

Association between viral infection and increased mucosal eosinophils and CD8⁺CD103⁺ T cells in chronic rhinosinusitis

To the Editor: Patients with chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) frequently present with extensive eosinophilic inflammation, severe symptom scores, and disease recalcitrance. Different immune cell types and their secreted products are associated with tissue eosinophilia¹; however, the initial insult promoting the recruitment of these cells in CRS remains unclear. CD8⁺ T cells have been found to be enriched in CRS patients in association with eosinophilic disease.² In response to alloantigen encounter at mucosal surfaces, CD8⁺ T cells can be induced to express CD103.³ CD103 expression is required for eosinophil recruitment in the context of allergic airway inflammation, and is associated with airway hyperresponsiveness.² Eosinophils are also recruited into the airways in response to viral infection to support innate host defense, while also contributing to the pathophysiology of disease.⁴ CD8⁺CD103⁺ T cells, also known as tissue-resident memory T cells (Trm cells), are thought to have a major role in viral immunity at mucosal surfaces.^{5–7}

As yet, CD103⁺ cell subsets, including CD8⁺CD103⁺ Trm cells, and their relationship to tissue eosinophilia and the presence of virus has not been investigated in CRS. In a recent study, we analyzed the sinonasal virome in 288 CRS and control patients, showing that viruses were more prevalent in CRS and were associated with significantly worse disease severity scores.⁸ The present study utilized tissue samples from virus-positive (VP) and virus-negative (VN) CRS and control patients to investigate the number of eosinophils, CD8⁺, CD103⁺, and CD8⁺CD103⁺ Trm cells in relation to viral infection. We hypothesized that these cell types would be more abundant in virus-positive CRS. Viral sampling was undertaken from consented patients using mucosal cytology brushings of the left and right middle and inferior meatuses under endoscopic visualization at the time of endoscopic sinus surgery. DNA/RNA extracts underwent polymerase chain reaction (PCR)/

real-time PCR (RT-PCR) testing for a panel of common respiratory viruses (adenovirus, bocavirus, coronavirus, enterovirus, influenza, metapneumovirus, parainfluenza, respiratory syncytial virus, and rhinovirus). Mucosal samples were taken at the same time as the aforementioned swabs and processed to undergo hematoxylin and eosin or immunofluorescence staining for eosinophils and CD8⁺ and CD103⁺ cells, respectively (the differing methodology resulting in the 2 study arms). Details of materials and methods are found in the online Supporting Materials and Methods.

A total of 58 patients were recruited to the study; 6 controls, 33 CRS without nasal polyps (CRSsNP), and 19 CRSwNP. Thirty patients were negative for any of the viruses tested (VN) and 28 were positive (VP). Patients in the different groups were matched for age, gender, disease phenotype, and the season in which samples were taken (Supporting Table 1). Numbers of eosinophils, CD8⁺ cells, and CD103⁺ cells were counted by 2 blinded observers RKG, SJ; these are summarized in Supporting Table 2. Viral species identified are summarized in Supporting Table 3.

Mean mucosal eosinophil, CD8⁺ T cells, CD103⁺ cells, and CD8⁺CD103⁺ Trm cell counts were significantly greater in VP CRSsNP and VP CRSwNP than in their VN counterparts. Eosinophils were significantly more abundant in VP CRSwNP than in VP controls. CD8⁺ T cells, CD103⁺ cells, and CD8⁺CD103⁺ Trm cells were also significantly more abundant in VP CRSsNP and VP CRSwNP than in VP controls (Figs. 1 and 2).

Bivariate permutation analysis of variance (ANOVA) models for each of the 4 cell types confirmed the significant association of disease phenotype and viral detection status with the outcome variables (inflammatory cell counts) in all 4 models. These models also showed a significant interaction between disease phenotype and viral status in determining CD8⁺, CD103⁺, and CD8⁺CD103⁺ Trm cell counts (Supporting Tables 4 to 7).

This is the first study linking viral infection with tissue eosinophilia and accumulation of CD8⁺ T cells, CD103⁺ cells, and CD8⁺CD103⁺ Trm cells in the context of CRS. Strikingly, all 4 cell-types were increased in VP CRS patients compared to VP controls, and our ANOVA multivariate analysis indicated that both viral status and CRS diagnosis positively affected inflammatory cell counts. The significant interaction terms in these models indicate that CRS patients might react to viral infection by inciting a more exacerbated immune response compared to controls and that viral infection might contribute to inflammatory cell infiltration in a subset of CRS patients.

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

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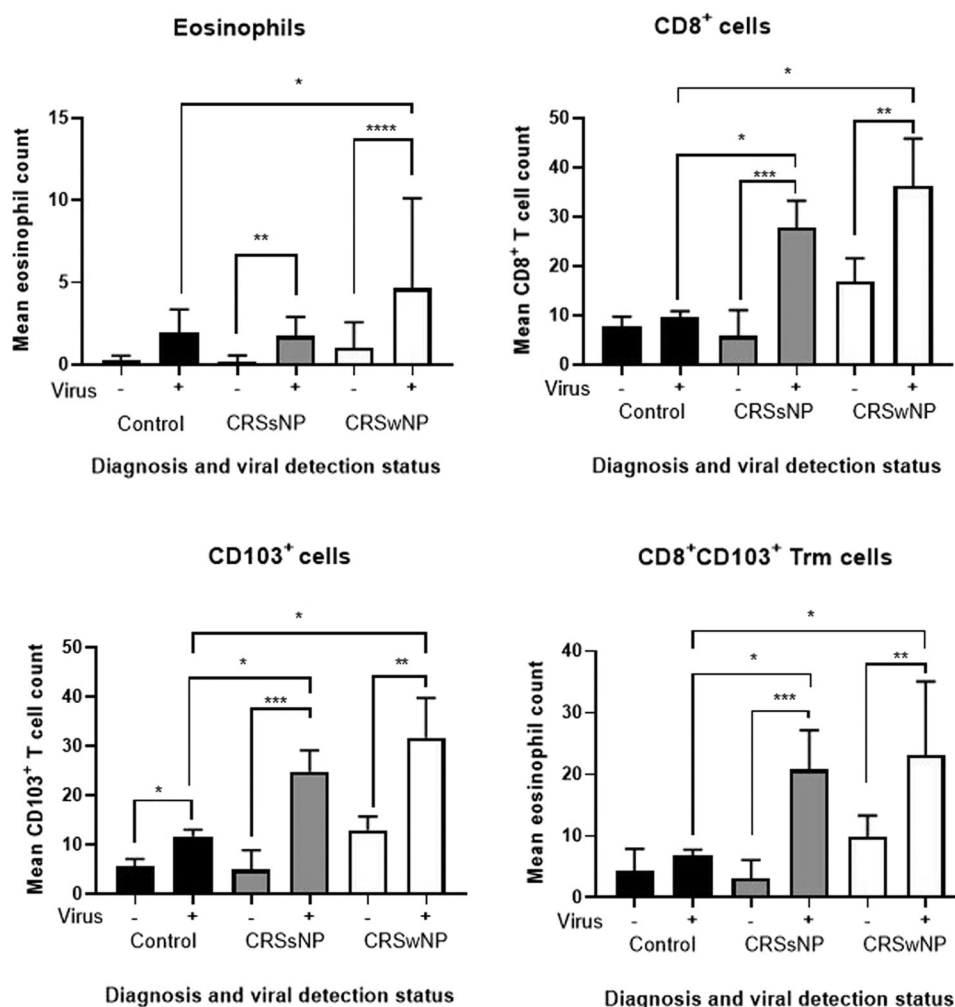


FIGURE 1. Mean cell counts of eosinophils (A), CD8⁺ cells (B), CD103⁺ cells (C), and CD8⁺ CD103⁺ Trm cells (D) in virus negative (–) and virus positive (+) controls (n = 6), CRSsNP (n = 17 top left, n = 16 elsewhere), and CRSwNP (n = 10 top left, n = 9 elsewhere) patients. **p* < 0.05, ***p* < 0.005, ****p* < 0.0005, *****p* < 0.0001. CRSsNP = chronic rhinosinusitis without nasal polyps; CRSwNP = chronic rhinosinusitis with nasal polyps; Trm cell = tissue-resident memory T cell.

Both allergens and viruses are known to induce tissue eosinophilia, and recent studies indicate CD103 expression plays a critical role in these processes.^{8,9} In line with these findings, this study also shows significantly increased CD103⁺ cell numbers in the presence of virus in CRS patients and controls. CD8⁺CD103⁺ Trm cells were also increased in VP CRS patients compared to VP controls and represented the majority of the total CD103⁺ cell population. Unlike Trm cells in the lung, Trm cells in nasal mucosa have been shown to develop independently of local antigen recognition and can persist for extended periods, potentially explaining their presence also in VN patients.¹⁰ Apart from expression on CD8⁺ T cell subpopulations, CD103 is also expressed on a subset of CD4⁺ T cells, natural killer cells, and dendritic cells.¹¹ These cells have been reported to play host-protective roles in the context of infection and detrimental roles in the context of chronic inflammation.¹¹

Our study has some limitations. First, the exact role of CD103⁺–expressing cells including CD8⁺CD103⁺ Trm cells and their role in protection against viral infection and/or contribution to chronic inflammation in CRS is unknown and warrants further investigation. Second, the potential for causal relationships between viral infection of the sinuses and influx of eosinophils and CD103⁺CD8⁺ Trm cells is not known. Third, the control group size in this study was small relative to the CRSsNP and CRSwNP groups; this is due to the rarity of viral positivity in control patients.

In conclusion, this study links viral infection in CRS with mucosal eosinophilia and accumulation of CD8⁺ T cells, CD103⁺ T cells, and CD8⁺CD103⁺ Trm cells, implicating viruses as possible inciting factors in the dysregulated immune response in CRS. More investigation is required to elucidate a causative relationship, should one exist.

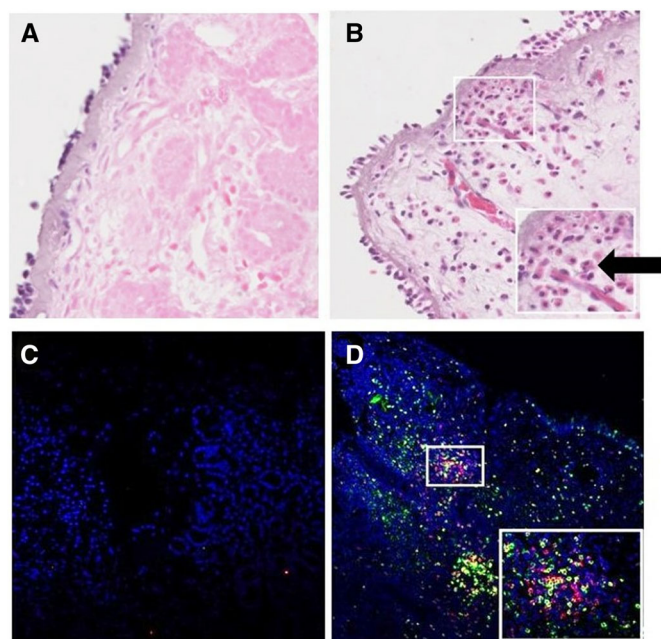


FIGURE 2. Sinonasal mucosa of representative virus-negative control (A,C) and virus-positive CRSwNP patient (B,D). (A,B) Hematoxylin and eosin-stained tissue with eosinophils evident in inset image B (arrow indicates eosinophil). (C,D) Immunofluorescence staining CD8⁺ T cells red, CD103⁺ cells green, and CD8⁺ CD103⁺ Trm cells yellow/orange. DAPI stains nuclei blue. CRSwNP = chronic rhinosinusitis without nasal polyps; CRSwNP = chronic rhinosinusitis with nasal polyps; DAPI = 4',6-diamidino-2-phenylindole; Trm cell = tissue-resident memory T cell.

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Supplementary material

Materials and methods

Study participants

Patients were recruited from the tertiary rhinologic practices of two of the senior authors (PJW and AJP). This study was carried out in accordance with the recommendations of the Central Adelaide Local Health Network Ethics Committee, and the protocol was approved by the same (HREC/15/TQEH/132). Subjects gave written informed consent in accordance with the Declaration of Helsinki. Patients were included in this study if they were older than eighteen years of age and were undergoing endoscopic surgery. No patients in this study suffered from asthma or aspirin-exacerbated respiratory disease, none were smokers, none were on oral or topical steroids in the two weeks before the sample collection day, and none reported symptoms of viral illness in the two weeks before the sample collection day. Control patients did not have any clinical or radiologic evidence of CRS. CRS patients fulfilled the diagnostic criteria for CRS as outlined in the American guidelines. (E (1)

Viral sampling, processing and analysis

Viral sampling, processing and analysis was undertaken using a previously published departmental protocol (E(2)). Briefly, endoscan cytology brushes (McFarlane Medical, Melbourne, Australia) were used to sample the left and right middle meatus (MM) and inferior meatus (IM) mucosa with an aseptic technique and under endoscopic visualisation. Samples were transported on ice and stored at -80°C. Samples were thawed for processing and underwent RNA and DNA extraction using an AllPrep DNA/RNA Mini Kit (Qiagen,

Hilden, Germany). These were again stored until real-time PCR batch testing for adenovirus (AdV), bocavirus (BoV), coronavirus (CoV), enterovirus (EnV), influenza, metapneumovirus (MPV), parainfluenza (PIV), respiratory syncytial virus (RSV) and rhinovirus (RV). An initial endogenous retrovirus 3 (ERV3) assay was undertaken in the DNA extract fractions prior to assays for the disease-causing viruses to ensure adequate sample quality, and to ensure detection of active, intra-cellular viral DNA/RNA. Specifics regarding PCR target genes, primer and probe sequences and cycling conditions have also been detailed previously. (2) Viral detection was defined as a cycle threshold (Ct) of forty or less.

Mucosal sampling, processing and analysis

Mucosal tissue was harvested by sharp dissection with an aseptic technique as a routine part of patients' endoscopic sinus surgery. Bony elements were removed from the tissue after which it was placed in transport medium (Dulbecco's Modified Eagle Medium, Gibco by ThermoFisher, Waltham, USA) on ice. Once in the laboratory it was divided into small pieces and placed in 10% neutral buffered formalin for at least 24 hours, after which it was embedded in paraffin. These samples were cut into 5µm-thick slices, mounted on histological slides, stained with haematoxylin and eosin (H&E) and scanned using a NanoZoomer 2.0HT digital whole-slide imager (Hamamatsu, Japan). The images were then assessed by two blinded observers for eosinophilia using previously described techniques. (E(3) In brief, images were scanned for areas of high cellular infiltration. Six areas of 0.035mm² were selected from the subepithelial layer from each slide, eosinophils were counted and a mean eosinophil count was determined for each patient.

A separate set of 5µm-thick slices were taken from a subset of the aforementioned paraffin blocks and mounted on histological slides for immunofluorescence microscopy. Some patients had no further tissue available and so were not able to be included in this arm of the study. The slides were de-paraffinised by sequential immersion in solutions of xylene, 100% ethanol, 90% ethanol and PBS. Antigen retrieval was performed using a pressure-cooker method with the slides immersed in sodium citrate. Slides were washed with PBS and then blocked with serum-free blocker (Dako, Glostrup, Denmark) for 60 minutes at room temperature. CD8 and CD103 antibodies (clones CD/144B and EPR4166(2) respectively, both Abcam, Cambridge, UK) were diluted in 10% FCS TBST (1:5 dilution for the former and 1:250 for the latter). 50-100µL was added to each slide and incubated overnight at 4°C. Slides were washed twice in PBST in preparation for secondary antibody addition. These were anti-mouse IgG 647 and anti-rabbit IgG CY3, both diluted in 10% FCS TBST at a ratio of 1:200, and 50-100µL per slide. The slides were incubated for one hour at room temperature in the dark, and then washed twice with PBST. 200µg/mL of 4',6-diamidino-2-phenylindole (DAPI; Sigma, Aldrich) was added to resolve nuclei, and allowed to incubate for ten minutes at room temperature in the dark. The slides were rinsed twice in PBS, after which 50-100µL of TrueBlack dye (Biotium, Fremont, USA, diluted 1:20 in 70% ethanol) was added to reduce autofluorescence. The slides were washed a final three times in PBS after which a drop of anti-fade mounting medium (Dako, Glostrup, Denmark) was added before cover-slipping. Samples were visualised by using a LSM700 confocal laser scanning microscope (Zeiss Microscopy, Jena, Germany). Images were assessed for T cell infiltration using the aforementioned eosinophil methodology.

71 Statistical analysis

72 Statistics were performed using R statistical software (R Foundation for Statistical
73 Computing, Vienna, Austria) and the scientific Python package (scipy). Mann Whitney tests
74 were used to compare means of inflammatory cell counts between various study groups and
75 subgroups, with False Discovery Rate corrections for multiple comparisons. Multivariate
76 permutation-based ANOVA models (as implemented in the R package ‘lmPerm’) were used
77 to investigate the effect of the clinical covariates (disease phenotype, viral status) as well as
78 their interaction terms, on the various mean inflammatory cell counts. Spearman tests were
79 used to investigate any correlation between the eosinophils and the two T cell types.
80 Statistical significance was defined as a p-value of < 0.05 .

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Tables

Study arm	Eosinophils		T lymphocytes	
Viral detection status	Virus-negative	Virus-positive	Virus-negative	Virus-positive
Diagnosis (control:CRSsNP:CRSwNP)	3:17:10	3:16:9	3:16:9	3:15:8
Mean age (years)	50.6	49.9	50.9	49.8
Sex (M:F)	19:11	19:9	17:11	17:9
Season (spring:summer:autumn:winter)	10:6:9:5	8:6:9:5	8:6:9:5	8:5:9:4

Table E1. Summary of patient demographics and characteristics for eosinophil and T lymphocyte analysis (H&E staining and immunofluorescence studies carried out on separate samples).

	Control		CRSsNP		CRSwNP	
	VN	VP	VN	VP	VN	VP
Mean eosinophil counts	0.28	1.94	0.20	1.76	1.02	4.65
Mean CD8 ⁺ T cell counts	7.72	9.56	5.86	28.29	15.48	36.29
Mean CD103 ⁺ T cell counts	5.69	11.69	4.96	24.80	12.94	31.70
Mean CD103 ⁺ CD8 ⁺ T _{rm} cell counts	4.25	6.89	3.08	20.47	9.86	23.13

Table E2. Mean cell counts. “VP” indicates virus-positive, “VN” indicates virus-negative

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Study arm	Eosinophils	T lymphocytes
Control	BoVx1, CoV x2	As for eosinophil arm
CRSsNP	BoV x2, CoV x4, influenza A x5, RV x5	BoV x2, CoV x4, influenza A x5, RV x4
CRSwNP	BoV x1, CoV x3, EnV x1, influenza A x1, RSV x1, RV x2	BoV x1, CoV x3, influenza A x1, RSV x1, RV x2

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103 **Table E3.** Viral species identified

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Poisson model: Eosinophil average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	-1.1125	0.4643	0.0166
Factor (phenotype) CRSsNP	-0.1320	0.4288	0.7583
Factor (phenotype) CRSwNP	0.9396	0.4142	0.0233
Factor (virus) 1	1.7509	0.3006	< 0.0001
- Null deviance: 141.491 on 53 degrees of freedom - Residual deviance: 72.907 on 50 degrees of freedom			

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107 **Table E4.** Poisson model of eosinophil average

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Poisson model: CD8 ⁺ T cell average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	1.23775	0.15030	< 0.0001
Factor (phenotype) CRSsNP	0.87423	0.14521	< 0.0001
Factor (phenotype) CRSwNP	1.34350	0.14609	< 0.0001
Factor (virus) 1	1.37696	0.06983	< 0.0001
- Null deviance: 826.09 on 53 degrees of freedom - Residual deviance: 216.02 on 50 degrees of freedom			

Table E5. Poisson model of CD8⁺ T cell average.

Poisson model: CD103 ⁺ T cell average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	1.14435	0.15383	< 0.0001
Factor (phenotype) CRSsNP	0.73168	0.14677	< 0.0001
Factor (phenotype) CRSwNP	1.19785	0.14782	< 0.0001
Factor (virus) 1	1.48061	0.07755	< 0.0001
- Null deviance: 725.42 on 53 degrees of freedom - Residual deviance: 162.89 on 50 degrees of freedom			

Table E6. Poisson model of CD103⁺ T cell average.

Poisson model: DP Trm cell average ~ factor (phenotype) + factor (virus)			
Term	Coefficient (β)	Standard Error	P value
Intercept	0.4693	0.2029	0.0207
Factor (phenotype) CRSsNP	1.0018	0.1934	< 0.0001
Factor (phenotype) CRSwNP	1.4286	0.1948	< 0.0001
Factor (virus) 1	1.6048	0.0952	< 0.0001
- Null deviance: 705.98 on 53 degrees of freedom - Residual deviance: 248.92 on 50 degrees of freedom			

Table E7. Poisson model of DP Trm cell average.

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