\Delta\Delta CT}$) compared to medium control. Data represent mean and SEM from five independent experiments from five different donors, performed in duplicate.

Supplementary Figure 1



A

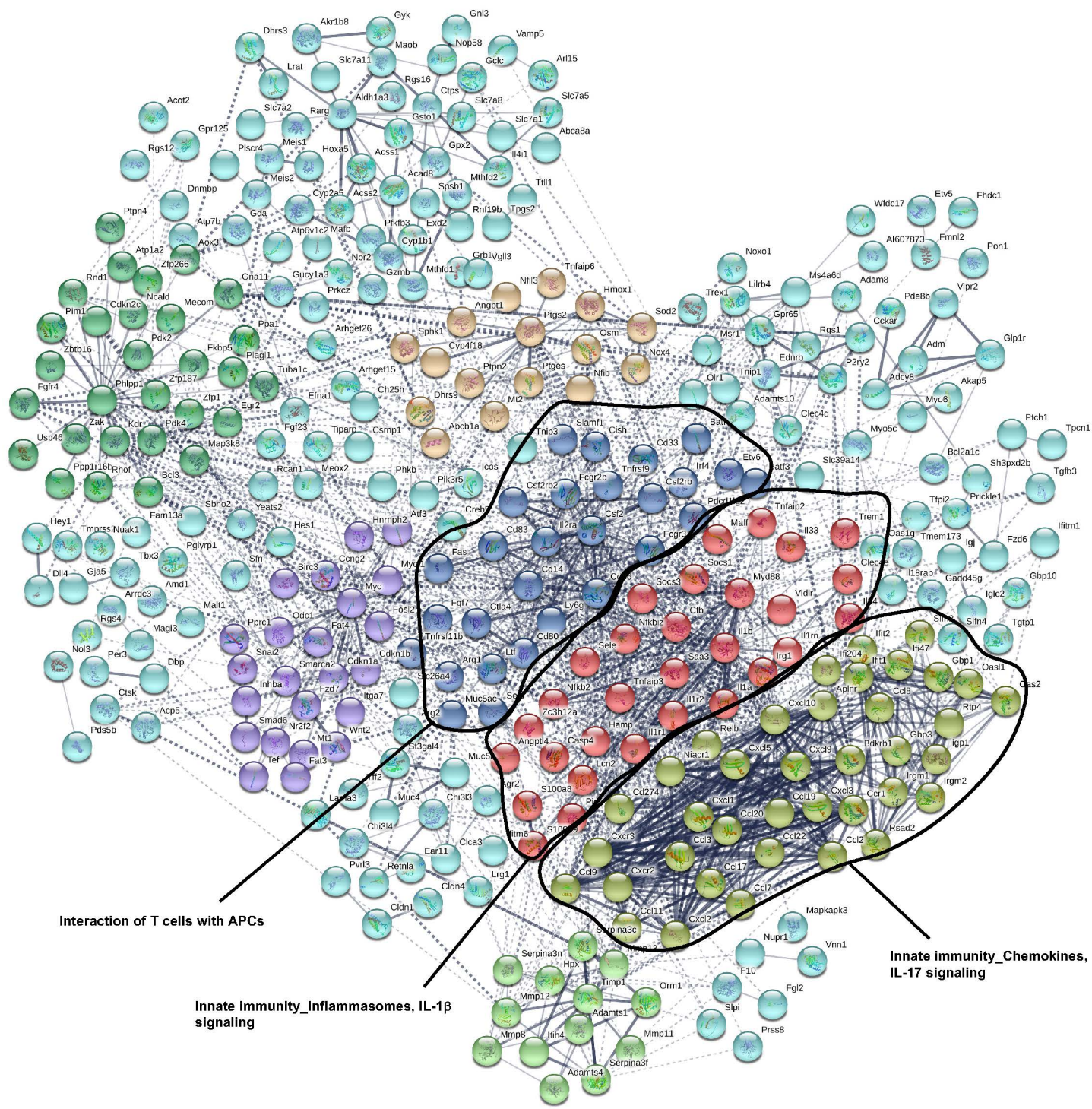
ERK, PKC and PI3K/Akt signaling

**Cytoskeleton reorganization,
muscle cell metabolism,
adherent junctions**

Supplementary Figure 2

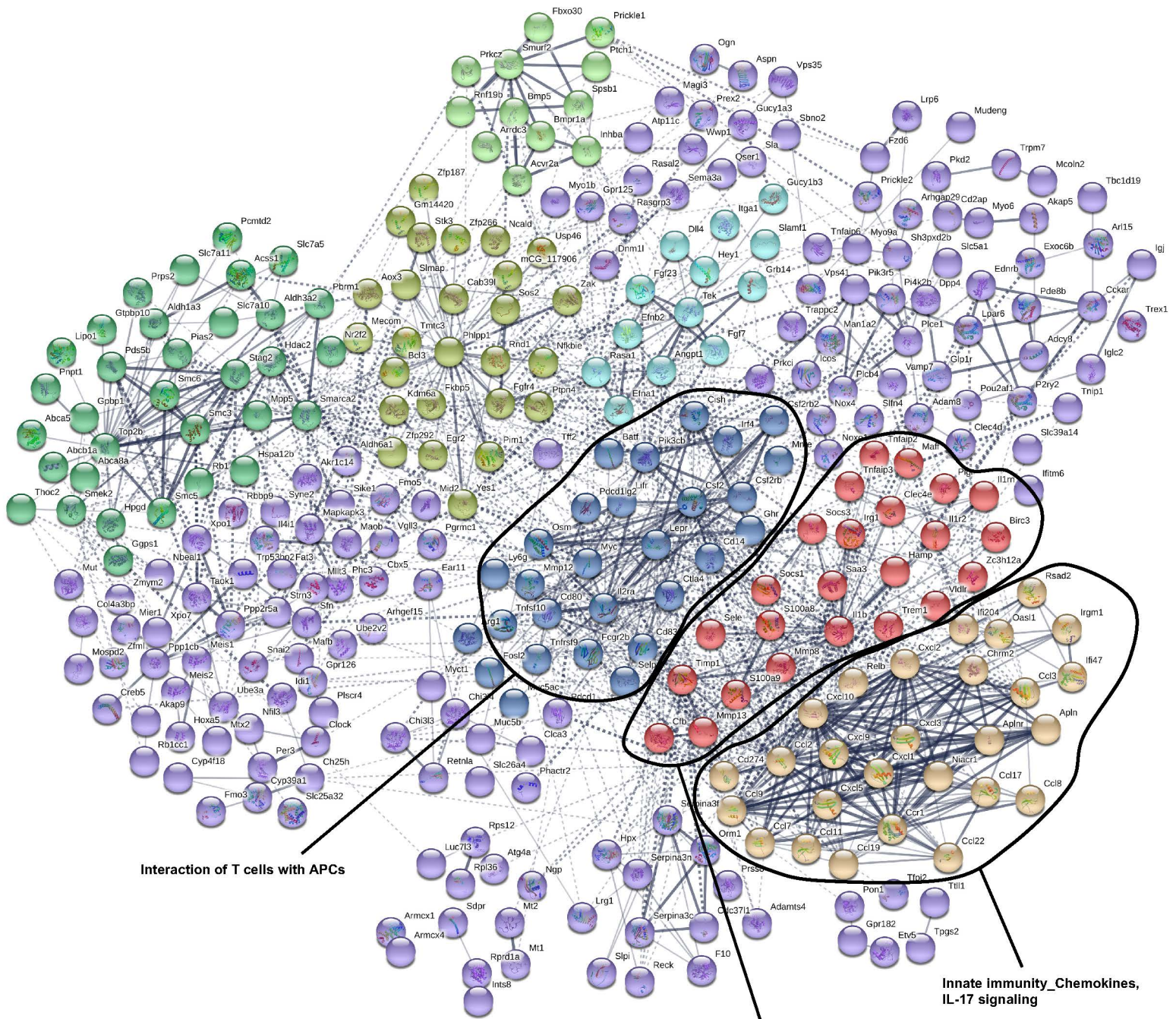
B

Mixed AAI

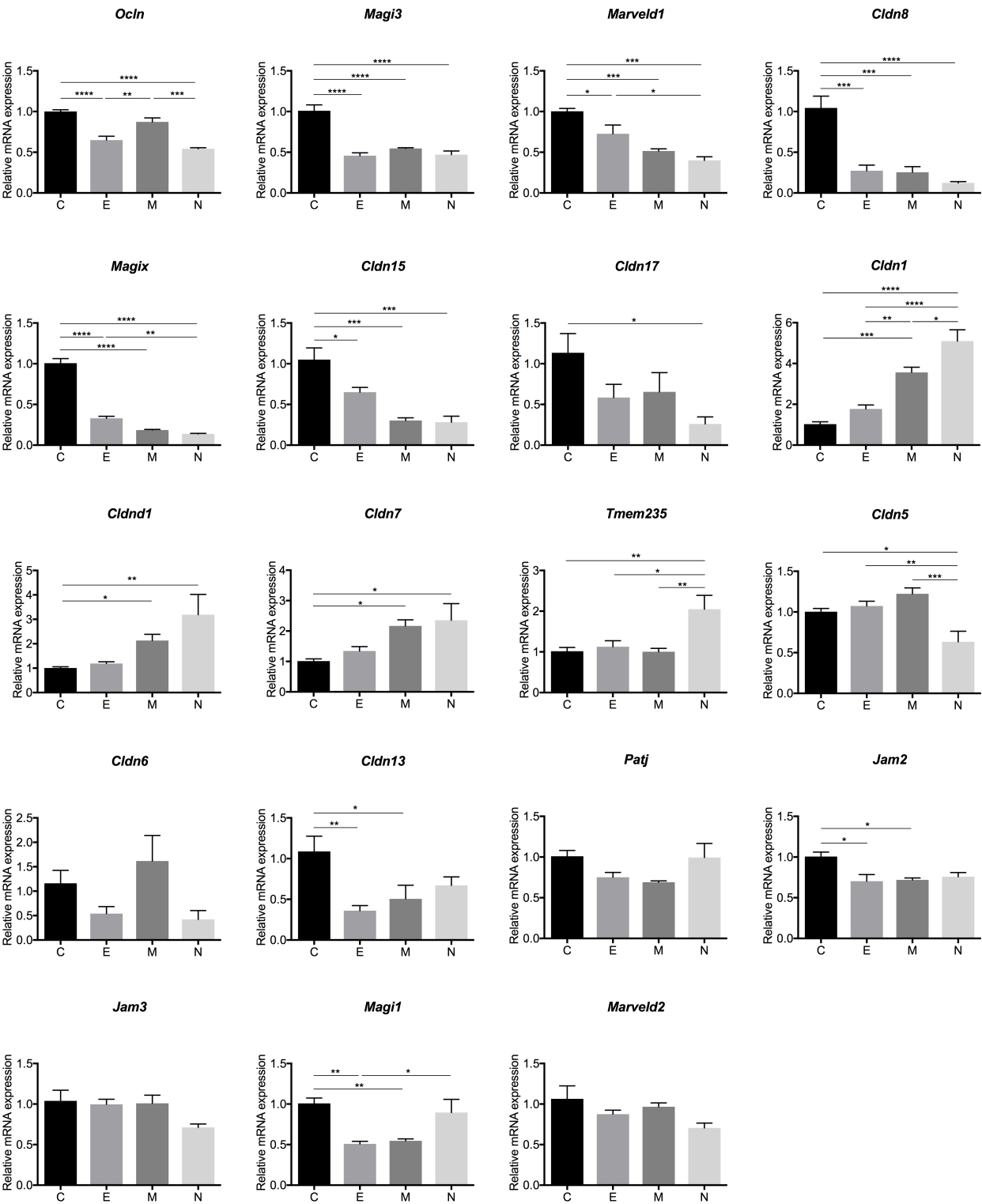


C

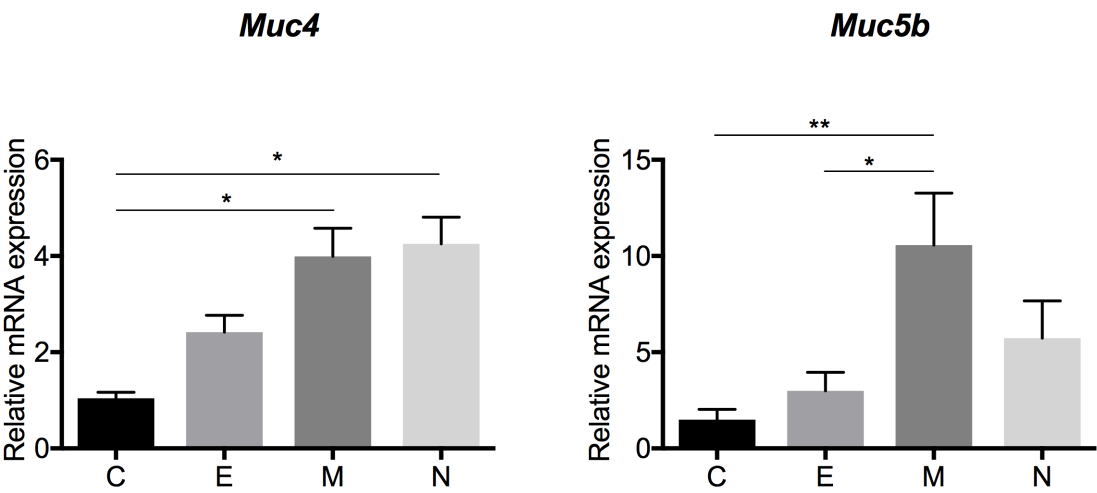
Innate immunity_Inflammasomes, IL-1 β signaling



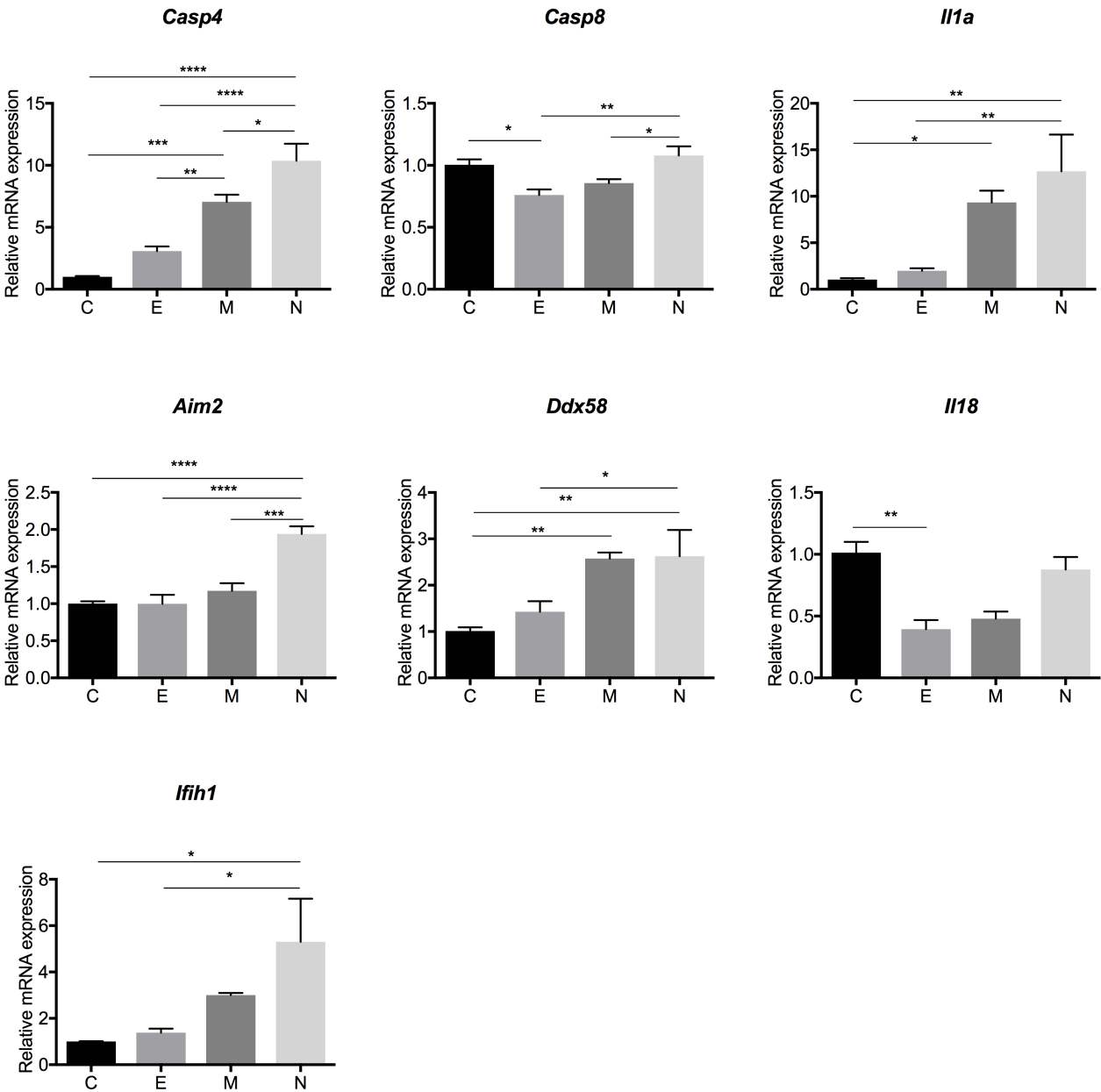
Supplementary Figure 3



Supplementary Figure 4

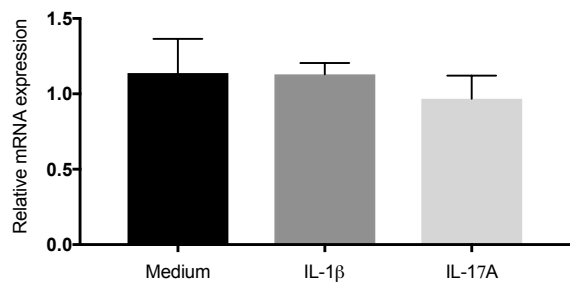


Supplementary Figure 5

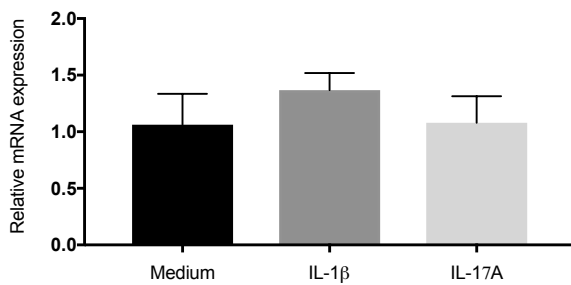


Supplementary Figure 6

CLDN4



CLDN1



TJP1 (ZO-1)

