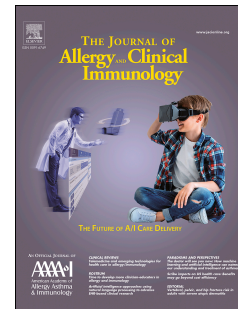


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NLRP1 variant M1184V decreases inflammasome activation in the context of DPP9 inhibition and asthma severity

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Abstract

Background: NLRP1 is an innate immune sensor that can form cytoplasmic inflammasome complexes. Polymorphisms in *NLRP1* are linked to asthma, however there is currently no functional or mechanistic explanation for this.

Objective: We aimed to clarify the role of *NLRP1* in asthma pathogenesis.

Methods: Results from the GALA II cohort study were used to identify a link between *NLRP1* and asthma in Mexican Americans. *In vitro* and *in vivo* models for NLRP1 activation were applied to investigate the role of this inflammasome in asthma at the molecular level.

Results: We document the association of an *NLRP1* haplotype with asthma for which the SNP rs11651270 (M1184V) individually is the most significant. Surprisingly, M1184V increases NLRP1 activation in the context of N-terminal destabilization, but decreases NLRP1 activation upon DPP9 inhibition. *In vitro* studies demonstrate that M1184V increases binding to DPP9, which can account for its inhibitory role in this context. Additionally, *in vivo* data from a mouse model of airway inflammation reveal a protective role for NLRP1 inflammasome activation reducing eosinophilia in this setting.

Conclusion: Linking our *in vitro* and *in vivo* results, the NLRP1 variant M1184V reduces inflammasome activation in the context of DPP9 inhibition and could thereby increase asthma severity. Our studies may have implications for the treatment of asthma in patients carrying this variant of NLRP1.

Key Messages:

- NLRP1 SNP M1184V is associated with asthma
- NLRP1 M1184V alters inflammasome formation depending on the method of activation
- NLRP1 activation decreases severity of mouse asthma model

Capsule Summary: Genetic deletion of *Nlrp1* in mice and the M1184V variant in humans both exacerbate asthmatic pathology, consistent with the mechanism of activation being related to DPP9 inhibition.

Key words: NLRP1, inflammasome, asthma, DPP9, SNP

Abbreviations: NLRP (nucleotide-binding oligomerization domain-like receptor containing a pyrin domain), SNP (single nucleotide polymorphism), DPP9 (dipeptidyl peptidase 9), IL (interleukin), BAL (bronchoalveolar lavage), GALA II (Genes-Environment & Admixture in Latino Americans)

Introduction

Asthma is a common chronic respiratory disease that according to WHO estimates affects 235 million people worldwide. It typically occurs in genetically predisposed individuals after inappropriate immune activation caused by antigen exposure, like viral infections and allergens, and subsequent epithelial damage^{1,2}. Patients suffer from airway inflammation, obstruction and remodelling, as well as airway hyperresponsiveness³. As asthma is an incurable disease, patients require continued treatment and management⁴.

Release of proinflammatory cytokines from the IL-1 family (IL-1 β , IL-18, IL-33) is a hallmark of asthmatic inflammation. IL-1 β and IL-33 activate Th2/Th17 cells and ILC2/3 cells leading to the release of proinflammatory cytokines (IL-4, IL-5, IL-9, IL-13, IL-17A)⁵⁻⁷. Subsequently, eosinophils, neutrophils, mast cells and Th2 cells infiltrate the lung to trigger inflammation and tissue damage^{8,9}. The other IL-1 family member, IL-18, is also linked to asthma, but with conflicting effects. On the one hand it is described to inhibit Th2 cell development together with IL-12, resulting in reduced airway hyper-responsiveness in a mouse model of asthma¹⁰. Moreover, significantly increased serum levels of IL-18 were found in patients during acute asthma episodes¹¹. On the other hand, reduced levels of IL-18 were found in the BAL fluid of asthma patients compared to healthy controls¹². To date, the exact role of IL-18 in asthma pathogenesis is not entirely clear¹³.

Inflammasomes form an important part of the innate immune system and are potent activators of procaspase-1. As such, they are able to induce the release of mature IL-1 β and IL-18 as well as a rapid form of cell death called pyroptosis¹⁴. The current model suggests that upon expression NOD-like receptors (NLRs) are localized in the cytosol of the cell as monomers. Immediately after activation they assemble into disc-like oligomers, providing a platform for the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD). In turn, ASC assembles into large filaments and recruits procaspase-1, ultimately leading to activation of procaspase-1 by self-cleavage. Active caspase-1 can process the pro-forms of IL-1 β and IL-18 into their active forms and thus induce inflammation^{14,15}. Further, active caspase-1 also cleaves gasdermin D, which in turn forms membrane pores inducing pyroptosis¹⁶.

NLRP1 was the first inflammasome described and has been shown to be expressed in various tissues including the respiratory epithelium, as opposed to

NLRP3, which is primarily expressed in the hematopoietic compartment^{17,18}. The 166 kDa NLRP1 protein also differs from other NLRPs in its domain composition. The NLRP1 N-terminus is formed by a common tripartite domain architecture that is shared by all NLRP proteins, comprised of a PYD, a NACHT domain and leucine rich repeats (LRR). However, an additional FIIND and CARD domain on the C-terminus are unique to NLRP1¹⁹. Autolytic proteolysis within the FIIND domain is a strict requirement for NLRP1 activity^{20,21}. A common single nucleotide polymorphism (rs11651270) resulting in the amino acid substitution methionine 1184 to valine (M1184V) was described to increase cleavage in the FIIND domain and has been associated with genetic predisposition to asthma^{21,22}. Furthermore, SNPs in *NLRP1* were found to be involved in other diseases, like vitiligo-associated autoimmune diseases²³. Several mutations in the *NLRP1* gene locus were identified to lead to constitutive inflammasome activation, causing skin autoinflammatory syndromes^{24,25}. These patient-derived mutations helped to understand the mechanistic role of NLRP1 PYD and LRR in keeping the protein in an autoinhibited conformation²⁴. The fact that the NLRP1 PYD is autoinhibitory differentiates this inflammasome from other members of the NLRP family, which require their PYD to directly bind the adaptor protein ASC and thus enable downstream activation of procaspase-1²⁶. In contrast, it is the C-terminal CARD domain of NLRP1 that triggers inflammasome activation^{21,24}.

To date, the exact mechanism by which NLRP1 is activated through pathogens is not fully understood. However, *Toxoplasma gondii* has been demonstrated to activate NLRP1 in mice and rats, and genetic variants in human NLRP1 increase susceptibility to congenital Toxoplasmosis²⁷⁻³⁰. Dense granule proteins of the parasite have been identified to be essential for triggering NLRP1-dependent pyroptosis in rats³¹. Lethal toxin from *Bacillus anthracis* cleaves and thereby activates certain alleles of mouse and rat Nlrp1^{32,33}. Recently, different pathogen enzymes were identified to induce N-terminal degradation of mouse NLRP1b. Cleavage within the FIIND domain provided, this degradation process led to activation of the NLRP1 inflammasome³⁴. Additionally, it has been shown that inhibition of the negative regulator dipeptidyl peptidase 9 (DPP9), which binds to the FIIND domain, leads to activation of NLRP1 in humans and mice^{35,36}. Inhibition of DPP9 leads to a dissociation of the peptidase from NLRP1 and results in N-terminal degradation and finally activation of the inflammasome^{37,38}. Moreover, one of the human mutations causing skin inflammatory symptoms and associated arthritis was identified to be

located within the FIIND domain (P1214R)²⁵. This mutation was shown to activate NLRP1 by preventing DPP9 association with NLRP1³⁶.

Here we confirm that the M1184V mutation within the FIIND domain of NLRP1 is associated with increased asthma severity. Surprisingly, we find that activation of NLRP1 M1184V *in vitro* via N-terminal destabilizing mutations has divergent effects compared to activation via DPP9 inhibition. Additionally, genetic manipulation of *Nlrp1* in mouse models of asthma shows that *Nlrp1*-deficiency exacerbates asthma models. Combined with the *in vitro* data, this aligns with a causative effect of M1184V in asthma to decrease NLRP1 activation in the context of DPP9 inhibition.

Methods

Plasmids and mutagenesis

Viral constructs including pRP-hASC-RFP (retroviral construct) and pTRIPz-NLRP1-HA-T2A-GFP-IRES-Puro (2nd generation lentiviral construct) were used to establish cell lines with stable expression of the transgenes. For immunoprecipitation, NLRP1 constructs were expressed from a pCIG2 vector backbone with a C-terminal 3xFLAG-tag, eGFP was expressed from an IRES on the same vector. Mutagenesis was carried out using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) following the manufacturer's instructions. Successful mutation was confirmed by sequencing.

Cell culture

HEK293T cells were cultured at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (FBS), 0.1% (w/v) streptomycin and 100 U/mL penicillin. FBS was heat-inactivated for 30 minutes at 55°C before supplementation. Continuous cultures were monitored for absence of mycoplasma by PCR.

Generation of stable cell lines

Production of viral particles for stable expression of transgenes was achieved by transfecting HEK293T with packaging plasmids pGag-pol and VSV-G along with the transgene hASC-RFP for retroviral system and psPAX2 and pMD2.G along with the transgene NLRP1-HA-T2A-GFP-IRES-Puro for 2nd generation lentiviral system, respectively. Plasmids were transfected into HEK293T cells, seeded at 3x10⁶ cells in 10 cm² dishes one day before the transfection was performed. Medium of the transfected cells was replaced 16 hours post transfection. The viral supernatant was collected and filtered through a 0.45 µm filter at 24 hours and 48 hours post medium change. The filtered viral supernatant was then added directly to HEK293T and incubate for 24 hours before the viral supernatant was entirely removed.

Successful retroviral transduction of hASC-RFP cells was confirmed and cells were isolated by fluorescence-activated cell sorting (FACS) for RFP⁺ non-speckling cells. These cells were used as a parental cell line for the subsequent lentiviral transduction of NLRP1-HA-T2A-GFP-IRES-Puro. Following the lentiviral transduction

of NLRP1-HA-T2A-GFP-IRES-Puro, the cells were subjected to 5 µg/mL puromycin selection before sorting for GFP⁺ non-specking cells. Expression of the NLRP1-T2A-GFP transgene was induced using 1 µg/mL doxycycline (Sigma Aldrich) and the expression was confirmed by flow cytometry and western blot analysis.

ASC speck assay

3.5x10⁴ HEK293T cells stably expressing human ASC-RFP and stably carrying the doxycycline-inducible wild type (WT) or mutant human NLRP1 transgene were seeded in a flat-bottom 96-well tissue culture plate. The cells were seeded in the presence of 1 µg/mL of doxycycline to induce the expression of the transgene. Cells were harvested and analysed for speck formation 12 hours post induction by flow cytometry.

For Talabostat treatment, the medium was replaced at 16 hours post doxycycline induction with medium containing increasing concentration of Talabostat (MedChemExpress HY-13233A) from 0.125 µM to 1 µM. The highest equivalent amount of DMSO was used as a control. Following Talabostat treatment, cells were incubated for 6 hours at 37°C. Cells were then harvested and analysed for speck formation by flow cytometry on a BD Bioscience LSR Fortessa X-20 as described previously³⁹.

Immunoprecipitation

2.5x10⁵ HEK293T cells were transfected with 500 ng of wildtype (WT) or mutant NLRP1 (IRES-GFP). 18 hours post transfection cells were washed once with 1xDPBS and harvested in NP40 lysis buffer (1% NP40 (v/v), 10% Glycerol (v/v), 20 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 10 mM NaPPI, 5 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF) freshly supplemented with 1x cOmplete protease inhibitor cocktail (Roche). After lysing cells for 20 minutes on ice, cell debris was spun down and the supernatant was collected. Immunoprecipitation was performed using anti-FLAG-M2-agarose resin (Sigma) for 4 hours or overnight at 4°C. Beads were washed three times with lysis buffer before elution by boiling in SDS sample buffer for 10 minutes. Immunoblots were prepared using 4-12% gradient gels (Novex, Invitrogen) and subsequently transferred to a PVDF membrane. Membranes were blocked in phosphate buffer saline/tween 20 (PBS-T) with 5% skim milk for 60 minutes at room

temperature and probed over night at 4°C. Antibodies: aNLRP1: AL176 (AdipoGen), aDPP9: ab42080 (Abcam), aFLAG: 9H1 (in house), aActin: sc47778 (SCBT).

Mice

WT, Nlrp1^{-/-}, Il1r^{-/-}, Il1r^{-/-}Neut1^{m/m}, Il1r^{-/-}Il18^{-/-}, and Il1r^{-/-}Il18^{-/-}Neut1^{m/m} mice described previously⁴⁰ were analysed between 1.5-3 months of age. Animal experiments complied with the regulations set by the Walter and Eliza Hall Institute of Medical Research (WEHI) Animal Ethics Committee.

Asthma model

Mice were immunised with sensitisation solution (50% (v/v) aluminum hydroxide (Sigma), 250 µg/mL ovalbumin (lyophilized chicken egg white albumin) (Sigma), 10% (v/v) type II water and 40% (v/v) normal saline (WEHI media department); 200 µL/mouse) via IP injection on days 1 and 14. On days 27, 28 and 29 mice were challenged via nebulization (inhalation) of challenge solution (MTPBS (WEHI media department), 5% (w/v) ovalbumin (Sigma)) for 20 minutes. Mice were sacrificed and prepared for assessment of asthma pathology on day 30.

Isolation and preparation of organs and fluids

Bronchoalveolar lavage fluid (BALF) was collected by performing a tracheotomy on the mice before flushing the lungs with 3 x 500 µL flow buffer (MTPBS (WEHI media department), 1% fetal bovine serum, 2.5 mM EDTA). Next, lungs were collected and the left lobe retained in 10% buffered formalin for histology. Lungs were diced with scissors and digested at RT for approximately 30 minutes in digestion buffer (RPMI (WEHI media department), 1 mg/mL Collagenase III (Worthington), 0.4 U Dispase, 1 µg/mL Dnase) with mixing. Single cell suspensions were obtained by passing digested lungs through a 70 µm sieve. Cells were collected by centrifugation. BALF supernatants were collected and stored at -20°C for later analysis, and red cells lysed in red cell removal buffer (WEHI media department) for 1 min at RT. Cells were then washed and resuspended in flow buffer. Cells were incubated with CD16/32 Fc block for 20 minutes prior to incubation with cell surface staining combination. Cells were then incubated with fixation buffer (eBioscience), permeabilization buffer (eBioscience), and intracellular antibodies, and washed between steps. Cells were resuspended in 200 µL of flow buffer. Spleen single stain controls were included for

all experiments. All centrifugations were performed at 1300 g for 5 minutes at 4°C and all incubations were on ice for 20 minutes.

Flow cytometry

Flow cytometry was performed on a BD Bioscience LSR Fortessa cell analyser and analysis was performed using FlowJo software. Granulocytes were defined as CD45.2⁺, B220⁻ or CD19⁻, CD11b⁺ cells and further defined as eosinophils or neutrophils based on Siglec-F⁺ Ly6G⁻ or Siglec-F⁻ Ly6G^{hi}, respectively. Macrophages/monocytes were defined as Siglec-F⁻ and Ly6G⁻ CD11b⁺. T lymphocytes were defined as CD45⁺ CD3⁺ CD4⁺ B220-cells and further classified as regulatory T lymphocytes (T_{reg}), Th2, or Th17 by expression of FoxP3, GATA3 or RORγt, respectively. ILCs were defined as CD45⁺ lineage negative (CD3⁻/CD4⁻) cells and further stratified into ILC2 or ILC3 by expression of GATA3 or RORγt, respectively. Single stain controls were included for all flow cytometry experiments to facilitate appropriate compensation. Representative dot plots for the gating strategies are shown in the supplement (Figure S1 and S2).

Histopathology

Lungs were collected in 10% buffered formalin. Sections were stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) and toluidine blue (TB) for detection of inflammatory cells, mucus production and mast cells.

Multiplex ELISA

Multiplex ELISAs were performed using the BioPlex Pro Mouse Cytokine Kit (BioRad) following the manufacturer's instructions. Absorbance was recorded at 450 nm using a microplate reader.

Statistics

Data was analysed using Prism software. Comparison of data was performed using a student's t-test or an ANOVA followed by a paired t-test. Values are displayed as mean ± standard error of the mean (SEM).

GALA II and SAGE II Cohort Study

The Genes-environments and Admixture in Latino Americans (GALA II) Study and the Study of African Americans, Asthma, Genes, and Environments (SAGE II) Study are two clinic-based multi-center asthma case-control studies, conducted using identical protocols and questionnaires, to examine the complex network of genetic and environmental factors contributing to asthma prevalence and severity among Latino and African American children^{41,42}. Full descriptions of the GALA II and SAGE II study protocols and recruitment, including inclusion and exclusion criteria have been previously described, in detail^{41,42}. Briefly, asthma cases and controls were recruited from community centers and clinics in the mainland U.S. and Puerto Rico (2006-present). Individuals were eligible to participate if they were 8-21 years of age and identified all four grandparents as Latino (GALA II) or African American (SAGE II). Participants were excluded if they had: (1) 10 or more pack-years of smoking; (2) any smoking within 1 year of recruitment date; (3) pregnancy in the third trimester; or (4) history of one of the following conditions: sickle cell disease, cystic fibrosis, sarcoidosis, cerebral palsy, or history of heart or chest surgery. Demographic data, including medical history and environmental exposure information, was collected from participants at study enrolment. All local institutional review boards of participating recruitment sites approved the study and all participants (or parents of participants younger than age 18 years) provided written informed consent.

Asthma case/control status was determined by physician assessment at study enrolment. Age- and sex-specific BMI percentiles (BMI-pct) were calculated as previously described and used to assign BMI categories⁴³. For subjects aged 20 years and over, BMI categories were defined as follows: non-obese (BMI < 30) and obese (BMI ≥ 30). For subjects under 20 years of age, BMI categories were defined as follows: non-obese (BMI percentile < 95) and obese (BMI percentile ≥ 95).

Study Population

Mexican American subjects from the GALA II study cohort with complete demographic data (age, sex, asthma status, obesity status) and available NLRP1 SNP were used as a discovery dataset in the current study (n = 905). Puerto Rican participants from the GALA II study (n = 1418), and African American participants from the SAGE study (n = 1256) were later examined to determine if associations between NLRP1 variants and asthma (both single variant and haplotype effects) present in the discovery dataset were also present in these non-Mexican populations.

Study demographics for all individuals in the discovery dataset (n = 905 Mexican Americans) and the two replication datasets (n = 1418 Puerto Ricans; n = 1256 African Americans) are presented in Supplemental Tables 1a-1c. All descriptive statistics presented in Supplemental Table 1 were generated using the R statistical software program base package.

NLRP1 Genotyping

Blood samples were collected from GALA II and SAGE II participants at study enrolment for DNA analysis. Axiom[®] Genome-Wide LAT 1 array (Affymetrix, Santa Clara, CA, dbGaP phs000921.v1.p1) was used for genotyping. SNPs were excluded if they failed the manufacturer's quality control, had genotyping call rates below 95%, and had a deviation from Hardy-Weinberg equilibrium ($p < 10^{-6}$) within controls. Additional SNP genotypes were imputed using the Michigan Imputation Server. The NHLBI Trans-Omics for Precision Medicine (TOPMed) program data (freeze 5) was used as the imputation reference panel⁴⁴. Imputed SNPs were excluded from the dataset if R^2 was below 0.3. Genotypes of several NLRP1 single nucleotide polymorphisms (SNPs), identified by prior studies as functionally relevant in relation to immunological diseases⁴⁵, were then extracted from the total genotype data (Supplemental Table 3) for downstream single variant and haplotype analyses. Principal component analysis using genotyped data for all participants was performed using the PLINK 2.0 software platform⁴⁶. The first three principal components were included in downstream association analyses to account for hidden substructure in the dataset. Genotype data for GALA II study participants is available on dbGaP under accession number phs001274, genotype data for SAGE II study participants is available on Dryad (<https://datadryad.org/stash/share/20Ma3lthxRaK5sxbTNHGpOMGeDnJVFTPxDIxECTh2is>).

Single Variant Analyses

We used logistic regression to assess the relationship between each variant and asthma susceptibility, separately. All regression models were adjusted for age, sex, obesity status, and the first three principal components (generated as described in the previous subsection). We also calculated allele frequencies for each variant in participants with and without asthma, separately and combined (Supplemental Table

2). All single variant genetic analyses were performed using the PLINK 2.0 software platform⁴⁶.

Multi-variant (Haplotype) Analyses

NLRP1 variant haplotype analyses were performed using the Haplo.stats R software package⁴⁷. Haplo.stats is a statistical program optimized to assess the relationship between a specified haplotype and case/control status in unrelated individuals, when haplotype phase is unknown, using regression-based analyses. We identified five NLRP1 haplotypes, containing a total of 14 SNPs, previously defined by Levandowski et al., and shown by the Author's to be significantly associated with vitiligo and other autoimmune disorders⁴⁵. Haplotype definition and haplotype frequencies in our study population are presented in Supplemental Tables 4 and 5a-c. The most commonly occurring haplotype, referred to as Haplotype 1, in both asthma cases and controls was selected as the reference haplotype for regression-based analyses performed in Haplo.stats. Presented odds ratios for haplotype effects describe the effect of the specified haplotype on asthma status as compared to Haplotype 1 (Table 1).

Multiple Testing Correction for Single Variant and Multi-variant (Haplotype) Analyses

We used the Bonferroni method to control the familywise error rate (FWER) and correct for multiple testing⁴⁸. Significance thresholds derived using the Bonferroni equation can also produce false negatives. To minimize type II error in our study, in addition to significant association thresholds, we also generated a more liberal suggestive association threshold^{43,49}. For multi-variant analyses, the effective number of tests was defined as the number of haplotypes assessed. Bonferroni FWER thresholds of 0.05 and 0.1 were used to generate significant and suggestive association thresholds, respectively⁵⁰. The suggestive and significant p-value thresholds for single variant analyses (3 variants) were 0.033 and 0.017, respectively. The suggestive and significant p-value thresholds for the multi-variant analyses (4 haplotypes) were 0.025 and 0.0125, respectively.

Results

***NLRP1* variant M1184V is associated with genetic predisposition to asthma**

In a discovery cohort of 905 Mexican American children with and without asthma from the GALA II study, we assessed the effect of select *NLRP1* single nucleotide polymorphisms (SNPs) on asthma susceptibility. Descriptive characteristics for all discovery study subjects are summarized in Supplemental Table 1a. Demographic variables were assessed for significant differences between asthma cases and controls. Significant differences were found for age ($P = 2.20 \times 10^{-16}$), sex ($P = 0.007$), and obesity status ($P = 0.01$).

A previous study by Levandowski *et al.* identified significant *NLRP1* haplotype effects associated with vitiligo and other immunologic conditions⁴⁵. We assessed the impact of these same haplotypes on asthma status in our study population. Regression-based haplotype analysis revealed one *NLRP1* haplotype significantly associated with asthma status, compared to the most common/reference haplotype (Table 1). This haplotype, Haplotype 2A, remained significantly associated with asthma after correction for multiple testing (Odds Ratio: 1.69; $P = 0.004$). Genotypes for all *NLRP1* SNPs included in Haplotype 2A are listed in Supplemental Table 4.

We then performed single variant association testing, using logistic regression, to assess the impact of the three non-synonymous *NLRP1* polymorphisms in Haplotype 2A L155H (rs12150220), V1059M (rs2301582), and M1184V, (rs11651270) on asthma status in our study population. Each regression analysis was adjusted for age, sex, obesity status, and the first three principal components. Two *NLRP1* variants, V1059M and M1184V, were associated with asthma status in our study; however only one variant, M1184V remained suggestively associated with asthma after correction for multiple testing ($P = 0.02$, Table 2). Specifically, increased copies of the C allele of M1184V were associated with increased asthma susceptibility (Odds Ratio: 1.28; $P = 0.02$).

After completing haplotype and single variant analysis in our Mexican American dataset, we attempted to further validate our findings in two independent datasets of Puerto Rican and African American children (Supplemental Tables 1b-c). Neither the significant 2B haplotype effect, nor the suggestive single locus effect at M1184V, were present in either of our validation datasets (Supplemental Tables 6a-b, 7a-b). Further assessment revealed differing allele and haplotype frequencies among the

three populations that may account, in part, for the lack of replication of significant and suggestive associations found in Mexican Americans.

Activation of NLRP1 is reduced by M1184V in the context of DPP9 inhibition

The NLRP1 M1184V variant has been reported to increase FIIND domain cleavage in a previous study²¹. Analysis of a homology model showing the ZU5 and part of the UPA subdomains of NLRP1 FIIND suggests that the respective methionine residue is located in proximity to the proposed catalytic triad consisting of amino acid residues S1213, H1186 and E1195 (Figure S3). These residues were previously described to be directly involved in facilitating autolytic proteolysis between F1212 and S1213^{20,21}. Exchanging methionine 1184 to valine could alter the positioning of the catalytic residues relative to each other, thereby allowing increased proteolysis. However, the exact molecular mechanism of how substitution of methionine 1184 affects proteolytic processing remains unclear. Additionally, the effect of M1184V has not yet been examined in the context of NLRP1 stimulation, either by activating mutations or DPP9 inhibition. To investigate the functional effect of M1184V on inflammasome activation, we reconstituted NLRP1 in HEK293T cells stably expressing ASC-RFP. ASC speck formation was used as a measure for NLRP1 inflammasome activation and was quantified by flow cytometric analysis as previously described³⁹. NLRP1 WT and M1184V showed similar baseline levels of ASC speck formation (Figure 1A). Western blot analysis for NLRP1 full length and N-terminal cleavage fragments (anti-NLRP1), as well as the NLRP1 C-terminal cleavage fragment (anti-Flag) confirmed increased FIIND domain cleavage by M1184V (Figure 1B).

NLRP1 inflammasome activation was triggered by introducing recently reported patient mutations into the WT and M1184V NLRP1 constructs. The A66V mutation was described to activate NLRP1 by destabilizing its PYD²⁴. As expected, introduction of this mutation into NLRP1 WT resulted in increased ASC speck formation. In the context of increased FIIND domain cleavage, induced by the M1184V polymorphism, this effect was enhanced as a further accumulation of ASC specks was detected (Figure 1A). Increased FIIND domain cleavage for NLRP1 A66V/M1184V was confirmed by western blot analysis (Figure 1B). Introduction of a different monogenic autoinflammatory disease mutation (P1214R), described to activate NLRP1 through a loss of DPP9 binding^{25,36}, also resulted in a strong increase in ASC speck formation. Interestingly, combining this mutation with M1184V

reduced NLRP1 activity significantly, as quantified by ASC speck formation (Figure 1A). Western blot analysis of the P1214R variant did not show a large increase in the C-terminal fragment, and combining P1214R with M1184V did not result in a further increase in C-terminal cleavage.

Thus, we concluded that increased cleavage of NLRP1 for the M1184V variant liberates more of the active C-terminal fragment, when in combination with mutations that destabilise the N-terminus, accounting for increased inflammasome activation. However, this cannot explain the decreased inflammasome activation observed when M1184V is present in conjunction with the P1214R mutation. Given that P1214R activates NLRP1 by destabilizing the interaction with DPP9, we performed immunoprecipitations to see if DPP9 binding was altered due to M1184V. Indeed, DPP9 binding was abolished by P1214R (Figure 1B). Compellingly, the M1184V variant increased binding to DPP9, even in the presence of P1214R, explaining how it inhibits NLRP1 activity in this context. As reported previously, A66V had no effect on DPP9 binding³⁶, and combination with M1184V also increased binding to DPP9 (Figure 1B). Therefore, when the N-terminus of NLRP1 is destabilised, inflammasome activation proceeds regardless of DPP9 binding.

To independently confirm the inhibitory effect of M1184V on NLRP1 activation induced by loss of DPP9 binding, we made use of the DPP9 inhibitor Talabostat. Consistent with the findings described for NLRP1 M1184V/P1214R, we found that activation by Talabostat was inhibited for NLRP1 M1184V, however only at lower thresholds of activation (Figure 2A). Increased DPP9 binding in NLRP1 M1184V after Talabostat stimulation was also confirmed by immunoprecipitation (Figure 2B). These results indicate that NLRP1 activation induced by impaired DPP9 binding is reversed by the M1184V variant, which stabilises the interaction with DPP9.

Nlrp1^{-/-} mice show increased eosinophil infiltration in asthma model

To better understand the role of Nlrp1 in asthma, we used an alum/OVA mouse model. Noteworthy, mice variants of Nlrp1 were reported to naturally carry a valine at the position corresponding to the human M1184²¹. We confirmed this finding by multiple sequence alignment of the amino acid sequence of NLRP1 in different organisms. Interestingly, we found that within the analysed sequences, including sequences from different primate species, only humans have methionine as the canonical amino acid at position 1184 (Figure S4).

Airway inflammation in mice was assessed by comparing immune cell infiltration into the lung and cytokine levels of WT and *Nlrp1*^{-/-} mice. Histological comparison of lung sections of WT and *Nlrp1*^{-/-} mice revealed no overt difference. *Nlrp1*^{-/-} mice showed no pathology at baseline (Figure 3A) and both groups developed alveolitis and showed immune cell infiltration into the airways and lung vessels following alum/OVA treatment. Immune cell infiltration into the lung was measured by flow cytometric analysis of the lungs after bronchoalveolar lavage of alum/OVA-treated mice. Interestingly, *Nlrp1*^{-/-} mice showed increased eosinophilia compared to WT mice, suggesting a protective effect of NLRP1 in asthma (Figure 3B). Levels of monocytes/macrophages, neutrophils, Th2 cells and ILC2s were comparable for both WT and *Nlrp1*^{-/-} mice (Figure 3B and C). A protective effect of *Nlrp1* in the asthma model was also indicated by an increase of IL-13 in the bronchoalveolar lavage (BAL) fluid of *Nlrp1*^{-/-} mice (Figure 3D). IL-4 and IL-5 levels were comparable for WT and *Nlrp1*^{-/-} mice (Figure 3D). Furthermore, IgE levels were comparable in the serum and BAL fluid of WT and *Nlrp1*^{-/-} mice (Figure 3E).

Active *Nlrp1* protects against asthma model in mice independently of IL-1

The results obtained from *Nlrp1*^{-/-} mice suggest a protective effect of *Nlrp1* activation in the asthma model. Therefore, we wanted to further investigate the role of the proinflammatory cytokines IL-1 β and IL-18 in mediating protection during asthma, since both cytokines are released upon *Nlrp1* activation. To this end, we first looked at immune cell infiltration and cytokine levels in *Il1r*^{-/-} and *Il1r*^{-/-}/*Neut1*^{m/m} mice during asthma. *Neut1*^{m/m} mice were described to have an NLRP1a variant carrying an activating mutation (Q593P)⁴⁰. Although *Neut1*^{m/m} mice have spontaneous lung inflammation, this is totally resolved in *Il1r*^{-/-}/*Neut1*^{m/m} mice, which can be used in models of disease to establish effects that are independent of IL-1R. *Il1r*^{-/-}/*Neut1*^{m/m} mice exhibited decreased eosinophilia in the lung during asthma (Figure 4A), indicating a protective effect of NLRP1 activity even in the absence of IL-1R. This was further supported by a decrease in IL-5 and IL-13 levels in the BAL fluid of alum/OVA-treated *Il1r*^{-/-}/*Neut1*^{m/m} mice compared to *Il1r*^{-/-} mice (Figure 4C). Levels of monocytes/macrophages, neutrophils, Th2 cells and ILC2s were comparable for both groups (Figure 4A and B). IL-4 levels in the BAL fluid of both groups were also similar (Figure 4C).

Since the protective effect of NLRP1 in asthma was independent of IL-1R signalling, we hypothesized that IL-18 plays a major role in mediating protection. We therefore investigated asthma pathology in *Il1r^{-/-}Il18^{-/-}* and *Il1r^{-/-}Il18^{-/-}Neut1^{m/m}* mice. Again, we looked at immune cell infiltration into the lungs of these mice and at cytokine levels in the BAL fluid. Interestingly, there were no differences in eosinophilia or IL-5 and IL-13 cytokine levels observed for both groups (Figure 4D and F). In addition, the levels of monocytes/macrophages, neutrophils, Th2 cells and ILC2s were also comparable for both groups (Figure 4D and E). These results indicate a role for IL-18 in mediating protection to asthma in an NLRP1-dependent manner.

Discussion

The herein presented data is consistent with literature covering genetic associations of the inflammasome and IL-18 to asthma and further advances our understanding of the role of NLRP1 in this chronic disease^{10-12,22,51,52}. One limitation of our approach was to interrogate NLRP1 haplotypes that were previously established in vitiligo and other autoimmune disorders⁴⁵, so we may not have captured haplotype effects that were specific to asthma. Additionally, a larger or more diverse asthmatic population than what we currently have available would be needed to replicate and validate these genetic associations. Our *in vivo* data establish that activation of Nlrp1a is protective in mouse models of this condition,⁵¹ via IL-18. Extrapolating from this, we provide evidence that the M1184V allele results in decreased inflammasome activation in the context of DPP9 inhibition. This would be consistent with the allele providing an increased disease risk, which we confirmed by genetic analysis of asthma patients.

Our findings are novel because until now, studies looking at the role of M1184V did not activate the NLRP1 inflammasome with a specific stimulus. Using patient mutations that destabilise the N-terminus, or DPP9 inhibiting mutations/molecules, we are the first to observe that there is a dichotomous response, with the former resulting in increased inflammasome activation, and the latter resulting in decreased inflammasome activation. Previously, it was documented that M1184V can increase autocatalytic cleavage of NLRP1²¹, and that this could account for increased activation in the presence of N-terminal destabilising mutations. This is consistent with the observation that, where genetic information was available, all patients with

dominantly inherited destabilising N-terminal mutations have also possessed the M1184V allele, thus increasing NLRP1 activity²⁴. On the other hand, we now show that M1184V also increases binding to DPP9. Consequently, this would prevent its activation by DPP9 inhibition, which we also observed (Figure 1 and 2).

Dichotomous effects of M1184V on NLRP1 activation are also consistent with dichotomous effects in human diseases. Gathering all known disease associations for this allele, it is clear that a protective/causative role is not always consistent with inflammasome inhibition/activation, respectively (Table 3). Therefore, we suggest that in conditions such as asthma, where M1184V contributes to disease but NLRP1 has a protective role, the underlying mechanism of activation for NLRP1 resembles DPP9 inhibition. In contrast, for conditions where M1184V and NLRP1 both contribute to disease, NLRP1 inflammasome activation would be mediated via destabilisation of the N-terminus.

Previously, deletion of *Nlrp1b* was found to protect mice against inflammation in the lung due to inhalation of anthrax lethal toxin⁵³. This is consistent with the mechanism of that disease model being dependent on IL-1b and neutrophil influx. In contrast, our data to demonstrate that activation of *Nlrp1* can prevent a model of asthma is consistent with results from a model of bleomycin induced lung fibrosis⁵⁴. In that model, Talabostat (PT100) inhibition of DPP9 reduced collagen deposition and inflammation, which would agree with *Nlrp1* activation providing a protective effect *in vivo*, presumably via IL-18⁵⁵. Given that insufficient NLRP1 exacerbated eosinophilia and IL-13 levels in the mouse model we studied, it is conceivable that blocking this axis with agents such as IL-4R α may work more effectively in carriers of the NLRP1 M1184V variant. However, there are critical differences between NLRP1 in humans and mice, so the conclusions from our work need to be viewed in this context.

Collectively, this work defines the role of NLRP1 in asthma at a molecular level, and explains how the M1184V risk factor decreases activation in the context of DPP9 inhibition. This has implications for targeted therapies in asthma, and broader considerations for the NLRP1 inflammasome in other diseases associated with the M1184V allele.

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Author Contributions

JM, PL, KC, MJW, CRH, CHY, SD, KHS, DH, CE, SH, DJC, JCH and MGerlic performed or assisted with experimentation. JM, PL, KC, MJW, CRH, CHY, SD, PMH, RJJOD, JPT, EGB, MGeyer, MGerlic and SLM were involved in experimental analysis and interpretation. All authors contributed to the writing of this manuscript.

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Figure Legends

Figure 1: M1184V has divergent effects on NLRP1 activation due to altered DPP9 binding. (A) 3.5×10^4 HEK 293T cells stably expressing a human ASC-RFP fusion protein were stably reconstituted with dox-inducible NLRP1-T2A-GFP (WT or indicated mutant). Cells were harvested 12h post doxycycline induction and analysed for ASC speck formation by flow cytometric measurement. (B) 2.5×10^5 HEK293T cells were transfected with 500 ng of a vector control or a plasmid encoding human NLRP1-3xFLAG (WT or indicated mutant). 18h post transfection cells were harvested, and immunoprecipitation was performed for analysis of protein expression by western blot. Data in A is 3 biological replicates, representative of 3 independent experiments. fl: NLRP1 full-length, Nt: N-terminal fragment, Ct: C-terminal fragment. Data are mean $\pm$ SEM. P values were calculated using un-paired t-test between two groups. *** $p < 0.001$, **** $p < 0.0001$.

Figure 2: Activation of NLRP1 by Talabostat is reduced for M1184V due to enhanced DPP9 binding. (A) 3.5×10^4 HEK293T cells stably expressing a human ASC-RFP fusion protein were stably reconstituted with dox-inducible NLRP1-T2A-GFP (WT or M1184V). 16 h post doxycycline treatment, cells were stimulated with fresh medium containing increasing concentrations of Talabostat or the highest equivalent amount of DMSO and incubated for 6 h. Cells were harvested and ASC speck formation analysis was performed by flow cytometry. (B) 2.5×10^5 HEK293T cells were transfected with 500 ng of a vector control or NLRP1-3xFLAG (WT or M1184V). 18 h post transfection, cells were stimulated with $2 \mu\text{M}$ Talabostat or an equivalent amount of DMSO as described in A. Following the treatment, cells were harvested, and immunoprecipitation was performed for protein expression by western blot. Data in A is 3 biological replicates, representative of 3 independent experiments. Data in A was pooled from 3 independent experiments. fl: NLRP1 full-length, Nt: N-terminal fragment, Ct: C-terminal fragment. Data are mean $\pm$ SEM. P values were calculated using un-paired t-test between two groups. *** $p < 0.001$, **** $p < 0.0001$.

Figure 3: OVA-treated *Nlrp1*^{-/-} mice show increased lung eosinophilia and elevated IL-13 levels. (A) Representative hematoxylin and eosin histology of left lung lobe from WT and *Nlrp1*^{-/-} mice show no overt differences, either before or after OVA treatment. (B) Numbers of eosinophils (Eos), monocytes/macrophages (Mono/Mac) and neutrophils (Neut), and of (C) Th2 and ILC2 cells in the lungs of OVA-treated WT and *Nlrp1*^{-/-} mice. (D) Cytokine levels in the bronchoalveolar lavage (BAL) fluid of OVA-treated WT and *Nlrp1*^{-/-} mice. (E) Serum and BAL fluid IgE levels of OVA-treated WT and *Nlrp1*^{-/-} mice. Data are mean $\pm$ SEM. Representative of 3 independent experiments. P values were calculated using un-paired t-test between two groups. * $p < 0.05$, ** $p < 0.01$.

Figure 4: Protective effect of Nlrp1 in asthma model requires IL-18 signalling. Mice carrying an activating mutation of Nlrp1a (Q593P), referred to as *Neut1^{m/m}* are overtly healthy on the *Il1r^{-/-}* background, and studied compared to *Il1r^{-/-}* as control in the alum/OVA asthma model for; **(A)** Numbers of eosinophils (Eos), monocytes and macrophages (Mono/Mac) and neutrophils (Neut) in the lungs; **(B)** Numbers of Th2 cells and ILC2s in the lungs; **(C)** Cytokine levels measured in the BAL fluid. *Il1r^{-/-}Il18^{-/-}Neut1^{m/m}* mice were next investigated compared to *Il1r^{-/-}Il18^{-/-}* as control and studied in the alum/OVA asthma model for; **(D)** Numbers of eosinophils (Eos), monocytes and macrophages (Mono/Mac) and neutrophils (Neut) in the lungs; **(E)** Numbers of Th2 cells and ILC2s in the lungs; **(F)** Cytokine levels measured in the BAL fluid. Data are mean $\pm$ SEM. A-C representative of 2 independent experiments. P values were calculated using un-paired t-test between two groups. *p<0.05.

Supplemental Table 1a. GALA II Mexican American Study Participant Demographics (Discovery Dataset)

	Asthma Cases	Asthma Controls	P-Value ²
N	610	295	*****
Age (Mean[SE])	12.6 [0.12]	15.0 [0.2]	2.20E-16
Sex (% Female)	42.60%	59.30%	3.45E-06 ³
Obese ² (N Obese / N non-Obese)	254/356	85/210	2.49E-04 ³

Supplemental Table 1b. GALA II Puerto Rican Study Participant Demographics (Replication Dataset)

	Asthma Cases	Asthma Controls	P-Value ²
N	1078	340	*****
Age (Mean[SE])	12.5 [0.1]	12.9 [0.1]	0.002
Sex (% Female)	45.92%	56.18%	0.0012 ³
Obese ² (N Obese / N non-Obese)	320/758	78/262	0.02 ³

Supplemental Table 1c. SAGE African American Study Participant Demographics (Replication Dataset)

	Asthma Cases	Asthma Controls	P-Value ²
N	877	379	*****
Age (Mean[SE])	14.1 [0.1]	16.2 [0.2]	2.20E-16
Sex (% Female)	48.35%	57.52%	0.0035 ³
Obese ² (N Obese / N non-Obese)	320/758	78/262	0.0055 ³

¹ Obesity Definition: Obese = BMI > 95th percentile; Non-Obese = BMI ≤ 95th percentile

² Values presented were produced using the Wilcoxon Rank Sum test unless otherwise indicated.

³ P-Value presented was generated using the X² test.

Supplemental Table 2a. Single Nucleotide Polymorphism (SNP) Allele Frequencies in Mexican Americans

Variant ID	rs ID	Effect Allele	Alternate Allele	Effect Allele Frequency		
				Asthma Cases	Asthma Controls	All Participants
L155H	rs12150220	T	A	0.44	0.39	0.42
V1059M	rs2301582	T	C	0.42	0.36	0.40
M1184V	rs11651270	C	T	0.47	0.42	0.45

Supplemental Table 2b. Single Nucleotide Polymorphism (SNP) Allele Frequencies in Puerto Ricans

Variant ID	rs ID	Effect Allele	Alternate Allele	Effect Allele Frequency		
				Asthma Cases	Asthma Controls	All Participants
L155H	rs12150220	T	A	0.29	0.30	0.30
V1059M	rs2301582	T	C	0.27	0.27	0.28
M1184V	rs11651270	C	T	0.42	0.40	0.42

Supplemental Table 2c. Single Nucleotide Polymorphism (SNP) Allele Frequencies in African Americans

Variant ID	rs ID	Effect Allele	Alternate Allele	Effect Allele Frequency		
				Asthma Cases	Asthma Controls	All Participants
L155H	rs12150220	T	A	0.11	0.11	0.12
V1059M	rs2301582	T	C	0.14	0.15	0.14
M1184V	rs11651270	C	T	0.52	0.50	0.52

Supplemental Table 3. Imputation Quality Control Metrics

Variant ID	rs ID	Alleles	Imputation R ²
R1366C	rs2137722	G/A	+++
*****	rs6502867	T/C	+++
V1241L	rs11653832	C/G	+++
M1184T	rs146932154	A/G	0.98
M1184V	rs11651270	T/C	0.99
M1119V	rs35596958	T/C	+++
V1059M	rs2301582	C/T	+++
T995I	rs34733791	G/A	+++
T878M	rs11657747	C/A	0.99
T782S	rs52795654	G/C	0.99
T246S	rs11651595	G/C	0.99
L155H	rs12150220	A/T	0.99
*****	rs2670660	A/G	0.99
*****	rs8182352	C/T	0.99

*No Variant ID

+SNP was directly genotyped on the LAT1 genotyping Array

Supplemental Table 4. NLRP1 Haplotype Definitions

Variant ID	rs ID	Alleles	NLRP1 Haplotype Definitions				
			1	1C	2A	2B	3
R1366C	rs2137722	G/A	G	G	G	G	A
*****	rs6502867	T/C	T	C	T	T	C
V1241L	rs11653832	C/G	C	C	C	C	G
M1184T	rs146932154	A/G	A	A	A	G	A
M1184V	rs11651270	T/C	T	T	C	C	C
M1119V	rs35596958	T/C	T	T	T	T	C
V1059M	rs2301582	C/T	C	C	T	T	C
T995I	rs34733791	G/A	G	G	G	G	A
T878M	rs11657747	C/A	G	G	G	G	A
T782S	rs52795654	G/C	G	G	G	G	C
T246S	rs11651595	G/C	G	G	G	G	C
L155H	rs12150220	A/T	A	A	T	T	T
*****	rs2670660	A/G	A	A	G	G	G
*****	rs8182352	C/T	T	T	C	C	C

Reference haplotype used for regression analyses is highlighted in **BOLD**.

Supplemental Table 5a. NLRP1 Haplotype Frequencies by Asthma Status in Mexican Americans

	Haplotype Frequency		
	Asthma Cases	Asthma Controls	Overall
Haplotype 1	0.31	0.38	0.33
Haplotype 1C	0.17	0.15	0.17
Haplotype 2A	0.14	0.11	0.13
Haplotype 2B	0.25	0.22	0.24
Haplotype 3	0.01	0.02	0.01

Reference haplotype used for regression analyses is highlighted in **BOLD**.

Supplemental Table 5b. NLRP1 Haplotype Frequencies by Asthma Status in Puerto Ricans

	Haplotype Frequency		
	Asthma Cases	Asthma Controls	Overall
Haplotype 1	0.31	0.30	0.31
Haplotype 1C	0.16	0.19	0.17
Haplotype 2A	0.17	0.17	0.17
Haplotype 2B	0.05	0.05	0.06
Haplotype 3	0.01	0.02	0.02

Reference haplotype used for regression analyses is highlighted in **BOLD**.

Supplemental Table 5c. NLRP1 Haplotype Frequencies by Asthma Status in African Americans

	Haplotype Frequency		
	Asthma Cases	Asthma Controls	Overall
Haplotype 1	0.35	0.35	0.35
Haplotype 1C	0.04	0.04	0.04
Haplotype 2A	0.06	0.06	0.06
Haplotype 2B	0.007	0.008	0.008
Haplotype 3	0.02	0.02	0.02

Reference haplotype used for regression analyses is highlighted in **BOLD**.

Supplemental Table 6a: NLRP1 Haplotypes Associated with Asthma Status in Puerto Rican Children from the GALA II Study.

	R1366C	rs6502867	V1241L	M1184T	M1184V	M1119V	V1059M	T995I	T878M	T782S	T246S	L155H	rs2670660	rs8182352	Odds Ratio	95% Confidence Interval	P-Value
Haplotype 1	G	T	C	A	T	T	C	G	G	G	G	A	A	T	REFERENCE HAPLOTYPE		
Haplotype 1C	G	C	C	A	T	T	C	G	G	G	G	A	A	T	0.84	0.64-1.10	0.21
Haplotype 2A	G	T	C	A	C	T	T	G	G	G	G	T	G	C	0.96	0.74-1.25	0.78
Haplotype 2B	G	T	C	G	C	T	T	G	G	G	G	T	G	C	0.91	0.60-1.36	0.63
Haplotype 3	A	C	G	A	C	C	C	A	A	C	C	T	G	C	0.65	0.31-1.35	0.24

Supplemental Table 6b: NLRP1 Haplotypes Associated with Asthma Status in African American Children from the SAGE II Study.

	R1366C	rs6502867	V1241L	M1184T	M1184V	M1119V	V1059M	T995I	T878M	T782S	T246S	L155H	rs2670660	rs8182352	Odds Ratio	95% Confidence Interval	P-Value
Haplotype 1	G	T	C	A	T	T	C	G	G	G	G	A	A	T	REFERENCE HAPLOTYPE		
Haplotype 1C	G	C	C	A	T	T	C	G	G	G	G	A	A	T	0.84	0.51-1.38	0.49
Haplotype 2A	G	T	C	A	C	T	T	G	G	G	G	T	G	C	1.16	0.78-1.73	0.45
Haplotype 2B	G	T	C	G	C	T	T	G	G	G	G	T	G	C	1.81	0.63-5.21	0.27
Haplotype 3	A	C	G	A	C	C	C	A	A	C	C	T	G	C	0.83	0.42-1.65	0.60

Supplemental Table 7a: NLRP1 Variant Association with Asthma Status in Puerto Rican Children from the GALA II Study.

Variant	rs ID	Effect Allele	Effect Allele Frequency	Odds Ratio	95% Confidence Interval	P-Value
L155H	rs12150220	T	0.30	0.94	0.78-1.14	0.55
V1059M	rs2301582	T	0.28	1.00	0.83-1.21	0.99
M1184V	rs11651270	C	0.42	1.04	0.88-1.24	0.64

Supplemental Table 7b: NLRP1 Variant Association with Asthma Status in African American Children from the SAGE II Study.

Variant	rs ID	Effect Allele	Effect Allele Frequency	Odds Ratio	95% Confidence Interval	P-Value
L155H	rs12150220	T	0.12	1.05	0.78-1.40	0.76
V1059M	rs2301582	T	0.14	0.99	0.77-1.28	0.97
M1184V	rs11651270	C	0.52	1.08	0.91-1.29	0.38

Table 1: *NLRP1* Haplotypes Associated with Asthma Status in Mexican American Children from the GALA II Study.

	R1366C	rs6502867	V1241L	M1184T	M1184V	M1119V	V1059M	T995I	T878M	T782S	T246S	L155H	rs2670660	rs8182352	Odds Ratio	95% Confidence Interval	P-Value
Haplotype 1	G	T	C	A	T	T	C	G	G	G	G	A	A	T	REFERENCE HAPLOTYPE		
Haplotype 1C	G	C	C	A	T	T	C	G	G	G	G	A	A	T	1.46	1.05 - 2.02	0.02
Haplotype 2A	G	T	C	A	C	T	T	G	G	G	G	T	G	C	1.69	1.18 - 2.43	0.004
Haplotype 2B	G	T	C	G	C	T	T	G	G	G	G	T	G	C	1.35	1.01 - 1.79	0.04
Haplotype 3	A	C	G	A	C	C	C	A	A	C	C	T	G	C	1.02	0.42 - 2.45	0.97

Haplotypes significantly associated with Asthma Status, after correction for multiple testing, are highlighted in **BOLD**.

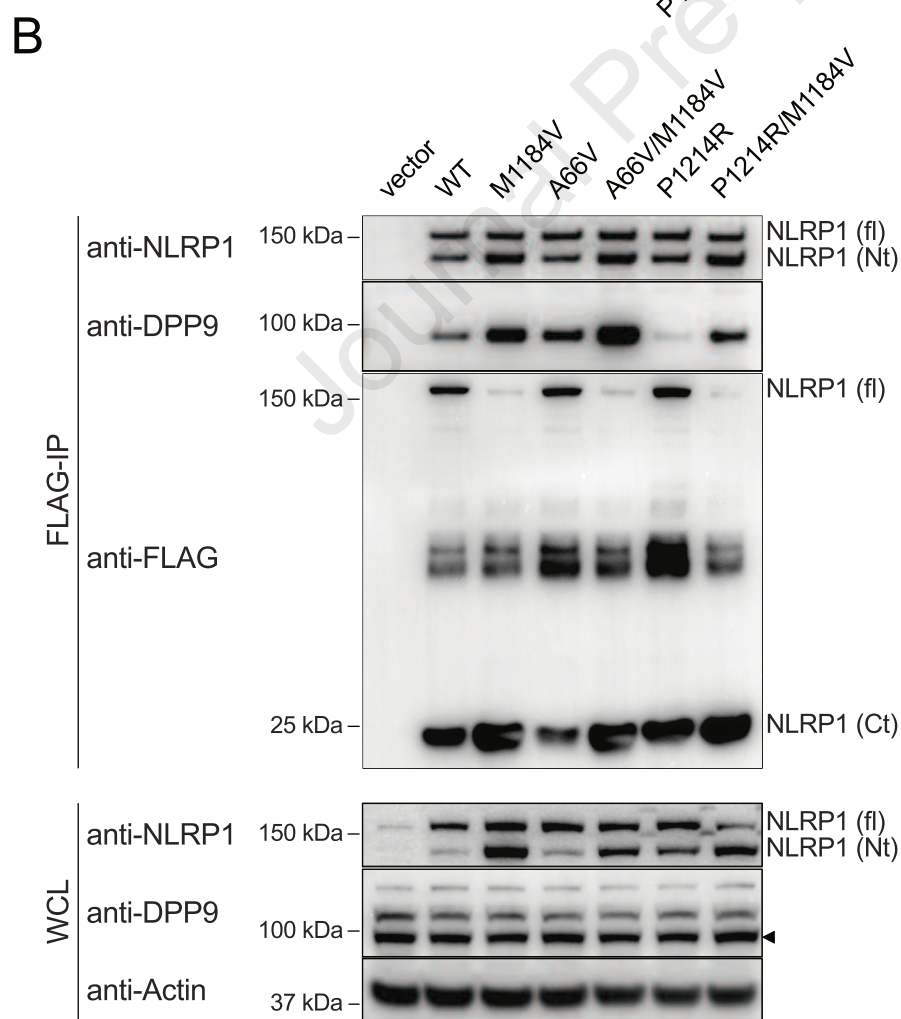
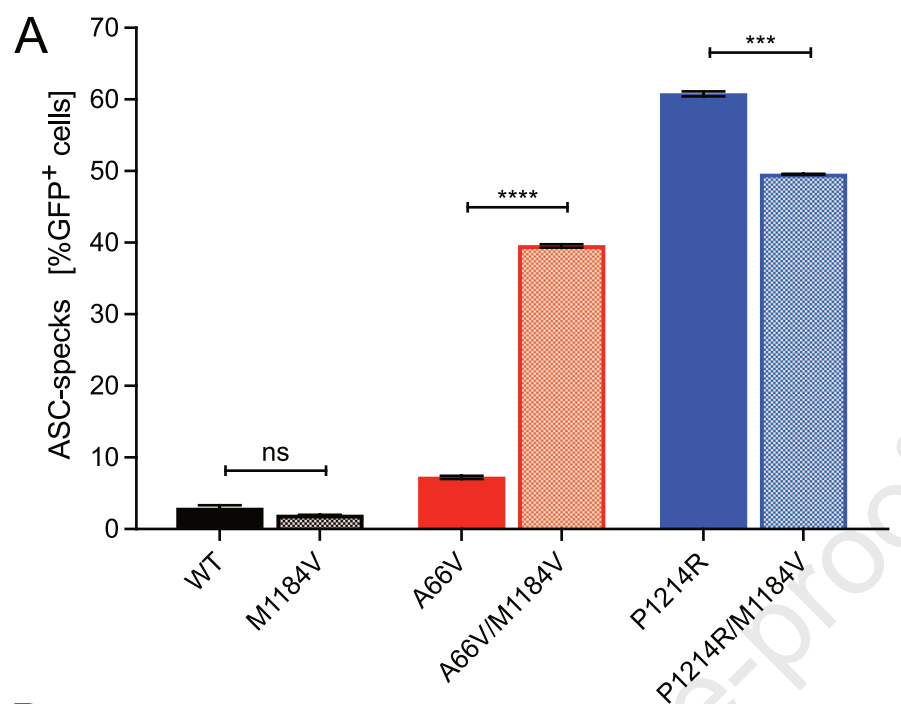
Table 2: *NLRP1* Variants Associated with Asthma Status in Mexican American Children from the GALA II Study.

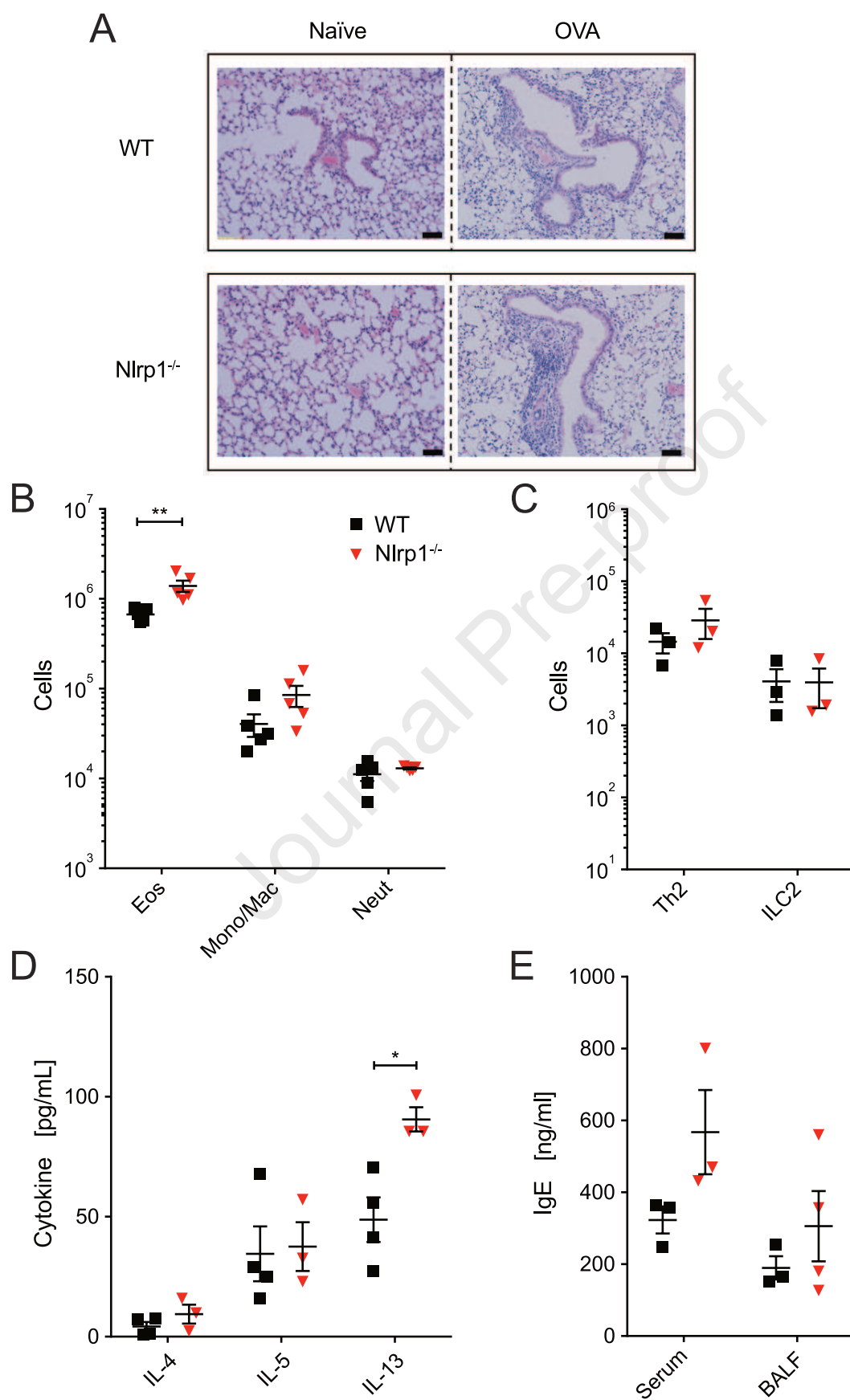
Variant	rs ID	Effect Allele	Effect Allele Frequency	Odds Ratio	95% Confidence Interval	P-Value
L155H	rs12150220	T	0.42	1.20	0.97 - 1.49	0.09
V1059M	rs2301582	T	0.40	1.28	1.03 - 1.59	0.03
M1184V	rs11651270	C	0.45	1.28	1.03 - 1.59	0.02

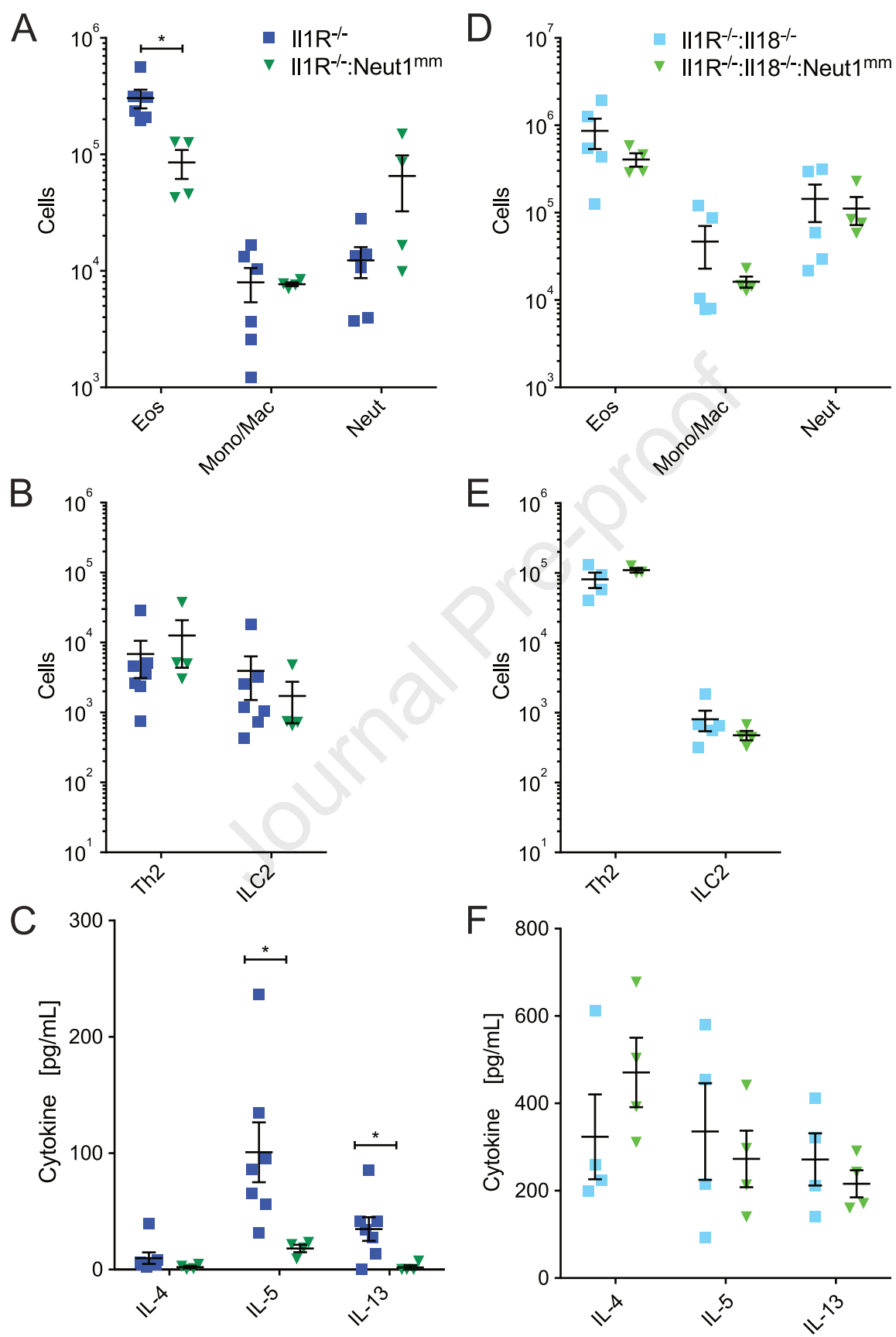
Variants significantly associated with Asthma Status after correction for multiple testing are highlighted in **BOLD**.

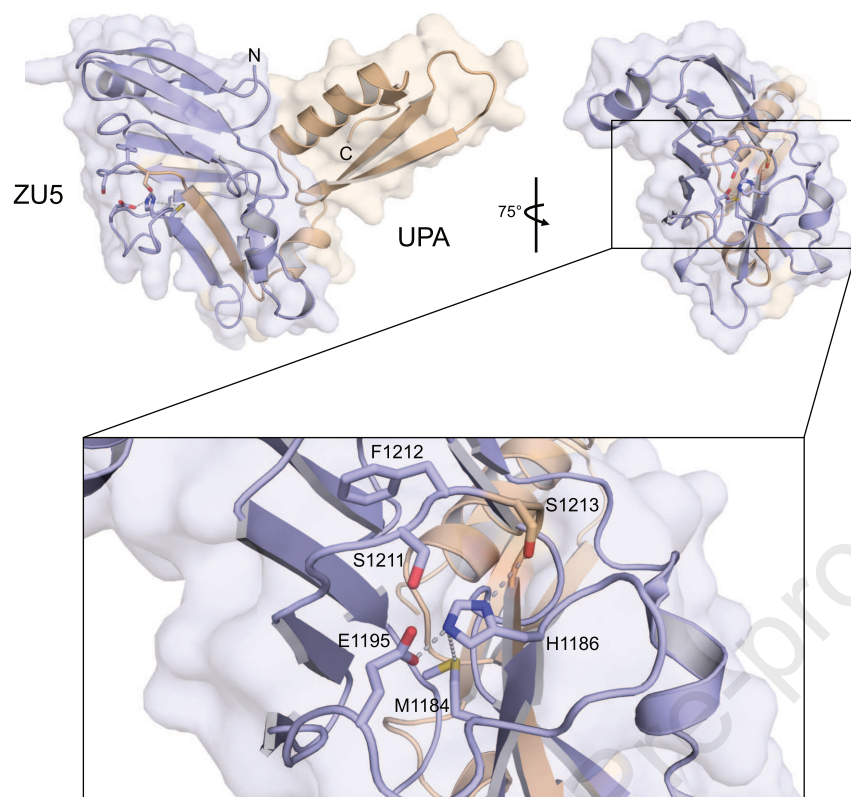
Table 3: Diseases associated with the M1184V polymorphism

Disease	Odds Ratio	P-value	M1184V (rs11651270) Contributes/Protects to/from Disease (PMID)	Inflammasome Contributes/Protects to/from Disease (PMID)
Vitiligo and associated autoimmunity	1.6	n/a	Contributes together with L155H/V1059M ⁴⁵ (23382179)	IL-1 β contributes ^{56,57} (28082234, 25221996)
Asthma	3.4	0.013	Contributes ²² (29154202)	IL-1 β contributes ⁵⁸⁻⁶⁰ (16210060, 23837489, 8527954) IL-18 protects ^{12,51,52} (12006423, 10629451, 11972614)
Breast Cancer	n/a	0.013	Potentially contributes ⁶¹ (23107584)	IL-1 β contributes ⁶² (30545915) IL-18 protects ⁶³ (29725393)
HPV infection and associated cervical cancer	0.43	0.003	Protects ⁶⁴ (26945813)	IL-1 β contributes to cancer ⁶⁵ (19904560) IL-18 protects from infection ⁶⁶ (11470273)
Crohn's disease	1.35	0.02	Contributes to inflammatory phenotype ⁶⁷ (20403135)	IL-1 β contributes ^{68,69} (22891275, 7817982) IL-18 contributes ^{70,71} (10352304, 10384110)
Chagas cardiomyopathy	n/a	0.036	Contributes ⁷² (29438387)	IL-1 β involvement unclear ⁷³ (30354432) IL-18 involvement unclear ⁷⁴ (25743241)
Type 1 Diabetes	0.643	0.002	Protects ⁷⁵ (31396539)	IL-1 β unclear ^{76,77} (23562090, 21518168) IL-18 contributes ^{78,79} (25576800, 18359638)
Diabetic Kidney Disease	0.36	0.01	Protects ⁸⁰ (29031829)	IL-1 β contributes ^{81,82} (27516236, 31191559) IL-18 contributes ⁸³⁻⁸⁵ (12759891, 16306550, 17425653)
Malaria (<i>Plasmodium vivax</i>)	n/a	n/a	Potentially contributes ⁸⁶ (26946405)	IL-1 β potentially contributes to severity ⁸⁷ (29602073) IL-18 reduces severity (with IL-12) ⁸⁸ (28615061)
Bacterial meningitis	2.32	0.023	Potentially contributes ⁸⁹ (23053059)	IL-1 β protects ⁹⁰ (12707352) IL-18 contributes to inflammation ⁹¹ (12742650)

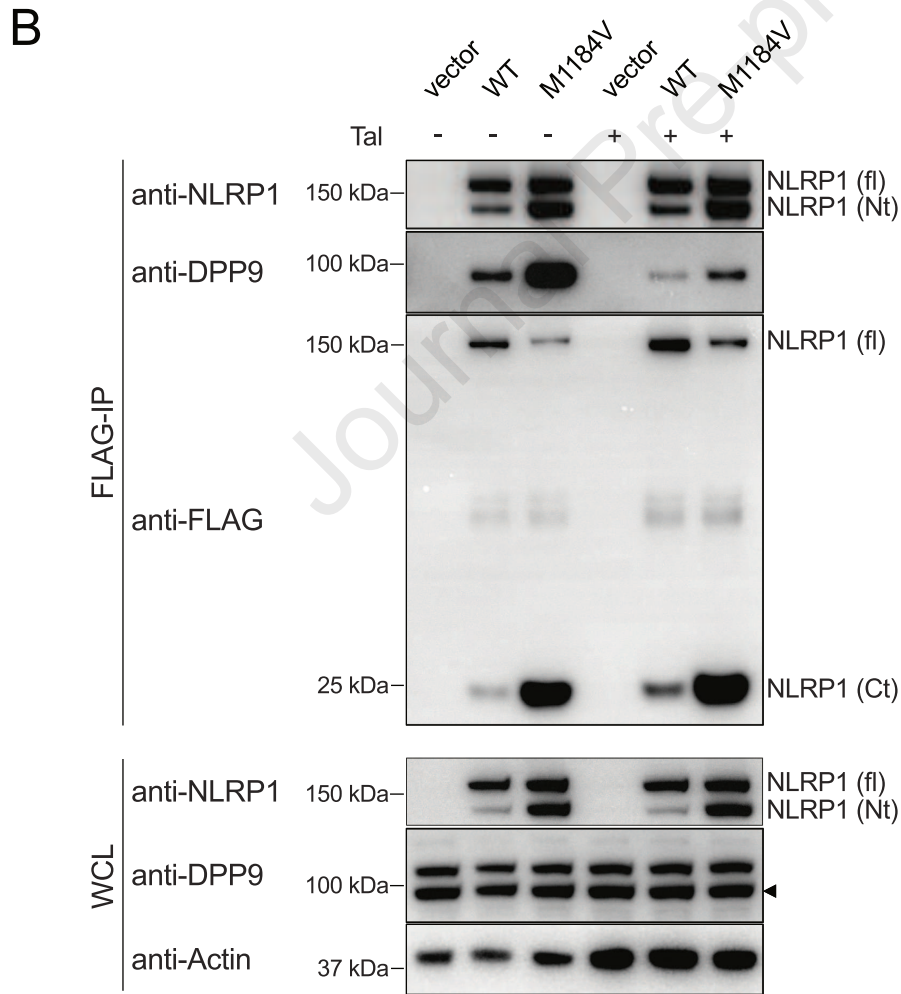
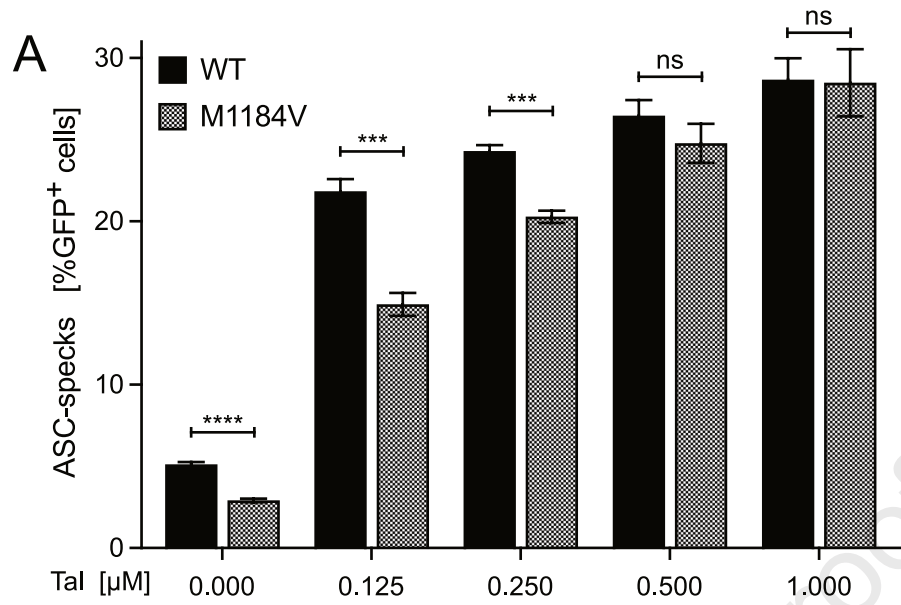








Lizard	{	Ophiophagus hannah	HTLCLNDQLSE--KAFK V FHFKKNCPEFEGS	183
Zebrafish	{	Danio rerio	HYMCLAESDPALTN V KL	LSVEDEGISLESV 1095
		Rattus norvegicus	HFVSLQEGKVD-SSLFH V AHFQDHGMVLETP	953
Rodents	{	Mus musculus (a)	HFVALQEGIVD-SSLFH V AHFQEHGMVLETP	917
		Mus musculus (b1)	HFVSLKDTKAS-TFDFK V AHFQEHGMVLETP	968
		Microcebus murinus	HFVDLQGGHVD-ISLFQ V AHFKKKEGMLLEKP	1147
		Aotus nancymae	HFVALQGGHMD-TSLFQ V AHFKEEGMLLEKP	1276
		Cebus capucinus im.	HFVALQGGHID-TSLFQ V AHFKEEGMLLEKP	1174
		Theropithecus gelada	HFVALQGGHVD-TSLFQ V AHFKEEGMLLEKP	1208
		Papio anubis	HFVALQGGHVD-TSLFQ V AHFKEEGMLLEKP	1260
Primates	{	Macaca nemestrina	HFVALQGGHVD-TSLFQ V AHFKEEGMLLEKP	1213
		Macaca mulatta	HFVALQGGHVD-TSLFQ V AHFKEEGMLLEKP	1199
		Pongo abelii	HFVALQGGHVD-TSLFQ V AHFKEEGMLLEKP	1196
		Homo sapiens	HFVALQGGHVD-TSLFQ M AHFKEEGMLLEKP	1197
		Pan troglodytes	HFVALQGGHVD-TSLFQ V AHFKEEGMLLEKP	1214
		Pan paniscus	HFVALQGGHVD-TSLFQ V AHFKEEGMLLEKP	1199
			* : * : *	



Supplementary Figure Legends

Figure S1: Gating strategy for inflammatory cell identification. Debris was gated out and beads were identified for quantification. Beads were further defined to remove contaminating cells. Doublets were removed and live leukocytes identified before gating for granulocyte populations. First, granulocytes were defined as CD45.2⁺, B220⁻ or CD19⁻, CD11b⁺ cells and afterwards further separated into eosinophils or neutrophils based on Siglec-F⁺ Ly6G⁻ or Siglec-F⁻ Ly6G^{hi}, respectively. Macrophages/monocytes were defined as Siglec-F⁻, Ly6G⁻ and CD11b⁺.

Figure S2: Gating strategy for T cell subset and ILC identification. Debris was gated out and beads were identified for quantification. Beads were further defined to remove contaminating cells. Doublets were removed and live T lymphocytes were defined as CD45⁺CD3⁺CD4⁺. T lymphocytes were further classified as regulatory T cells (T_{reg}), Th2 cells or Th17 cells by expression of FoxP3, GATA3 or RORγt, respectively. ILCs were defined as CD45⁺CD3⁻CD4⁻ cells stratified into ILC2 or ILC3 by expression of GATA3 or RORγt, respectively.

Figure S3: Homology model of the NLRP1 FIIND domain. The model of the NLRP1 FIIND domain (residues 1084-1278) was generated using Swiss Model^{E1}. Secondary structure elements are shown in cartoon representation. Amino acid side chains of the cleavage site residues S1211, F1212 as well as the side chains of the proposed catalytic triad S1213, H1186 and E1195 and the methionine affected by the polymorphism (M1184) are shown in stick representation. M1184 is not in direct proximity of the cleavage site but near the catalytic H1186. Potentially, substitution of M1184 affects cleavage by changing the positioning of the catalytic histidine, allowing it to induce cleavage more efficiently. Images and distance measurements were generated using PyMOL. The structure of the cytoplasmic domain of Unc5b (PDB: 3G5B)^{E2} served as a template for structure modelling.

Figure S4: Sequence alignment of NLRP1 from different species. Sequence alignment of the amino acid sequence of NLRP1 from different organisms. Sequences were taken from NCBI. Multiple sequence alignment was performed using the Clustal Omega online tool with default settings^{E3}. Methionine 1184 in human NLRP1 and corresponding residues in other species are highlighted. Stars/dots in the bottom line indicate similarity (dots) or identity (stars) of the amino acids in the respective position.

References

- E1 Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R *et al.* SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Res* 2018; **46**: W296–W303.
- E2 Wang R, Wei Z, Jin H, Wu H, Yu C, Wen W *et al.* Autoinhibition of UNC5b Revealed by the Cytoplasmic Domain Structure of the Receptor. *Mol Cell* 2009; **33**: 692–703.
- E3 Madeira F, Park Y, Lee J, Buso N, Gur T, Madhusoodanan N *et al.* The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res* 2019; **47**: 636–641.