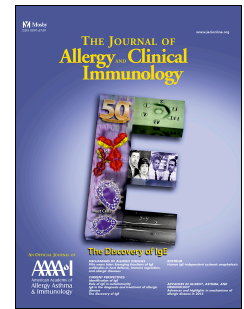


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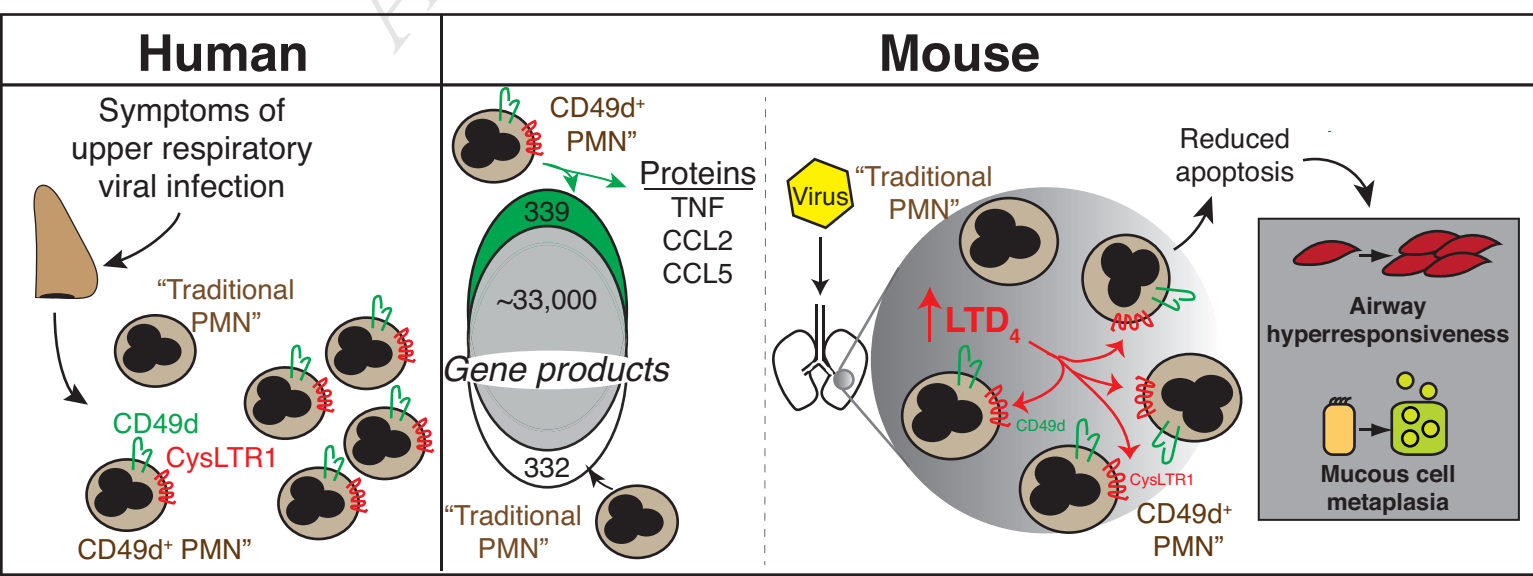
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CysLTR1-expression identifies a subset of neutrophils during the antiviral response that contributes to post-viral atopic airway disease

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contributed to subject enrollment, sample collection, flow cytometry, and data analysis.
PMS and KY contributed to the statistical analyses. S-RAH, JLS, EJB, DAH, and MR
contributed to carrying out of experimental protocols. DP contributed to subject
enrollment. MHG provided overall study supervision. All authors contributed to
interpretation of the study results, read, critically revised, and approved the final report
before submission.

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Condensed title: CysLTR1-expressing PMN during respiratory viral infections
contributes to post-viral atopic airways disease

ABSTRACT

Background: Respiratory viral infections increase risk of development and exacerbation of atopic disease. We previously demonstrated the requirement for a neutrophil (PMN) subset expressing CD49d to drive development of post-viral atopic airway disease in mice.

Objective: To determine if human CD49d⁺ PMN are present in the nasal mucosa during acute viral respiratory tract infections and further characterize this PMN subset in humans and mice.

Methods: Sixty subjects (5-50 years old) were enrolled within 4 days of acute onset of upper respiratory symptoms. Nasal lavage for flow cytometry and nasal swab for viral PCR were performed at enrollment and during convalescence. The Sendai virus mouse model was used to investigate the phenotype and functional relevance of CD49d⁺ PMN.

Results: CD49d⁺ PMN frequency was significantly higher in the nasal lavage during acute respiratory symptoms in all subjects (2.9% versus 1.0%, $n=42$, $p<0.001$). In mice, CD49d⁺ PMN represented a “pro-atopic” neutrophil subset that expressed CysLTR1 and produced TNF, CCL2, and CCL5. Inhibition of CysLTR1 signaling in only the first days of a viral respiratory infection was sufficient to reduce accumulation of CD49d⁺ PMN in the lungs and development of post-viral atopic airway disease. Similar to the mouse, human CD49d⁺ PMN isolated from nasal lavage during a respiratory viral infection expressed CysLTR1.

Conclusion: CD49d and CysLTR1 co-expressing PMNs are present during symptoms of an acute respiratory viral infections in humans. Further study is needed to examine

selective targeting of “pro-atopic” neutrophils as a potential therapeutic strategy to prevent development of post-viral atopic airway disease.

Abstract Word Count: 250

Key Messages:

- A “pro-atopic” CD49d and CysLTR1 co-expressing neutrophil subset contributes to development of post-viral airway disease in a mouse model.
- LTD4, presumably through CysLTR1, enhances survival of CD49d expressing mouse neutrophils both *in vitro* and *in vivo*.
- Humans with acute viral respiratory symptoms demonstrate increased CD49d expressing neutrophils in their nasal lavage, and CD49d expressing neutrophils from confirmed viral respiratory infections co-express CysLTR1 like their murine counterparts.

Capsule Summary: Human CD49d/CysLTR1 neutrophils are in nasal lavage during symptoms of acute viral respiratory infections. CysLTR1 blockade in mice inhibits recruitment of these neutrophils and post-viral disease.

Keywords: asthma, viral infection, nasal lavage, neutrophils, cysteinyl leukotrienes

Abbreviations:

BAL = bronchoalveolar lavage

CysLT = cysteinyl leukotriene

CysLTR = cysteinyl leukotriene receptor

101 FcεRI = high affinity receptor for IgE
102 FDR = false discovery rate
103 HPIV = human parainfluenza virus
104 HMPV = human metapneumovirus
105 HRV = human rhinovirus
106 IgE = immunoglobulin E
107 IL = interleukin
108 IQR = interquartile range
109 LPS = lipopolysaccharide
110 PCR = polymerase chain reaction
111 PI = propidium iodide
112 PMN = neutrophil
113 qPCR = quantitative PCR
114 RSV = respiratory syncytial virus
115 SEM = standard error of the mean
116 SeV = Sendai virus
117 TNF = tumor necrosis factor

INTRODUCTION

The prevalence of atopic diseases such as asthma and allergic rhinitis continue to increase (1, 2). These diseases represent a major burden contributing to rising health care utilization and expenditures (3, 4). Despite these trends, our understanding of the mechanisms involved in the development of allergic diseases, like asthma, remains limited. Several epidemiological studies have demonstrated severe respiratory viral infections early in life greatly increase the risk for development of atopic disease including asthma (5-9), yet mechanism(s) translating a respiratory viral infection into atopic disease and asthma are not fully known. Animal models have revealed several potential mechanistic pathways, but whether these pathways are operative in humans is less well known (10).

We have demonstrated that mice infected with Sendai virus (SeV), the murine parainfluenza virus type 1, develop post-viral atopic disease through a distinct pathway (11, 12). Infection with SeV drove recruitment of a CD49d-expressing subset of neutrophils (CD49d⁺ PMN), which induced expression of FcεRI, the high-affinity receptor for IgE, on lung conventional dendritic cells (cDC) (12). Subsequently, anti-SeV IgE was produced and crosslinking of the cDC bound IgE led to production of CCL28, a chemoattractant for Th2 cells (11). These Th2 cells produced IL-13, which drove the subsequent mucous cell metaplasia and airway hyperreactivity—i.e., the development of post-viral asthma – which all depended upon the presence of PMNs in the airway (12, 13).

Animal and human studies suggest an important role for the PMN in both respiratory viral infections and asthma. Robust PMN influxes have been observed in the airways of infants hospitalized with Respiratory Syncytial Virus (RSV) induced bronchiolitis or those having wheezing illnesses from other respiratory viruses, such as parainfluenza, influenza, and rhinovirus (14). Interestingly, many of these viral infections have been associated with development of asthma and/or atopy (15). PMNs also are elevated in extant asthma, although their precise pathogenic role is unclear. Our mouse data would suggest that they play an important role in translating viral infections into atopic disease.

The mechanistic pathway we documented in mice appears to function, at least in part, in humans. CD49d⁺ PMN are present in human peripheral blood and nasal lavage, and associate with allergic disease (16). Crosslinking IgE on human peripheral blood cDCs leads to production of CCL28 (17). FcεRI expression increases on peripheral blood dendritic cells during a respiratory viral infection, as does total serum IgE (18). While we did demonstrate the presence of CD49d⁺ PMN in humans, we did not assess whether viral respiratory infections were capable of driving accumulation of these CD49d⁺ PMN as we had seen in our mouse model.

We therefore sought to determine if pro-atopic CD49d⁺ PMN found in our model are recruited to the airway mucosa during a viral respiratory infection in humans. We further explored the genetic signature of CD49d expressing and non-expressing PMN, and determined whether specific manipulation of PMN subsets can mitigate development of post-viral atopic airway disease in our mouse model.

METHODS

Detailed methods are described in the online supplement.

Human neutrophil subsets in viral upper respiratory infection study

Asthmatic and non-asthmatic subjects between the ages of 5-50 years with symptoms of an upper respiratory tract infection within 4 days of onset were eligible to participate in the study. Individuals with a history of sinus surgery, confirmed immunodeficiency or autoimmune disease were excluded. The use of an intranasal corticosteroid or antihistamine within 48 hours of presentation, systemic corticosteroid within 4 weeks, or any biologic or investigational agent also excluded participation. Smoking was not specifically excluded but participants were instructed to maintain the same smoking status throughout the study. All individuals were enrolled either at the Medical College of Wisconsin Asthma and Allergy Clinic, Children's Hospital of Wisconsin Emergency Department, or Froedtert Memorial Lutheran Hospital Emergency Department. The Children's Hospital of Wisconsin Institutional Review Board approved the protocol, and all participants and/or their parent/guardian provided written consent. An outline of the study design is shown in Figure 1A.

Nasopharyngeal viral swab and nasal lavage were obtained at the initial (ill) visit and repeated at least 4 weeks later during convalescence (well). Viral swabs were processed for detection of influenza virus A, influenza virus B, respiratory syncytial virus (RSV), human metapneumovirus (HMPV), human rhinovirus (HRV), and human parainfluenza virus (HPIV) 1-4 using a PCR-based assay. Single cell suspensions from

nasal lavages were analyzed for CD49d, CD16, and CysLTR1 expression using flow cytometry.

Mouse model of post-viral asthma

The Sendai virus model was utilized to characterize the properties of neutrophil subsets (including gene array analysis) and the response to CysLTR1 blockade. Detailed experimental methods are in the supplement.

Statistical Analyses

Human study:

Unless stated otherwise, all data are presented as median [interquartile range, IQR].

The human data were not normally distributed, so the Wilcoxon signed rank test was used to compare the medians of the percent or absolute numbers of CD49d⁺ PMN and the percent CD16⁺ granulocytes during illness versus upon convalescence. The Mann-Whitney U-test was used to compare the atopic versus non-atopic, virus detected versus virus not detected, and asthma versus non-asthma subjects. Human CysLTR1 expression levels were compared using a Student's t-test, as these data were normally distributed. A significance level of 0.05 was used for each test. The step-down Bonferroni method was used to correct for the multiple comparisons we made within each figure. Statistical software employed were Cytel StatXact, SAS version 9.2, Prism 5.0 and 7.0, and SPSS version 19.0.

Mouse experiments:

Since mouse data were normally distributed, all mouse data are presented as mean $\pm$ SEM unless stated otherwise. Unpaired two-sided Student's *t*-test assessed statistical significance of differences between two means for normally distributed data. For airway hyperreactivity data repeated measures ANOVA with Tukey's multiple comparison test was used. A significance level of 0.05 was used for each test.

For GeneChip, Rank Product statistics were conducted and false discovery rates (FDR) were determined using RankProd from Bioconductor (<http://www.bioconductor.org/packages/2.12/bioc/html/RankProd.html>). Differentially expressed probe sets were defined as those possessing a $\log_2$ fold change between samples of > 2 and a *p* value corrected for false discovery rate (FDR) of ≤ 0.01 . Data have been submitted to the NCBI Gene Expression Omnibus (GEO accession number GSE61137).

RESULTS

Participants

60 subjects were recruited from March 2012 through March 2014. Follow-up visits were obtained from 42 of the 60 subjects through May 2014. Participant flow is summarized in Figure 1B. Table I shows a summary of subject characteristics of the 42 individuals with paired analyses (i.e., both ill visit and follow-up convalescence). There were no significant differences in any demographic information between the atopic (*n* = 31) and non-atopic subjects (*n* = 11). Subjects with virus detected were younger (*n*=23, median age 17, *p* = 0.046) compared with subjects in whom no virus was detected (*n* = 19,

median age 28). Asthmatic subjects (n = 17) were younger (median age 10 versus 29 years, $p < 0.001$) and predominantly male (65% versus 24%, $p = 0.0083$) compared to non-asthmatic subjects (n = 25).

Nasal lavage CD49d⁺ PMN frequency increases during acute respiratory illness

The frequency of CD16⁺ PMN expressing CD49d was determined in the nasal lavage of all subjects during illness and convalescence. Compared to convalescence, CD49d⁺ PMN were found more frequently in the nasal lavage during an acute respiratory illness among all 42 subjects (2.9% [1.04-4.42] vs. 1.0% [0-2] of PMN expressing CD49d, respectively; $p < 0.001$; Figure 2; see Supplemental Figure 1 for absolute numbers for all data shown in Figure 2). Among the 23 subjects with a positive viral PCR (HRV (n=19), RSV (n=3), or influenza virus B (n=1)), CD49d⁺ PMN frequencies were higher during illness compared to convalescence (2.0% [1-3.33] vs. 0.0% [0-1], respectively; $p < 0.001$; Figure 2). Atopic status had no effect in the virus positive subjects, as both virus positive atopic (n=16) and non-atopic (n=7) subjects demonstrated increased CD49d⁺ PMN frequency during the viral illness (atopic: 2.0% vs. 0.16%, $p = 0.002$; and non-atopic: 2.76% vs. 0%, $p = 0.016$; “ill” vs. “well”, respectively; Figure 2). In contrast, subjects with no virus detected demonstrated an increase in CD49d⁺ PMN during illness only if they had a history of a positive skin test or sIgE (atopic, n=15) (atopic: 3% vs. 1.34%; $p = 0.013$; and non-atopic: 4.69% vs. 2.2%; $p = 0.25$; n=4; “ill” vs. “well”, respectively; Figure 2); however, the lack of significance in the non-atopic group may relate to the small number of subjects (n=4) in this group and the fact that a lack of atopy was not verified in this study. Although we hypothesized that subjects with

asthma would demonstrate a greater viral infection induced increase in CD49d⁺ PMN than those without asthma, our data actually showed no difference in these two populations (asthmatic: 3.1% vs. 1.4%, $p \leq 0.05$; and non-asthmatic: 3.1% vs. 1.1%, $p \leq 0.05$; “ill vs. “well”, respectively; Figure 2). Further, the presence or absence of virus on nasal swab had no effect on the increase in CD49d⁺ PMN seen with or without asthma (Figure 2). Similar results were seen when comparing absolute numbers of CD49d⁺ PMN between all of these groups (Supplemental figure 1).

Subjects who had asthma were noted to have lower FEV1 at the time of respiratory symptoms (Supplemental figure 2a), and this difference was most marked in those subjects undergoing an exacerbation of their asthma at the time of illness (Supplemental figure 2b). While the absolute numbers of CD49d⁺ PMN in the nasal lavage were significantly different between illness and convalescence for those undergoing an asthma exacerbation, the frequency of CD49d⁺ PMN was not noted to be different (Supplemental figure 2c). Subjects without an asthma exacerbation demonstrated no significant difference in frequency or absolute number of CD49d⁺ PMN.

The frequencies of total PMN (%CD16⁺ of total cells) were also determined. Unlike CD49d⁺ PMN frequency, where viral swab status (i.e., identification of a viral etiology) was not associated with the changes in CD49d⁺ PMN, total CD16⁺ PMN were associated with viral swab status – with a significantly greater increase in the nasal lavage during an acute respiratory illness only if the subject had a virus detected in the

nasal swab (90.9% vs. 74.6%, ill versus well, respectively; $p < 0.001$; $n=23$ for virus detected; 77.8% vs 84%; $p = 0.49$; $n=19$ for virus not detected; Table II; Wilcoxon signed-rank test for comparison of median %CD16 frequencies).

This study was designed to compare the frequency of CD49d⁺ PMN in the nasal lavage of subjects during and after symptoms of an upper-respiratory viral illness (i.e., a paired analysis, as described above). However, using unpaired analyses, we can examine the effect of viral detection, atopy, and asthma status on the frequency (Figure 3) and absolute number (Supplemental Figure 3) of CD49d⁺ PMN during the acute illness and upon convalescence.

The identification of a virus by PCR did not differentiate the frequency or absolute number of CD49d⁺ PMN during the acute illness (Figure 3a and Supplemental Figure 3a); however, upon convalescence there were fewer (frequency and absolute numbers) CD49d⁺ PMN in the lavage of subjects for whom a virus was detected by PCR. This difference held regardless of atopic status for the frequency; however, in non-atopic subjects the absolute numbers of CD49d⁺ PMN upon convalescence did not vary with viral status (Supplemental figure 3a).

Our previous publication found the frequency and total number of CD49d⁺ PMN differentiated atopic and non-atopic subjects at baseline (16). However, in our current study, no significant difference was found between the numbers (Figure 3b) or frequency (Supplemental Figure 3b) of CD49d⁺ PMN in atopic and non-atopic subjects.

The detection of virus during the URI symptoms did not change this lack of discrimination. Similarly, asthma status did not affect the frequency (Figure 3c) or absolute numbers (Supplemental Figure 3c) of CD49d⁺ PMN in the nasal lavage, regardless of detection of virus or not.

Characterization of murine PMN subsets based on CD49d expression

To better characterize differences between CD49d expressing and non-expressing PMNs, we turned to our mouse model of post-viral atopic disease. PMNs were isolated from the bronchoalveolar lavage (BAL) fluid 3 days post inoculation (PI) with SeV and flow-sorted based on CD49d expression, with resulting populations >95% pure. The transcriptomes of the resulting CD49d⁺ and CD49d⁻ PMNs were then compared. The gene product expression of the two groups differed by only ~670 gene products (Figure 4a). Figure 4b lists gene products relevant to our model that had the largest fold difference between the two groups. Interestingly, classical PMN gene products (i.e., *Mmp9* and *Cxcr2*) were expressed most in the CD49d⁻ subset (19). CD49d⁺ PMNs differed from “classical” PMN (i.e., CD49d⁻ PMN) with higher expression of *Ccl2*, *Ccr2*, and *Cysltr1*. Using qPCR, we validated the microarray data (Figure 4c).

We next assessed whether there was differential protein expression between these two PMN subsets. We examined cytokines produced by cytokine array, and, as shown in Figure 4d, CD49d⁺ PMN produced significantly higher TNF, CCL2, and CCL5. Despite significant cell death after 24 hours of culture (viability by Trypan Blue exclusion, 29±6% for CD49d⁺ PMN and 5±1% for CD49d⁻ PMN), there were sufficient functional PMNs in

both groups for similar production of CCL3. However, using a DHR assay we found that in PMN freshly isolated from a SeV infected mouse, the oxidative burst was significantly greater for CD49d⁺ PMN (Figure 4e and f). The difference appears to be a deficient oxidative burst in the CD49d⁻ PMN from SeV infected animals, since PMN from LPS inoculated mice, all of which were CD49d⁻ (data not shown and (12)), demonstrated an oxidative burst similar to that seen with CD49d⁺ PMN from SeV infected animals.

CysLTR1 blockade reduces CD49d⁺ PMN accumulation in SeV infection

The expression of *Cysltr1* on CD49d⁺ PMN was intriguing, since a prior study had shown that a fraction of human PMN in the nasal lavage during seasonal allergic rhinitis expressed both message and protein for CysLTR1 (20). Therefore, we examined whether CysLTR1 was actually expressed on the surface of CD49d⁺ PMN isolated from BAL using flow cytometry. As shown in figure 5a, CysLTR1 was expressed exclusively on CD49d⁺ PMN. Importantly, the CD49d⁺ PMN did not express the eosinophil markers CCR3 or Siglec F (data not shown).

RSV infection has been reported to induce significant amounts of cysteinyl leukotrienes (CysLTs) (21), so we next examined whether CysLTs were generated during SeV infection. CysLT levels were elevated in the BAL by day 3 PI SeV and followed the increase in CD49d⁺ PMN in the BAL (Figure 5b). Further, the viability of CD49d⁺, but not CD49d⁻, PMN was increased with LTD₄ addition *in vitro* (Figure 5c). To examine whether LTD₄ could reduce apoptosis and increase viability of CD49d⁺ PMN *in vivo*, mice were given LTD₄ or PBS i.n. 36 hours PI SeV, and lung PMN examined 12 hours

later (i.e., 12 hours post LTD₄ or PBS administration). As shown in figure 5d, administration of LTD₄ led to increased viability and reduced apoptosis of CD49d⁺ PMN but not CD49d⁻ PMN. Further, treatment on days 1 and 2 PI SeV with the CysLTR1 antagonist, pranlukast, led to a significant increase in apoptosis (i.e., Annexin V/PI staining) of the CD49d⁺ PMN on day 3 PI SeV, but had no effect on apoptosis of CD49d⁻ PMN (Figure 5e).

If CysLTR1 functioned as a survival or recruitment signal for CD49d⁺ PMN, we hypothesized that blockade of this receptor would reduce the frequency and number of CD49d⁺ PMN post SeV infection. Therefore, using pranlukast, we examined the effect of CysLTR1 blockade during SeV infection. Pranlukast administration during the first two days PI SeV led to decreased absolute number and frequency of CD49d⁺ PMN in the BAL (Figure 6a, left panel). However, we saw no change in the lung frequency or number of CD49d expressing PMNs (Figure 6a, right panel). These data indicate CysLTR1 signaling is important for airway accumulation of CD49d⁺ PMN during SeV infection. Repeating the experiment using Ly6G rather than Gr-1 expression to identify PMN generated similar results (Fig. 6b and data not shown).

CysLTR1 blockade attenuates post-SeV lung disease

Since CysLTR1 blockade reduced the frequency and number of CD49d⁺ PMN in the BAL during SeV infection, we hypothesized that a similar blockade early during the antiviral response (i.e., when PMN are being recruited (22)) would decrease development of post-viral asthma. Indeed, when pranlukast was administered during

just the first 5 days PI SeV, both post-viral mucous cell metaplasia (Figure 6c) and airway hyperreactivity (Figure 6d) were attenuated.

Since Th2 cells can respond to CysLT, treatment with pranlukast might have inhibited their migration or function (23). To affirm that the effects of CysLTR1 blockade were not related to adaptive immune cells or stromal cells such as airway smooth muscle cells, which may also respond to CysLT (24), we administered pranlukast daily from day 12 to 18 PI SeV – the time points when blockade of IL-13 and Th2 cells have been shown to attenuate post-SeV atopic disease (13). Treatment with pranlukast at these later time points failed to alter development of post-viral disease (Figure 6e and f). Therefore, we conclude that CysLT through innate cells -- most likely CD49d⁺ PMNs – are necessary for the development of post-viral atopic airway disease in our model.

Human CD49d⁺ PMN express CysLTR1

Finally, we wanted to examine whether CysLTR1 expression can be seen on CD49d⁺ PMN in the human. As shown in figure 6g, CD49d⁺, but not CD49d⁻, PMN from nasal lavage of virally infected individuals (3 with RV, 1 with RSV) expressed CysLTR1. Because all of the PMN tested came from atopic subjects (3 male, 1 female), we are unable to determine if CD49d⁺ PMN expression of CysLTR1 is an atopic phenomenon or a general feature of the human CD49d⁺ PMN..

DISCUSSION

Our prior work outlined a mechanistic pathway translating a respiratory viral infection into atopic disease (11, 12). In this report we have further characterized the CD49d⁺ expressing PMN cohort and found that while most of their gene products are similar with traditional CD49d⁺ PMN, there were several marked differences. Key amongst these is the expression of the receptor for CysLT, CysLTR1. We found that this receptor is expressed on both murine and human CD49d⁺ PMN, and that in the case of mice, signaling through this receptor increases viability of the CD49d⁺ PMN. Further, CysLTR1 signaling early in a viral infection is critical for the translation of the viral infection into atopic disease, as treatment with a CysLTR1 antagonist early, but not late, in the SeV model prevented post-viral airway hyperreactivity and mucous cell metaplasia. These studies further define the mediators involved in translation of a respiratory viral infection into post-viral atopic disease, including asthma, and they provide rationale for studying these pathways in humans.

Interestingly, in our human study, we were able to demonstrate that CD49d⁺ PMN increase in the nasal lavage during acute upper respiratory symptoms, but that unlike in the mouse, this did not require documentation of a respiratory viral infection. Since we previously reported that CD49d⁺ PMN are associated with atopic responses, this is not that surprising – and may explain why in virus PCR negative subjects the increase in CD49d⁺ PMN was only significant in atopic individuals (16). In fact, since subjects were enrolled based on symptoms, it is possible that some of the atopic individuals were having allergic rhinitis symptoms rather than a viral infection. However, our subgroup analysis in this study found that CD49d⁺ PMN do not differentiate atopic and non-atopic

subjects. This is in contrast to our prior study, which showed significantly increased CD49d⁺ PMN in the lavage (and blood) of atopic subjects at baseline (16). A possible explanation for this discrepancy is how non-atopic status was defined in the two studies. In our prior study we verified the non-atopic status of subjects, something that was not done for this study (i.e., the lack of any history of a positive skin test or sIgE was equated with non-atopic, while in the prior study all subjects underwent skin testing as part of the study). Further, we showed in the previous publication that CD49d⁺ PMN increase in the nasal lavage following an allergen challenge. Therefore, it is possible that some of our subjects in this study were exposed to allergens to which they were allergic, and that exposure might have been different between the initial presentation (i.e., the “ILL” visit) and 6 weeks later. We acknowledge that there are limitations to this study. For example, the number of non-atopic subjects with negative viral assays was small, the study was not powered to test the role of atopy, asthma, or viral status on the CD49d⁺ PMN frequency/numbers, and subjects without confirmed respiratory viral infection may have had other triggers of symptoms such as bacterial infection or viral infection not captured by the PCR assay.

An additional limitation of our study is the lack of transcriptome analysis of human PMN subsets. We ended up focusing on the mouse model because the yield of viable PMN from nasal lavage was too low to allow for useful sorting (data not shown). Further, our mechanistic model is based on the airway (upper and/or lower) and we did not believe that human peripheral blood PMN transcriptomes would accurately reflect that of the airway.

439

440 Utilizing our mouse model we demonstrated significant functional differences between
441 CD49d⁺ and CD49d⁻ PMN. These findings provide additional evidence that PMN
442 subsets are distinct, and in the mouse that CD49d⁺ PMN are more “pro-atopic”. In fact,
443 the cytokines/chemokines exclusively produced by CD49d⁺ PMN (TNF, CCL2, and
444 CCL5) have all been associated with atopic disease. TNF has been shown to be a key
445 mediator for Th2 development in a model of viral PAMP induced airway inflammation
446 (25), and both CCL2 and CCL5 are Th2 chemoattractants that increase during
447 respiratory viral infections (26, 27). The differential transcriptome and
448 cytokine/chemokine profiles further argue for the idea that CD49d⁺ PMN are “pro-
449 atopic”, while CD49d⁻ PMN are “classical” PMN.

450

451 Leukotriene (LT) B₄ is a well-known chemotactic agent for PMN (28), but cysteinyl
452 leukotrienes (CysLTs – LTC₄, LTD₄, and LTE₄) are not known to be important PMN
453 chemotactic agents. Thus, expression of CysLTR1 on CD49d⁺ PMN was surprising.
454 Eosinophils also express CD49d and CysLTR1, however, our CD49d⁺ PMN did not
455 express either of the eosinophil markers CCR3 or Siglec F (29). Moreover, our
456 previously published cytopins clearly demonstrate PMN morphology in both CD49d⁺
457 and CD49d⁻ PMN (12). Interestingly, CysLTR1 expression has been noted on a subset
458 of nasal lavage PMNs in humans suffering from allergic rhinitis (20). However, whether
459 these PMN express CD49d is unknown. Therefore, we conclude that there is a subset
460 of PMN, both in the mouse and human, that expresses CysLTR1 and, at least in the
461 context of a viral infection, CD49d.

462

463 The CysLTR1 receptor seems to be functionally important, since pranlukast
464 administration during the first two days PI SeV decreased absolute number and
465 frequency of BAL CD49d⁺ PMN. However, this effect was limited to the BAL, since the
466 number of CD49d⁺ PMN in the lung was unchanged with CysLTR1 blockade. The
467 disparate effect of pranlukast on BAL versus lung suggests that reduction of BAL
468 CD49d⁺ PMN is not completely due to decreased recruitment to the BAL, as we would
469 expect this to have increased lung CD49d⁺ PMNs. One explanation could be that
470 CysLT provide a survival signal that keeps BAL CD49d⁺ PMN alive once they have
471 migrated out of the lung. Supporting this view are our data that demonstrate increased
472 viability in CD49d⁺ PMN when cultured with LTD₄. Our prior work demonstrated that
473 CD49d⁺ PMN drive FcεRI expression on lung cDC after a 48 hour cognate interaction
474 (12). Therefore, it is possible that the loss of post-viral atopic disease with pranlukast
475 treatment resulted from reduced viability of CD49d⁺ PMN, preventing them from fully
476 interacting with cDC.

477

478 The role of CysLTR1 and PMN appears to depend upon the stimulus used to drive
479 airway disease. Unlike our viral model where CysLTR1 appears to increase the level of
480 disease through CD49d⁺ PMN, a model of irritant-induced asthma demonstrated the
481 apparent opposite (30). Mice exposed to Cl₂ developed lung neutrophilia that was
482 associated with increased CysLT. Unlike our model, inhibition of CysLTR1 signaling led
483 to increased airway disease, which could be prevented by depletion of PMN. While the
484 authors did not examine PMN subtypes, it is intriguing to speculate that Cl₂ induced

disease is driven by CD49d⁻ PMN, with the CD49d⁺ PMN possibly being protective.

Perhaps not surprising, the type of stimulus driving airway disease may well tune the PMN response.

CD49d⁺ PMNs have been reported in human disease (31) and CysLTs have been shown to increase β 2-integrin activation and adhesion in human PMN *in vitro* (32). But no human studies have addressed whether CysLTR1 is exclusively expressed on CD49d⁺ PMN. We examined the nasal lavage of human subjects within 4 days of onset of upper respiratory tract viral infections and found expression of CysLTR1 on CD49d⁺ but not CD49d⁻ PMN. These results, combined with our prior work demonstrating CD49d⁺ PMN in the peripheral blood and nasal lavage of atopic individuals, support further investigation into the role these PMN play in human disease (16).

To our knowledge, this is the first report documenting functional differences between two PMN subsets during a respiratory viral infection. Development of post-viral atopic disease requires the “alternative” CD49d⁺ PMN, whereas “classical” CD49d⁻ PMN do not appear to play a critical role in our SeV model. We have demonstrated the feasibility of manipulating pro-atopic PMN subset accumulation during a respiratory viral infection to significantly diminish development of post-viral airway disease. This may have clinical implications, since current therapies either do not target the PMN or else affect their activities in a nonspecific way that can lead to potential harm (33).

Given that other components of the SeV model are intact in humans, we believe the demonstration of CysLTR1 expression on human CD49d⁺ PMN may have therapeutic implications. One human study showed a clinical benefit to starting CysLTR1 antagonism (montelukast) early in RSV bronchiolitis in children without pre-existing asthma (34). However, later trials using montelukast in post-viral wheezing or asthma exacerbations failed to show efficacy (35, 36). We would expect these different responses, since, in our model, CysLTR1 blockade only prevented post-viral disease when given *before* symptoms of disease (i.e., days -1 to 5 PI SeV); whereas, treatment after viral clearance (days 12-18 PI SeV) had no effect on post-viral disease.

In summary, our study begins to define an “alternative” PMN subset (based on expression of CD49d, CysLTR1, TNF, CCL2, and CCL5) that is needed for full development of post-viral atopic disease. Expression of CysLTR1 on the CD49d⁺ PMN is functional, as culture with LTD₄ increased their *in vitro* survival, and specific CysLTR1 antagonism reduced accumulation of these cells in the BAL and blocked subsequent development of post-viral disease. In humans, CD49d⁺ PMN increased in the nasal lavage during an acute respiratory viral infection and co-expressed CysLTR1. Our work, therefore, suggests further studies are needed to identify strategies that alter accumulation of CD49d⁺ PMN in the airways and to ultimately determine their role in development of post-viral atopic disease.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1: Role of human neutrophil subsets in post-viral induced asthma study.

(a) Experimental design. **(b)** Participant flow diagram.

Figure 2: CD49d⁺ PMN are found in increased frequency in the nasal lavage during acute upper respiratory infection symptoms compared to convalescence.

The frequency of CD49d⁺ PMN was calculated during illness ("ILL") and convalescence ("WELL") in all subjects (n=42) as well as those only with a positive viral PCR (n=23; virus detected) or a negative viral PCR (n=19; virus not detected). The frequency of CD49d⁺ PMN increased during illness compared to convalescence in all subjects, including those with a positive or negative viral PCR. This increase was also seen in both atopic (n=31) and non-atopic (n=11) individuals that were confirmed to have a virus detected (n=7 virus detected and non-atopic; n=16 virus detected and atopic), however among subjects in whom a virus was not detected this increase occurred only among atopic individuals (n=4 virus not detected and non-atopic; n=15 virus not detected and atopic). Asthma status (n=17 with asthma; n=25 non-asthma) did not alter the increase in CD49d⁺ PMN with illness, nor did the detection of virus (n=11 with asthma; n=12 non-asthma). However, among subjects in whom a virus was not detected, this increase occurred only among individuals who did not have asthma (n=6 with asthma; n=13 non-asthma). Boxes represent the 25th-75th percentile of data with the median indicated by a line. Whiskers plotted with Tukey's method and outliers shown as individual circles. For all panels, the Wilcoxon signed-rank test was used to compare median frequency of

CD49d⁺ PMN between ill and well. $P < 0.05$ was considered significant; * results were still significant after multiple test adjustment using the step-down Bonferroni method.

Figure 3: Correlation of CD49d⁺ PMN frequency with viral, atopic, and asthma

status. Frequency of CD49d expressing PMN were determined as in Figure 2. (a) Comparison of viral status and CD49d⁺ PMN frequency during illness (“ILL”) and convalescence (“WELL”) in all subjects (top panel) in whom PCR detected no virus in the lavage (“Virus Not Detected”; n=19) or in whom a virus was detected (“Virus Detected”; n=23), in atopic subjects (middle panel; n=15 virus not detected, n=16 virus detected), and in non-atopic subjects (lower panel; n=4 virus not detected, n=7 virus detected). (b) Comparison of CD49d⁺ PMN frequency based on atopic status in all subjects (top panel, n=11 non-atopic; n=31 atopic), or in those in whom a virus was detected (middle panel, n=7 non-atopic; n=16 atopic) or was not detected (lower panel, n=4 non-atopic; n=15 atopic). (c) Comparison of CD49d⁺ PMN frequency based on asthma status in all subjects (top panel, n=25 non-asthma; n=17 asthma), or in those in whom a virus was detected (middle panel, n=12 non-asthma; n=11 asthma) or was not detected (lower panel, n=13 non-asthma; n=6 asthma). Boxes represent the 25th-75th percentile of data with the median indicated by a line. Whiskers plotted with Tukey’s method and outliers shown as individual circles. For all panels, Mann-Whitney U-test used to compare median frequency of CD49d⁺ PMN between the two groups on each panel. $P < 0.05$ was considered significant; * results were still significant after multiple test adjustment using the step-down Bonferroni method.

694

695 **Figure 4: CD49d expression defines unique PMN phenotype.** (a) Venn diagram
696 showing differential gene products between CD49d⁺ and CD49d⁻ PMN from BAL 3 days
697 PI SeV. Gene product profiles of PMN subsets were determined by Affymetrix
698 microarray (n=3 separate experiments; cells for each experiment pooled from 10 mice).
699 (b) The 5 inflammatory gene products with most significant fold change from (a) that
700 appeared most relevant to our model. Rank Product statistics were conducted and
701 false discovery rates (FDR) were determined using RankProd from Bioconductor.
702 Differentially expressed probe sets were defined as those possessing a false discovery
703 rate (FDR) <1% and log₂ ratio > 0.05 between compared samples. (c) qPCR validation
704 of gene products from (B) (n=3 separate experiments; cells pooled from 5 mice;
705 Student's *t*-test). (d) Cytokine/chemokine production from CD49d⁺ and CD49d⁻ PMN.
706 BAL PMN from day 3 PI SeV were sorted for CD49d expression and cultured for 24
707 hours. Supernatant cytokines were determined by ELISA-based cytokine array (n=3
708 separate experiments; cells pooled from 5 mice; Student's *t*-test). (e) Representative
709 DHR assay histograms of day 3 PI SeV freshly isolated (solid line) CD49d⁺ and CD49d⁻
710 PMN or isolated and cultured with PMA (dotted line). Representative data from 4
711 separate experiments. (f) Quantification of DHR assay as in (e) (PMN isolated from day
712 3 PI SeV, CD49d⁺ PMN in black histogram, CD49d⁻ PMN in white histogram)
713 compared with DHR from CD49d⁻ PMN isolated day 1 PI LPS (hatched bar). PMA
714 treatment was performed to maximize respiratory burst (n≥2 separate experiments;
715 *p<0.05; Student's *t*-test).

716

Figure 5: CD49d⁺ PMN survival is CysLTR1 dependent. (a) CysLTR1 expression is limited to CD49d⁺ PMN. Representative plots and histograms of BAL cells day 3 PI SeV, staining for Gr-1, CD49d, and CysLTR1 (n=6); Student's *t*-test. (b) CysLTs are expressed in the BAL of SeV infected mice. CysLTs (black bars, left axis) and CD49d⁺ PMN (open bars, right axis) were measured in the BAL of mice at the indicated days PI SeV (n≥2 per timepoint; Student's *t*-test). (c) LTD₄ in culture prolongs CD49d⁺ PMN survival. Gr1⁺ CD49d⁺ and CD49d⁻ BAL cells from day 3 PI SeV were cultured with indicated concentrations of exogenous LTD₄. Viability determined by Trypan Blue exclusion at 24 hours (n=4-8 per group; Student's *t*-test). (d) LTD₄ reduces CD49d⁺ PMN apoptosis *in vivo*. Mice were given either PBS or LTD₄ (40pg/mL) i.n. 36 h PI SeV, and 12 hours later lung CD49d⁺ (black bars) or CD49d⁻ (white bars) PMN were isolated and viability by Trypan Blue exclusion and apoptosis by Annexin V/PI staining was determined. Viability is presented as percent viable compared to PBS control (mean ± SEM from 2 separate experiments of n≥2 mice per condition per experiment). Apoptotic PMN were determined by Annexin V and PI staining, data are shown as mean ± SEM Annexin V⁺/PI⁺ percent of CD49d⁺ and CD49d⁻ PMN (n=3-4 mice per treatment group, from 2 separate experiments; Student's *t*-test). (e) CysLTR1 blockade *in vivo* increases CD49d⁺ PMN apoptosis. Mice were inoculated with SeV i.n. and on days 1 and 2 PI vehicle or pranlukast (25mg/kg in 200μL) was given by gavage. On day 3 PI SeV lung CD49d⁺ and CD49d⁻ PMN were purified and apoptosis assessed by Annexin V/PI staining (data are presented as mean ± SEM from n=3-4 mice per treatment group; Student's *t*-test).

Figure 6: SeV mediated CD49d⁺ PMN accumulation and atopic disease are CysLTR1 dependent. (a) Pranlukast, a specific CysLTR1 antagonist, given on days 1 and 2 PI SeV only is sufficient to reduce CD49d⁺ PMN in the BAL (left panel), but not lungs (right panel) at day 3 PI SeV. Frequency and absolute number of CD49d⁺ PMN are shown (n≥5). (b) Mice were treated as in (a) but BAL PMN identified by Ly6G (instead of Gr-1) and CD49d staining (n=6). (c, d) Pranlukast from day -1 to day 5 PI SeV significantly reduced post-viral atopic disease as evidenced by (c) mucous cell metaplasia and (d) airway hyper-reactivity at day 21 PI SeV (n=5-6 per group). This effect was not seen in mice inoculated with UV-inactivated SeV. (e, f) Pranlukast treatment after viral clearance (day 12-18 PI SeV) failed to reduce (e) mucous cell metaplasia or (f) airway hyper-reactivity by day 21 PI SeV (n=5 per group). (g) Human CD49d⁺ PMN express CysLTR1. Representative plot and histograms, with fold change mean florescence intensity for CysLTR1 staining of nasal lavage cells from human subjects within first 4 days of viral upper respiratory tract infection symptoms (n=4). Student's *t*-test used for panels (a), (b), (c), (e), and (g); ANOVA with Tukey's multiple comparison test used for panels (d) and (f).

Table I. Demographics of study participants.

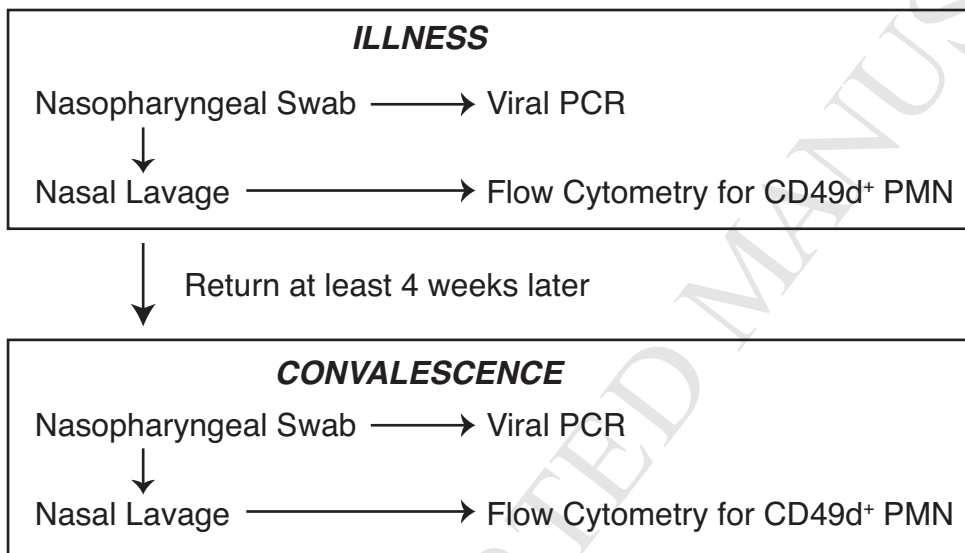
	Atopic	Non-atopic	Asthma	No Asthma	Virus Detected	
					Yes	No
Male (n (%))	14 (45.2)	3 (27.3)	11 (64.7)	6 (24.0)	10 (43.5)	7 (36.8)
Female (n (%))	17 (54.8)	8 (72.7)	6 (35.3)	19 (76.0)	13 (56.5)	12 (63.2)
Age (median, range)	23 (5 – 48)	26 (5 – 42)	10 (5 – 42)	29 (5 – 48)	17 (5 – 45)	28 (8 – 48)

Table II. Change in frequencies of total neutrophils and CD49d-expressing neutrophil subsets.

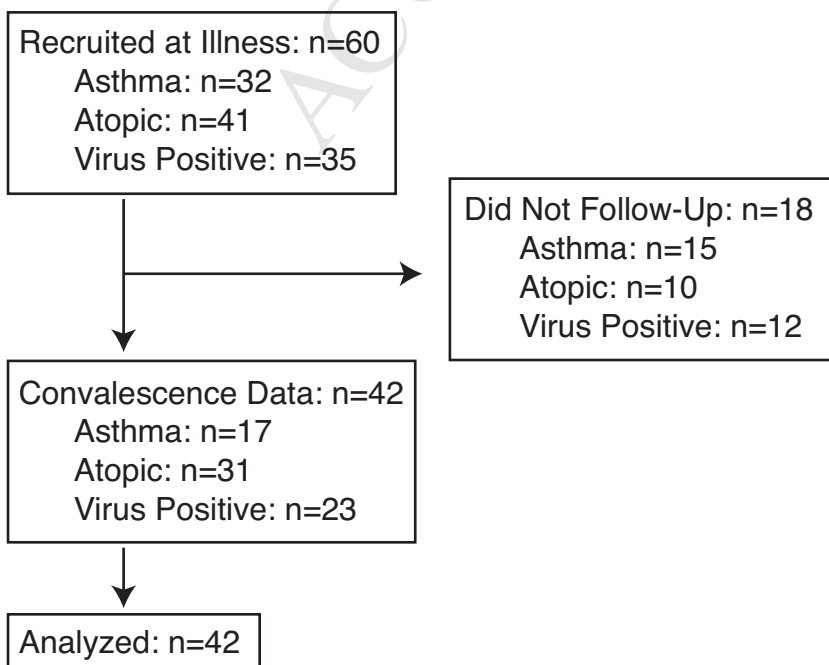
	Virus Detected	Visit	n	Median %	p-value*
Total PMN (%CD16 ⁺ of total cells)	Yes	ILL	23	90.9	<0.001
		WELL	23	74.6	
	No	ILL	19	77.8	0.490
		WELL	19	84.0	
CD49d ⁺ PMN (%CD49d ⁺ of total PMN)	Yes	ILL	23	2.0	<0.001
		WELL	23	0.0	
	No	ILL	19	3.0	0.003
		WELL	19	2.0	

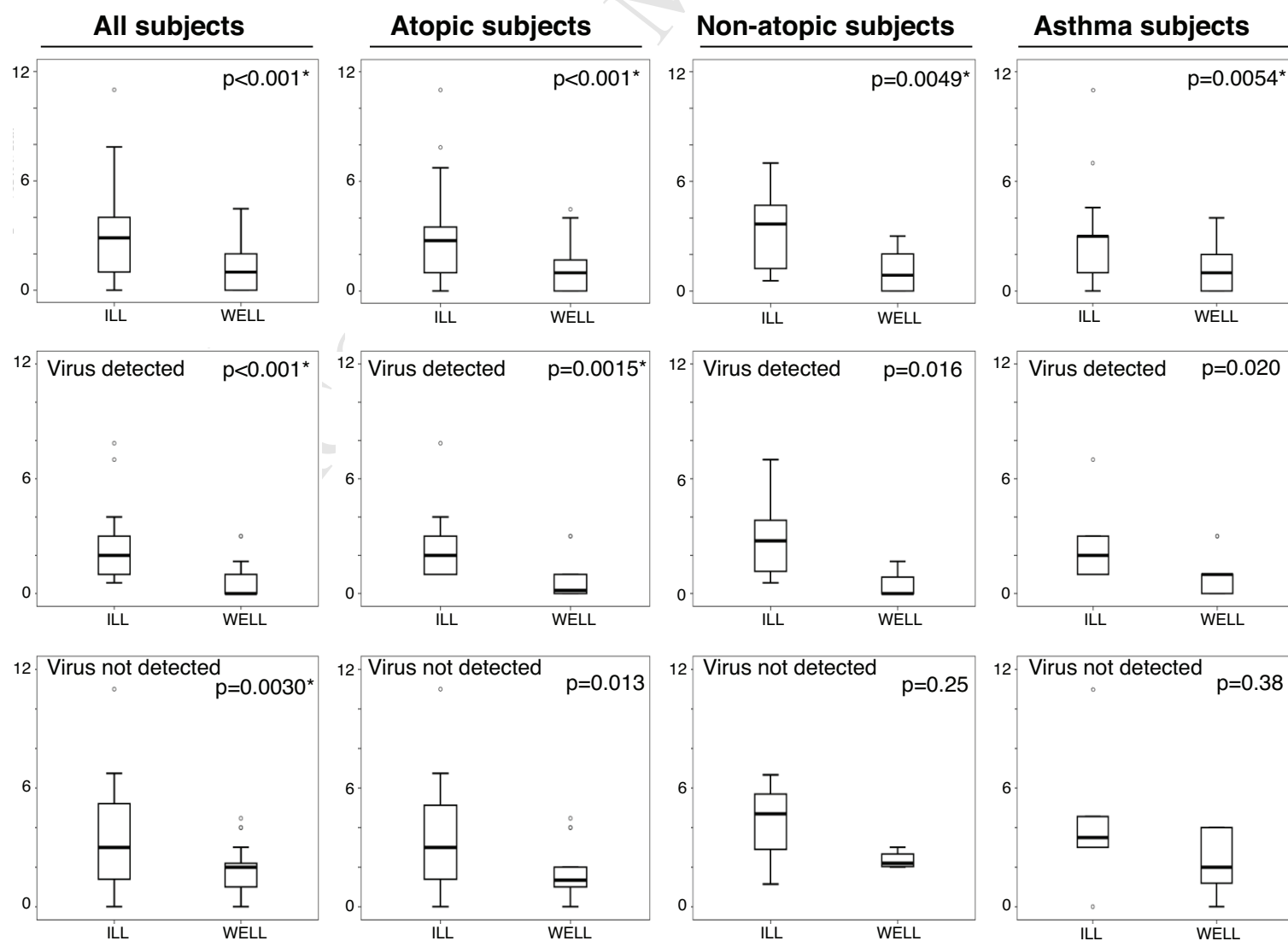
* Wilcoxin signed rank comparing "Total PMN" or "CD49d⁺ PMN" between ILL and WELL.

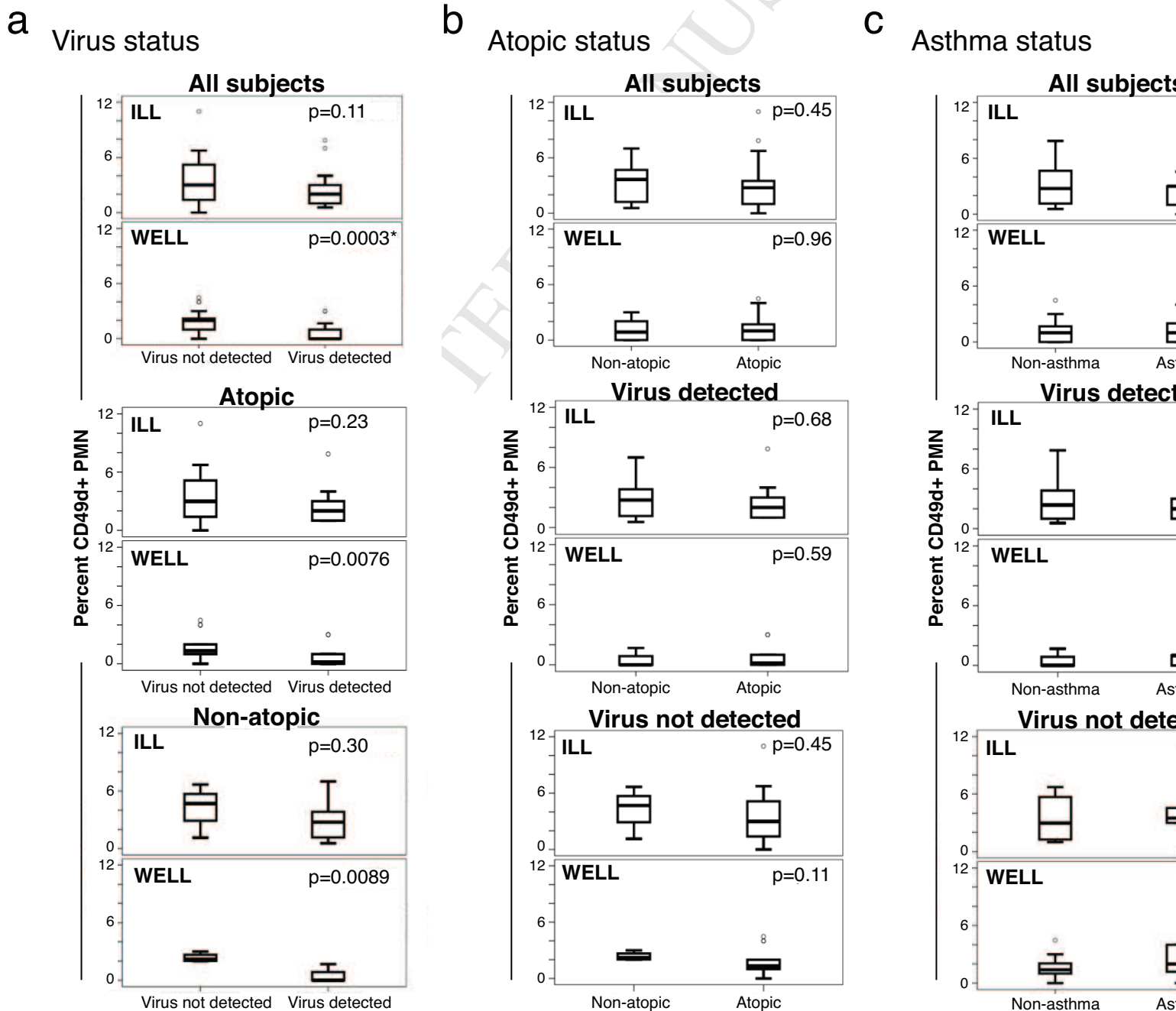
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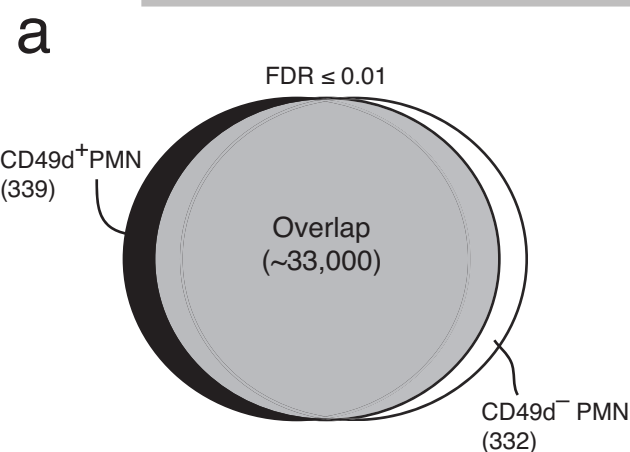


b



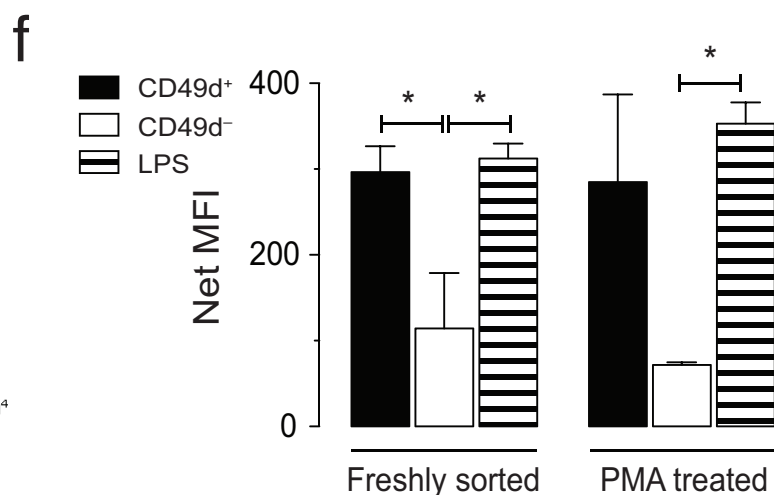
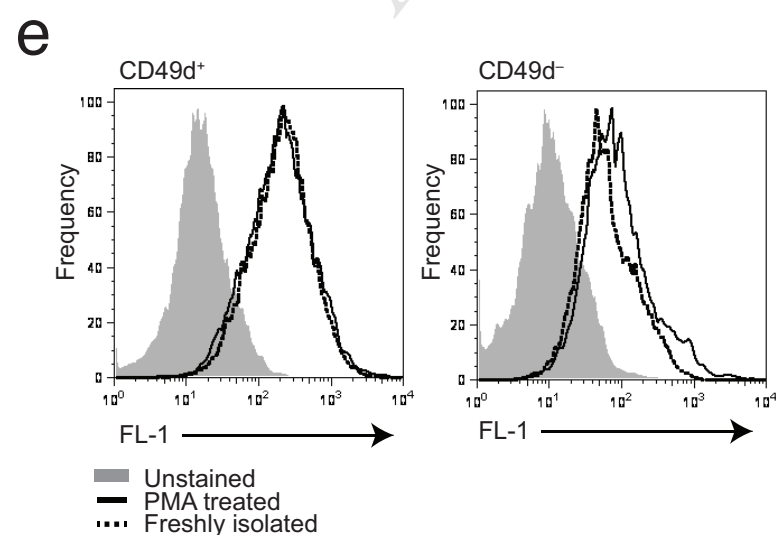
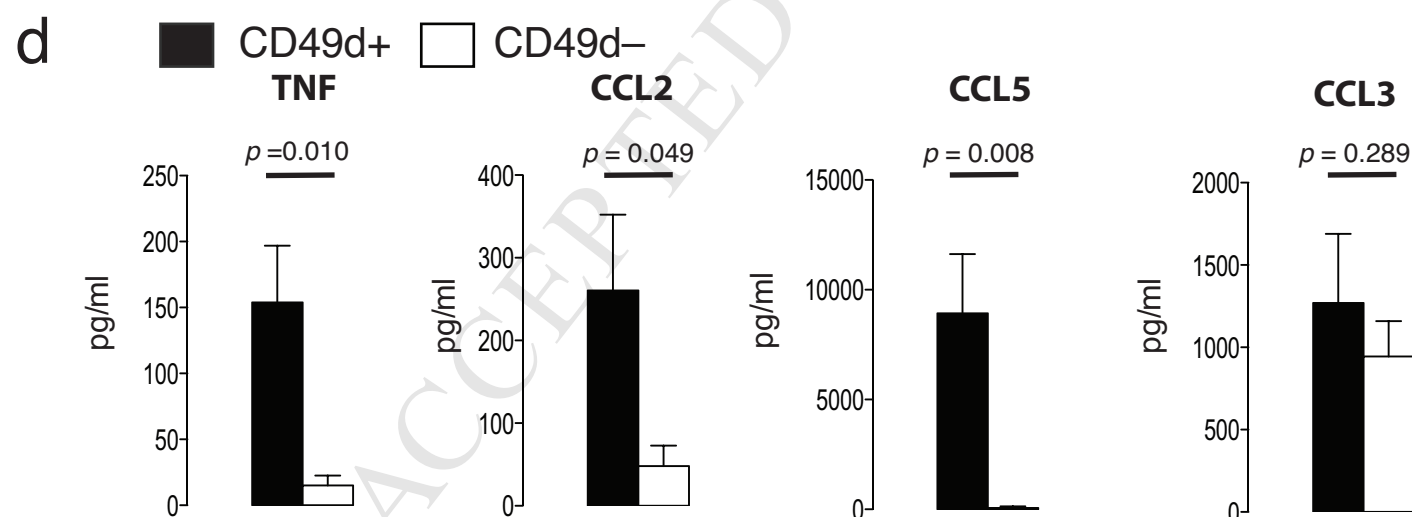
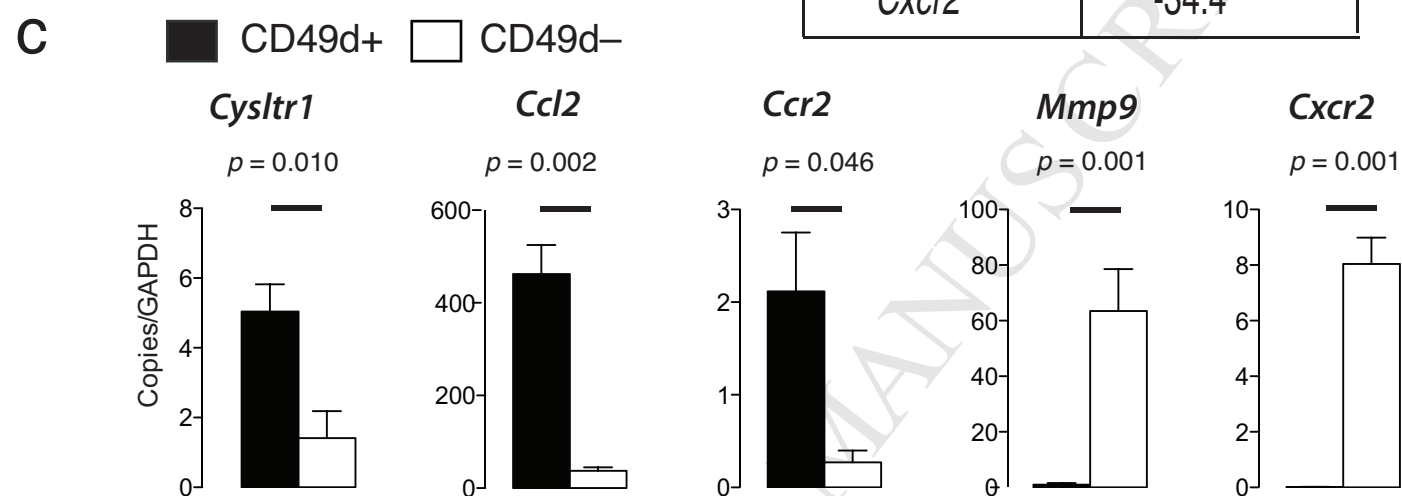


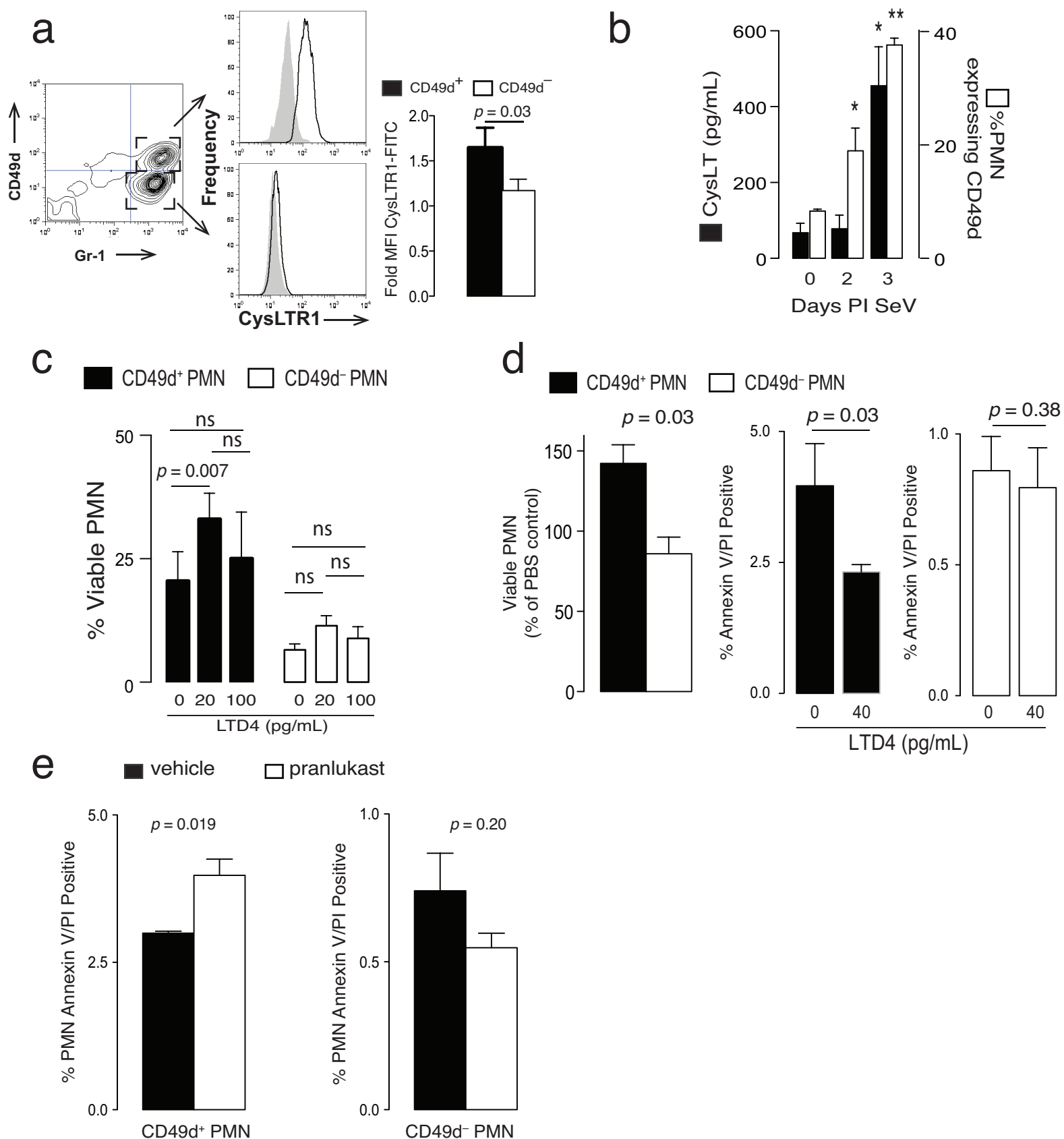




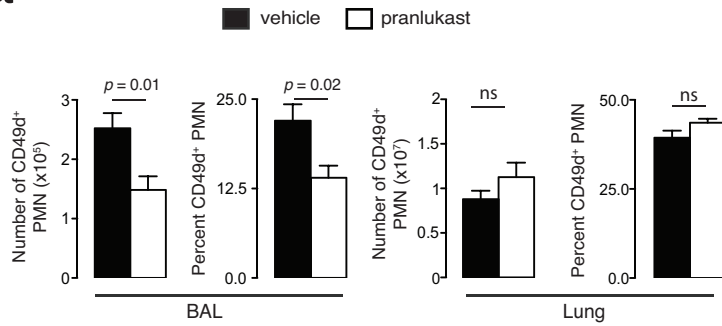
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Gene	Fold Change
<i>Cysltr1</i>	8.5
<i>Ccl2</i>	8.3
<i>Ccr2</i>	12.2
<i>Mmp9</i>	-28.0
<i>Cxcr2</i>	-34.4

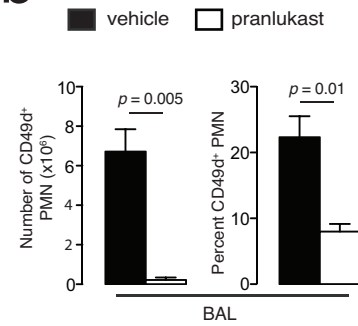




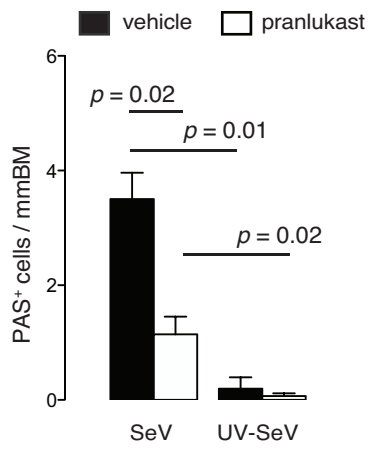
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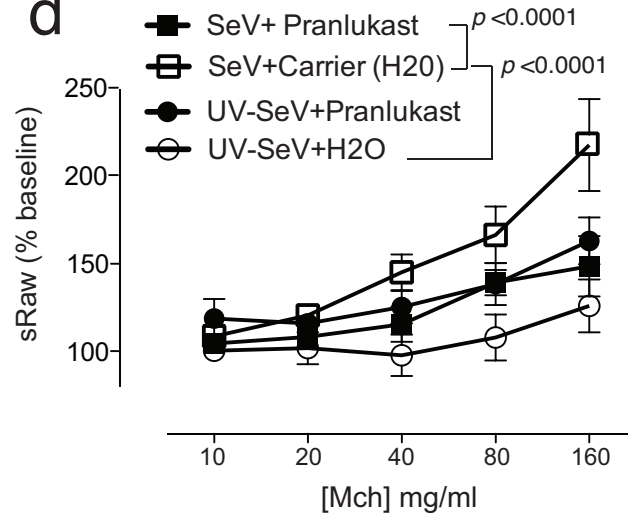
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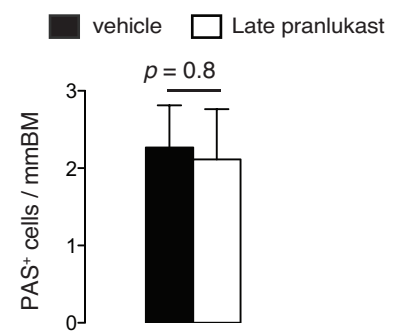
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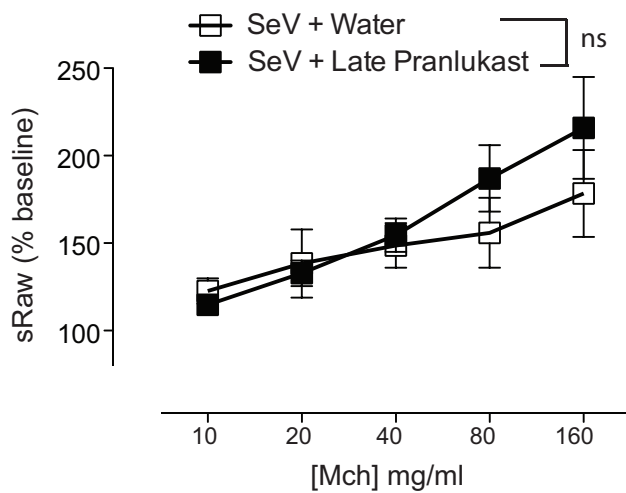
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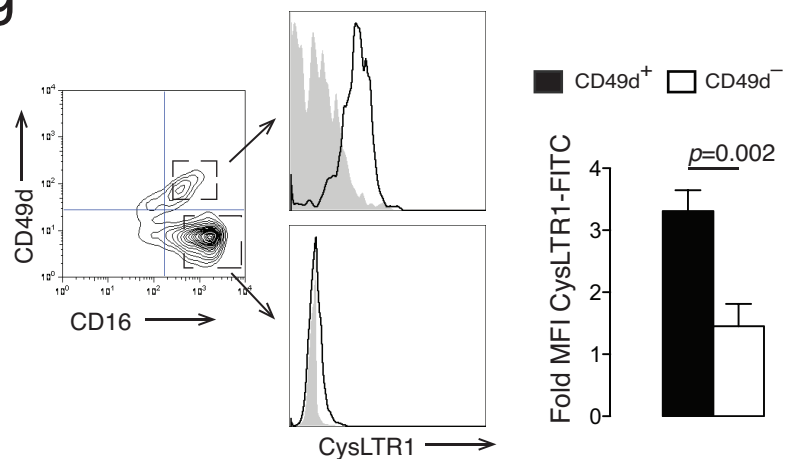
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f



g



SUPPLEMENT**CysLTR1-expression identifies a subset of neutrophils during the antiviral response that contributes to post-viral atopic airway disease**

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SUPPLEMENTAL METHODS

Personal Medical History

Subjects were classified as “atopic” by having a positive aeroallergen skin or serum specific IgE testing to at least one allergen with clinical correlation as documented in the medical record. Subjects were classified as “asthmatic” by physician diagnosis as documented in the medical record. Asthma exacerbation was defined as a decrease in FEV1 from baseline, symptoms as documented at the time of enrollment requiring frequent beta-agonist use or subsequent treatment with a systemic corticosteroid, or by telephone interview one week after enrollment, since symptoms of an asthma exacerbation could have been delayed after our enrollment timeframe (i.e., within 4 days of upper respiratory symptom onset). Of the 12 subjects that met criteria for an asthma exacerbation, only 2 subjects were not prescribed oral corticosteroids within 1 week of their “ILL” visit.

Nasopharyngeal Swab

A nasopharyngeal swab was performed as per manufacturer’s instructions and collected in transport media (M4, Remel). Swabs obtained were stored at -80°C and sent to the Children’s Hospital of Wisconsin clinical lab in batches for PCR-based viral assays. Viruses tested were: Influenza A, Influenza B, Respiratory Syncytial Virus, human Metapneumovirus, human Parainfluenza Virus 1, human Parainfluenza Virus 2, human Parainfluenza Virus 3, human Parainfluenza Virus 4, and human Rhinovirus. This technique of collecting swab samples in transport

media, freezing, and analyzing in batches is an approach validated by our clinical laboratory to preserve sensitivity of the viral assays.

Nasal Lavage

A nasal lavage was performed on all subjects at enrollment in order to assess the neutrophilic presence in the respiratory mucosa, as previously reported¹. The collected fluid was processed immediately and analyzed by flow cytometry for determination of CD49d⁺ PMN as outlined below.

Convalescent Visit

All subjects were asked to return at least 4 weeks after the initial visit (“convalescence”) for identical testing which included a SNOT-20 questionnaire, spirometry, nasopharyngeal swab, and nasal lavage.

Lavage Processing

Nasal lavage fluid was processed immediately to yield a single cell suspension as previously published¹.

Mouse studies

Mouse generation and handling. Male C57BL6 mice 6-20 wks old (The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Mice were housed and handled and experiments performed according to protocols approved by the

Institutional Animal Care and Use Committees at both the Medical College of Wisconsin and the Research Institute at Nationwide Children's Hospital.

Mice were inoculated i.n. with 2×10^5 pfu (in 15 μ l) SeV (Strain 52; American Type Culture Collection) or UV-inactivated SeV. UV-inactivated SeV was used as a control, as the UV exposure makes the virus incapable of infecting mice, but provides a way to administer the same amount of initial protein as live SeV. Mice in all groups were monitored daily for weight and activity. In some experiments, pranlukast (25mg/kg, Sigma) in dH₂O was administered by gavage daily from day -1 to 5 post-inoculation (PI) SeV or from day 12 to 18 PI SeV; control mice were given dH₂O by gavage (200 μ l). On day 21 PI SeV, airway resistance was measured and mice were euthanized with lungs harvested for evaluation of mucous cell metaplasia.

BAL and single cell suspensions from lung digest were obtained on day 3 PI SeV as previously described². Briefly, mice were euthanized, the inferior vena cava severed, and the right cardiac ventricle injected with PBS, before bronchoalveolar lavage (BAL) performed with 1ml PBS. Lungs were removed, minced, and incubated in digest media for 1 h at 37°C, EDTA was added to the media (2mM final concentration) for the last 15 min. The single cell suspension was filtered through 40- μ m pore cell strainers before removing erythrocytes by hypotonic lysis.

To determine the effect of LTD₄ administration on PMN apoptosis *in vivo*, mice were given 30μL PBS or LTD₄ (40pg/mL) i.n. 36 h PI SeV, and 12 hours later lung PMN isolated and viability determined by either Trypan Blue (Gibco, NY) exclusion or Annexin V/PI staining by flow cytometry. For effect of CysLTR1 blockade on PMN apoptosis, mice were inoculated with SeV i.n. and on days 1 and 2 PI SeV 200μL vehicle or pranlukast (25mg/kg) was administered by gavage. On day 3 PI SeV lung PMN apoptosis was assessed by Annexin V/PI staining.

Antibodies and flow cytometry

Phycoerythrin-, allophycocyanin-, fluorescein isothiocyanate (FITC), or Alexa Fluor 647- labeled antibodies against mouse CD49d (clone R1-2), Gr1 (clone RB6-8C5), Ly6G (clone 1A8), human CD16 (clone 3G8) and human CD49d (clone 9F10), and isotype control IgGs were obtained from eBioscience, BD Pharmingen, Biolegend, and/or Invitrogen. Rabbit polyclonal Ab against mouse CysLTR1 and FITC labeled goat Ab against rabbit IgG were obtained from Abcam. Stained cells were analyzed with a FACSCalibur flow cytometer (BD) and data analyzed with FlowJo software (Tree Star, Inc). In some experiments, cells were stained with mAb and then sorted through the FACSria cell sorter to obtain PMN subsets (purity of both subsets was >95%).

Oxidative burst

BAL PMN were isolated from mice 3 days PI SeV or 1 day PI LPS and flow sorting used to isolate Gr-1⁺/CD49d⁺ (CD49d⁺) and Gr-1⁺/CD49d⁻ (CD49d⁻) PMN. Using a commercial DHR assay (Cayman) according to the manufacturer's instructions, the oxidative burst was determined by flow cytometry. For some samples, phorbol myristic acid (PMA; 200nM) was added for 45 min. to maximize oxidative burst.

Transcriptome

RNA was amplified/labeled using the Express Kit (Affymetrix, Santa Clara, CA), and hybridized to the Affymetrix Mouse Genome 430_2.0 Array per the manufacturer's protocol. Image data were quantified with Affymetrix Expression Console Software and normalized with Robust Multichip Analysis (RMA; www.bioconductor.org/) to determine signal log ratios.

Real-time PCR assay

mRNA was isolated with Trizol (Sigma-Aldrich) from which cDNA was generated with the QuantiTect reverse transcription kit (Qiagen) as per manufacturer's instructions. Quantitative real-time PCR (qPCR) assays were performed using TaqMan Fast Universal PCR Master Mix and the StepOnePlus PCR system (Applied Biosystems). All qPCR data are presented as copy number normalized to *Gapdh* copy number. TaqMan primer and probes for rodent *Gapdh* (4352339E), *Cysltr1* (Mm00445433), *Ccl2* (Mm00441242), *Ccr2* (Mm00438270),

Mmp9 (Mm00442991), and *Cxcr2* (Mm00438258) were obtained from Applied Biosystems.

Cell culture experiments for cytokine or viability measurements

Flow-sorted populations of PMN (purity >95%) were cultured in complete RPMI 1640 supplemented with penicillin/streptomycin, gentamycin, L-glutamine (Invitrogen), and 10% fetal calf serum (Atlanta Biologicals) for 24 hours at 37°C and 5% CO₂. Cells were plated at 10⁵ cells/well in 200µL media in 96-well round bottom plate (Corning, NY). In some experiments, LTD₄ was added at 0, 20, or 100 pg/mL (Sigma-Aldrich, MO). Viability was determined by Trypan Blue stain exclusion (Gibco, NY) and/or Annexin V/PI staining.

Cytokine Microarray

Supernatants from cultured PMNs were plated in duplicate on an ELISA based cytokine array plate to measure IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, TNF, IFNγ, GM-CSF, CCL2, CCL3, CCL5, CCL17, and CXCL1 (Cat#104750GR, Quansys Biosciences, Logan, Utah). The array was developed on the Li Cor Odyssey according to manufacturer's instructions and analyzed with QView software (Quansys Biosciences, UT).

Histochemistry

Mouse lung (at 25 cm H₂O pressure) was fixed in 10% zinc formalin and processed by the Children's Research Institute histology core for standard

Hematoxylin and Eosin (H & E) and Periodic acid-Schiff (PAS) staining.

Quantification of mucous cells was performed by a blinded observer counting the number of PAS⁺ cells per mm of basement membrane using Image-J software (NIH) as previously reported ³.

Airway reactivity measurements

Non-invasive measurements of airway hyper-reactivity (specific airway resistance [sRaw]) after exposure to nebulized vehicle (phosphate-buffered saline [PBS]) or doubling concentrations of methacholine (10 –160 mg/ml) were performed using the FinePointe NAM double chamber plethysmography system according to the manufacturer's instructions (Data Sciences International, MN) and as we have previously reported ⁴. Briefly, FinePointe NAM system utilizes a restrained two-chamber design. Following daily calibration of the system, a collar is placed around the neck of the conscious mouse, which is then inserted into the chamber (our system has 4 chambers, and can analyze 4 mice at once). The collar divides the chamber into two sections – essentially separating the head from the body. This allows the NAM system to independently measure nasal and thoracic flow, and the phase difference between the two, which provides the measurement of airway resistance in conscious animals. Once placed in the chamber, the mice are exposed to 10µL nebulized PBS or various concentrations of methacholine over 30 sec. Data (sRaw) are then collected over 3 minutes following the nebulization. After a 30 sec recovery period, the next dose of methacholine is administered. The FinePointe NAM software then exports the

mean sRaw for each dose (data averaged over the 3 minute period of acquisition). We acknowledge that these measurements of sRaw may not reflect airflow in the lower airways, as changes in the upper airways potentially could add to the measured sRaw. However, the two-chamber design has been shown to provide discrimination between upper and lower airway flow and to correlate with other airway measurements, including the forced oscillation technique^{5, 6}.

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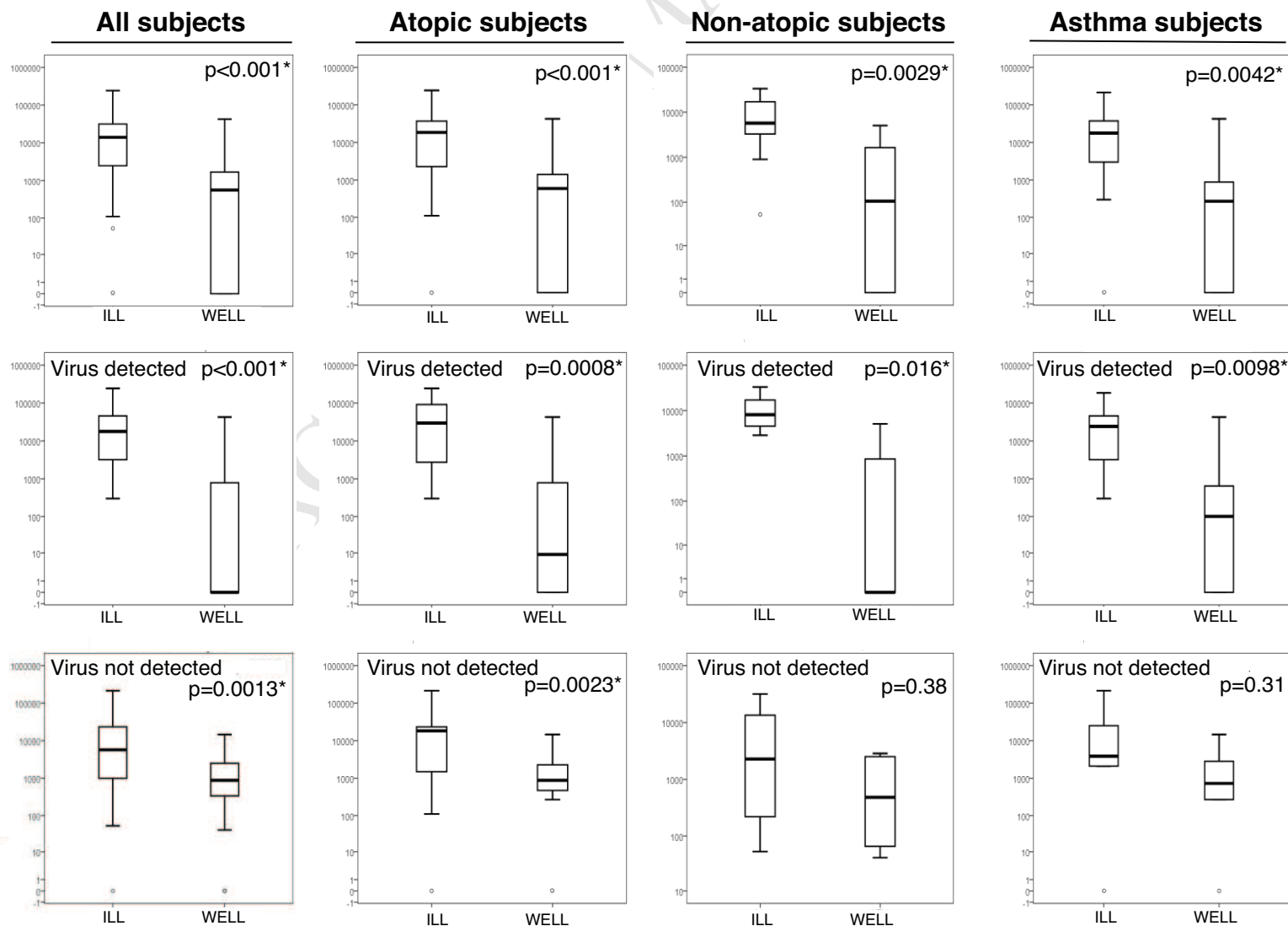
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: CD49d⁺ PMN are found in increased absolute numbers in the nasal lavage during acute upper respiratory infection symptoms compared to convalescence. The absolute number of CD49d⁺ PMN was calculated during illness ("ILL") and convalescence ("WELL") in all subjects (n=42) as well as those only with a positive viral PCR (n=23). The absolute number of CD49d⁺ PMN increased during illness compared to convalescence in both atopic (n=31) and non-atopic (n=11) individuals that were confirmed to have a virus detected (n=7 virus detected and non-atopic; n=16 virus detected and atopic), however among subjects in whom a virus was not detected this increase occurred only among atopic individuals (n=4 virus not detected and non-atopic, n=15 virus not detected and atopic). Asthma status (n=17 asthma, n=25 non-asthma) did not alter the increase in CD49d⁺ PMN with illness, nor did the detection of virus (n=11 with asthma; n=12 non-asthma). However, among subjects in whom a virus was not detected, this increase occurred only among individuals who did not have asthma (n=6 with asthma; n=13 non-asthma). Boxes represent the 25th-75th percentile of data with the median indicated by a line. Whiskers plotted with Tukey's method and outliers shown as individual circles. For all panels, the Wilcoxon signed-rank test was used to compare median frequency of CD49d⁺ PMN between ill and well. P<0.05 was considered significant; * results were still significant after multiple test adjustment using the step-down Bonferroni method.

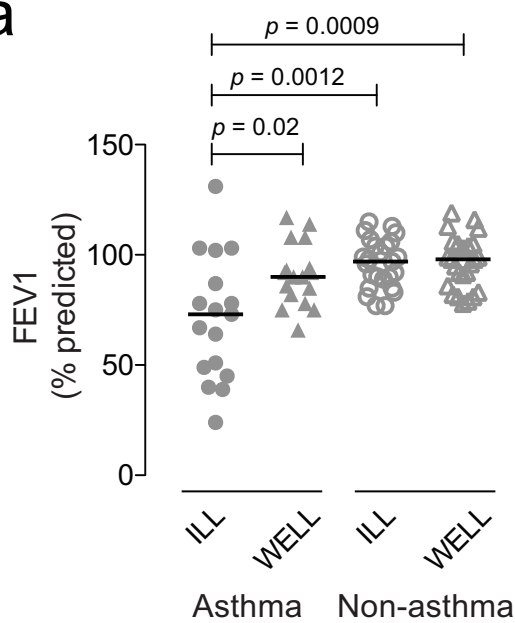
Supplemental Figure 2: Effect of asthma exacerbation on airway function and CD49d⁺ PMN in the nasal lavage. (a) Percent predicted FEV1 in subjects with (n=17) or without (n=25) asthma during the acute illness ("ILL") or upon convalescence ("WELL"). Lines indicate median values. (b) Percent predicted FEV1 in subjects with asthma who had an exacerbation during illness (n=12). (c) The frequency and absolute numbers of CD49d⁺ PMN in the nasal lavage as calculated during illness ("ILL") and convalescence ("WELL") in asthmatic subjects (n=17) with (n=12) or without (n=5) an exacerbation. Wilcoxon signed-rank used to compare median FEV1 (% predicted) for panels (a) and (b), and to compare median %CD49d⁺ PMN between ill and well for panel (c).

Supplemental Figure 3: Absolute numbers of CD49d⁺ PMN and correlation with viral, atopic, and asthma status. Absolute numbers of CD49d expressing PMN were determined as in Supplemental Figure 1. (a) Comparison of viral status and CD49d⁺ PMN numbers during illness ("ILL") and convalescence ("WELL") in all subjects (top panel) in whom PCR detected no virus in the lavage ("Virus Not Detected"; n=19) or in whom a virus was detected ("Virus Detected"; n=23), in atopic subjects (middle panel; n=15 virus not detected, n=16 virus detected), and in non-atopic subjects (lower panel; n=4 virus not detected, n=7 virus detected). (b) Comparison of CD49d⁺ PMN numbers based on atopic status in all subjects (top panel, n=11 non-atopic; n=31 atopic), or in those in whom a virus was detected (middle panel, n=7 non-atopic; n=16 atopic) or was

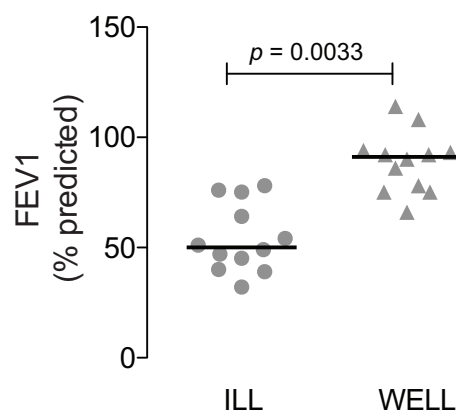
not detected (lower panel, n=4 non-atopic; n=15 atopic). (c) Comparison of CD49d⁺ PMN numbers based on asthma status in all subjects (top panel, n=25 non-asthma; n=17 asthma), or in those in whom a virus was detected (middle panel, n=12 non-asthma; n=11 asthma) or was not detected (lower panel, n=13 non-asthma; n=6 asthma). Boxes represent the 25th-75th percentile of data with the median indicated by a line. Whiskers plotted with Tukey's method and outliers shown as individual circles. For all panels, Mann-Whitney U-test used to compare median number of CD49d⁺ PMN between the two groups on each panel. P<0.05 was considered significant; * results were still significant after multiple test adjustment using the step-down Bonferroni method.



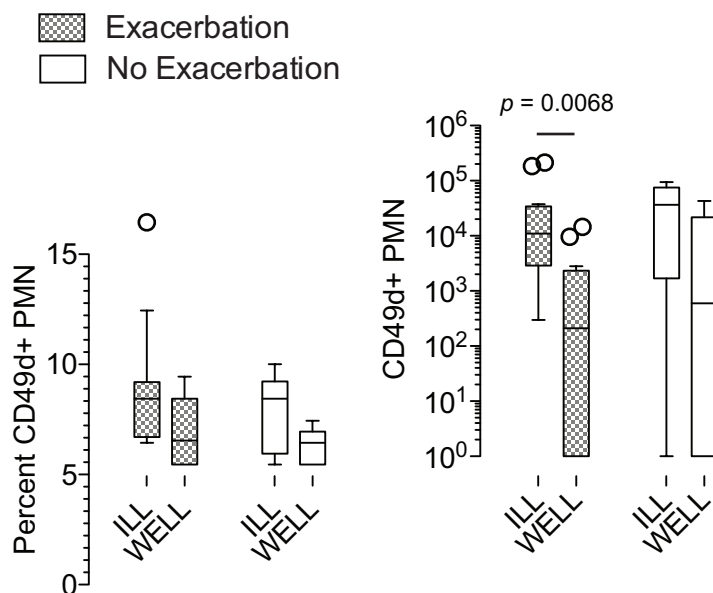
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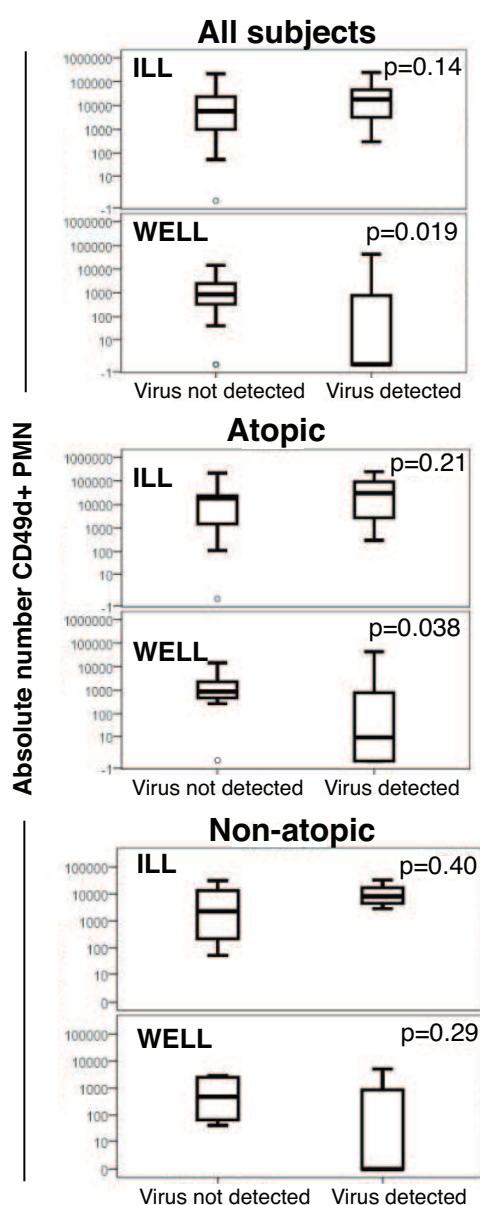
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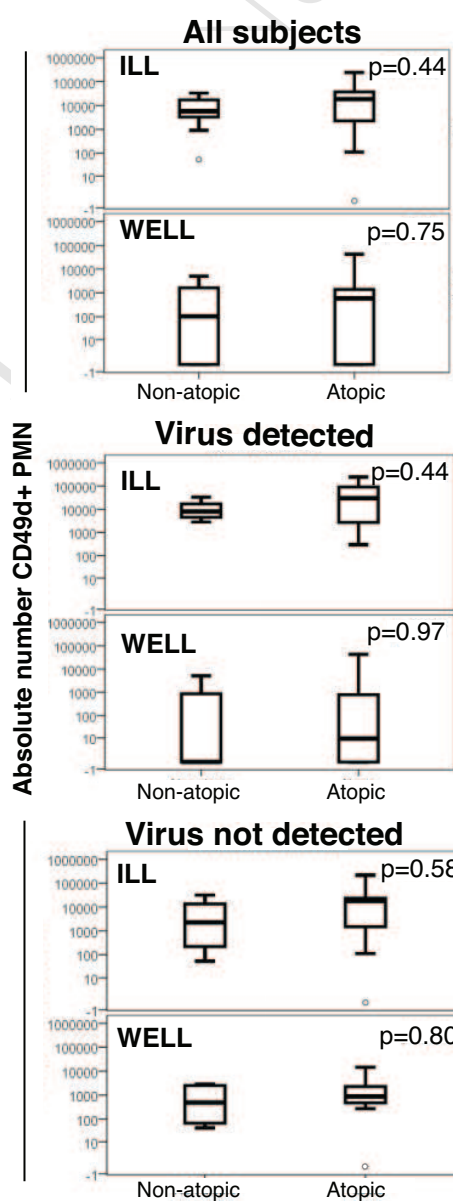
c



a Virus status



b Atopic status



c Asthma status

