CD48 Expression on Eosinophils in Nasal Polyps of Chronic Rhinosinusitis Patients

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Keywords

Rhinosinusitis · Nasal polyp · Allergy · Asthma · Eosinophil · CD48

Abstract

Introduction: The pathogenesis of chronic rhinosinusitis (CRS) with nasal polyps (CRSwNPs) is not yet completely understood. Based on current knowledge, the infiltration of mast cells and eosinophils in nasal polyps (NPs) plays an important role. This study aimed to investigate the interplay of asthma and allergy etiopathology in CRSwNPs patients by specifically studying tissue mast cells and eosinophils and the pro-inflammatory marker CD48. **Methods:** Immunohistochemistry was used to assess eosinophils, mast cells, and CD48 expressing eosinophils infiltrating NPs, and flow cytometry was used to assess surface receptors expression on eosinophils from digested NPs. **Results:** Immunohistochemical analyses showed that mast cell infiltration in NPs is higher in allergic patients in comparison to nonallergic patients; eosinophils infiltration in asthmatic NPs was significantly elevated in comparison to the nonasthmatic NPs, and membrane CD48 (mCD48) expression on eosinophils infiltrating nonallergic asthmatic NPs was highly elevated in compari-

son to the other subgroups. Similarly, mCD48 and its high-affinity ligand m2B4's expression on eosinophils from enzymatically digested NPs were significantly higher in nonallergic asthmatics in comparison to allergic asthmatics. **Conclusions:** Eosinophil infiltration in NPs for asthmatic patients, and mast cell infiltration for allergic patients, may be used as reliable biomarkers for endotyping CRSwNPs. In addition, CD48 in asthmatic patients who developed CRSwNPs could be regarded as a potential target for treatment.

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Published by S. Karger AG, Basel

Introduction

Chronic rhinosinusitis (CRS) affects about 10% of the adult population in industrialized countries. CRS is a multifactorial disease, characterized by inflammation of the sinonasal mucosa for at least 12 consecutive weeks. Current clinical consensus divide the disease into 2 dis-

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Edited by: H.-U. Simon, Bern.

tinct phenotypes based on nasal endoscopic and computed tomography findings: CRS with nasal polyps (CRSwNPs) and CRS without nasal polyps [1]. CRSwNPs affected subjects should be evaluated for their inflammatory endotype [1–3]. Type-2 inflammation CRSwNPs patients often suffer from comorbidities such as inhalational allergies and asthma [2, 3].

CRSwNPs, with or without allergy, is usually associated with infiltration of inflammatory effector cells, including eosinophils and mast cells [1], which may influence each other's survival and activity via soluble mediators and via physical contact, forming an "Allergic Effector Unit" [4]. A significant association between nasal polyps (NPs) and the presence of tissue eosinophilia has been described [5]. The incidence of eosinophils in NPs from CRSwNPs patients is markedly increased in the presence of coexisting asthma and/or positive allergy skin prick tests [6]. Moreover, the degree of eosinophil infiltration in NPs has a significant impact on the patients' clinical characteristics, surgical timing, drug efficacy, and prognosis [2, 7]. Concordant blood and tissue eosinophilia are associated with a higher likelihood of poor disease control in CRSwNPs than isolated blood or tissue eosinophilia after adjustment of potential confounders [8, 9].

Tryptase-positive mast cells have been demonstrated to be higher in the epithelium and glands of NPs of CRSwNPs patients [10]. It was shown that mast-cell deficient mice have limited development of CRS [11]. When looking at the mechanism of action of the new generation biological drugs in CRSwNPs, it is seen that infiltration, activation, and mediator release of eosinophils, mast cells, and basophils are important in the formation of NPs [12]. There is accumulating evidence for a role of CD48 in allergic inflammation, especially in asthma, as expressed both on eosinophils and mast cells [13–15].

CD48 is a glycosylphosphatidylinositol-anchored receptor lacking an intracellular domain, and it is the high-affinity ligand for 2B4 an activating receptor on several blood cells and notably on human eosinophils. Since CD48 lacks an intracellular domain, it is considered a co-activating receptor. CD48 is expressed on all the hematopoietic cells in a membrane-bound form (mCD48). In addition, it exists in a soluble form in the serum (sCD48) [16, 17].

Regarding mast cells and eosinophils, the main effector cells of allergy, both express CD48. On both cells, CD48 serves as a receptor for *S. aureus* and its exotoxins, inducing their activation. CD48 on murine mast cells has also been found to bind *E. coli* fimbria and *M. tuberculosis*. Moreover, we have previously found that mast cell

CD48 binds to eosinophils' 2B4, and their interaction contributes to the formation of the Allergic Effector Unit pro-inflammatory properties [18, 19].

Interestingly, neutralization of CD48 using specific mAb significantly reduced eosinophilic inflammation in murine "allergic" airway inflammation models [20]. Importantly, mCD48 and sCD48 are expressed differently in asthmatic patients of varying disease severity. For example, mCD48 in mild/moderate asthma is mostly expressed by eosinophils, while in severe asthma by NK cells, B cells, and T cells. Moreover, sCD48 is significantly elevated in patients with mild asthma as compared to controls and decreased in severe asthma [21]. Notably, sCD48 levels were further found to be elevated significantly in the serum of patients with nonallergic asthma [13].

The mechanisms driving the pathogenesis of CRSwNPs are not clearly understood. Defects in the sinonasal epithelial cell barrier, exposure to pathogenic and colonized bacteria, and host immune system dysregulation, may contribute to disease pathogenesis [22]. Analyses of NPs and adjacent normal nasal mucosa reveal that they share a similar immunologic profile, demonstrating chronic inflammation, which may explain the potential for new NP formation and recurrence following surgical removal [23].

We hypothesized that CD48 expression on eosinophils in NPs of patients with coexisting asthma and/or allergy could be increased, in addition to increased mast cells and increased eosinophils infiltration. In the present study, we investigated the interplay of asthma and allergy etiology in CRSwNP patients, mostly concentrating on NP eosinophil infiltration and on CD48 expression.

Materials and Methods

Patients

Immunohistochemical Study of NPs

The study was designed as an analytical, experimental, retrospective controlled study. NPs obtained from a total of 27 retrospective patients suffering from CRSwNPs who underwent nasal polypectomy at the Department of Otolaryngology/HNS (ENT), Hadassah Ein-Kerem, were chosen after excluding patients with diabetes, ischemic heart disease, immunologic illness, cancer, liver disease, kidney disease, BMI > 27, aspirin sensitivity, and tobacco smoking. All patients did not receive systemic steroids and/or antihistaminic pharmacotherapy at least 1 month prior to surgery.

Data on relevant clinical variables, such as asthma and allergy status (prior to surgery), were obtained from the medical records. Allergy status was determined via the skin prick test, and asthma status was determined according to lung function test including a methacholine challenge. Patients were divided into 4 groups: allergy⁺ asthma⁺ (group 1; control group; $n = 5$; 5 M/0 F; 39 ± 21

years), allergy⁻ asthma⁺ (group 2; $n = 6$; 3 M/3 F; 42.1 ± 18.3 years), allergy⁺ asthma⁻ (group 3; $n = 5$; 3 M/2 F; 41.2 ± 12.9 years), allergy⁺ asthma⁺ (group 4; $n = 11$; 4 M/7 F; 48.2 ± 12.5 years) (see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000515918 in online suppl. material).

NP Eosinophils Surface Receptors Study

Nine patients who underwent polypectomy were included in this part of the study. All patients had negative history of allergy (negative skin prick test). Exclusion criteria included patients with diabetes, ischemic heart disease, immunologic illness, cancer, liver disease, kidney disease, BMI > 27, aspirin sensitivity, tobacco smoking, and receiving systemic steroids 6 months prior to surgery. Patients were divided into allergy⁻ asthma⁻ ($n = 4$; 4 M/0 F; 55 ± 4.24 years; % peripheral blood eosinophil 2.65 ± 1.08) and allergy⁻ asthma⁺ ($n = 5$; 2 M/3 F; 52.4 ± 8.47 years; % peripheral blood eosinophil 5.92 ± 2.53). These patients NPs were used for the isolation of cells (as described below).

Peripheral Blood Cell CD48 Receptor and Soluble CD48 Evaluation

Peripheral blood was collected from 22 healthy controls (i.e., allergy⁻ asthma⁻ peripheral blood eosinophils $2.7 \pm 0.8\%$, 0.23 ± 0.2 [$10^9/L$], total IgE 63.0 ± 114.3 [IU/mL]) and from 11 allergic rhinitis (AR) patients not associated with asthma (i.e., allergy⁺ asthma⁻, peripheral blood eosinophils $3.3 \pm 2.4\%$, 0.2 ± 0.2 [$10^9/L$], total IgE $64.5 \pm 1,977.5$ [IU/mL]); negative skin prick tests and PC20 >8 mg/mL. AR patients (allergy⁺ asthma⁻) and healthy controls were defined according to established clinical guidelines [1]. Patients were untreated for 2 weeks before the study.

Histochemistry and Immunohistochemistry for Mast Cells, Eosinophils, and CD48

Consecutive sections (4 μ m) of formalin-fixed-paraformaldehyde-embedded tissue specimens were prepared and stained by hematoxylin and eosin (H&E) or by immunohistochemistry for mast cells, eosinophils, and CD48 identification. For H&E staining, the tissue sections were deparaffinized in xylene and rehydrated in ethanol and distilled water (DDW), stained with hematoxylin 3G, destained with DDW and acid alcohol, and then stained with eosin, washed with DDW, and dehydrated in rising concentrations of ethanol and xylene.

For immunohistochemistry staining, each tissue section was deparaffinized in xylene and rehydrated in ethanol and DDW. Antigen retrieval was performed by microwave treatment in citrate buffer (HIER citrate buffer pH = 6; Zytomed Systems, Berlin, Germany) for 15 min. The sections were then incubated overnight at 4°C with 2 μ g/mL antihuman EPX monoclonal antibody (isotype IgG2a, clone: MM25-82.2; kind gift from Dr. J. Lee Lab, Mayo Clinic, Scottsdale, AZ, USA) or 2 μ g/mL mouse anti-human tryptase monoclonal antibody (isotype IgG1a, clone: AA1; Agilent, Cambridge, UK; Dako). For isotype control, some sections were incubated with 2 μ g/mL of either Mouse IgG1 isotype control (eBioscience) or Mouse IgG2 isotype control (Agilent, Cambridge, UK; Dako) antibody.

On the next day, sections were incubated with alkaline-phosphatase (AP) conjugated secondary antibody polymer for 30 min (Zytochem Fast [AP] One-Step Polymer anti-mouse/Rabbit/Rat, Zytomed, Germany). ALK magenta chromogen was then added for 20 min to visualize the reaction (Polydetector ALK magenta,

BioSB, Heidelberg, Germany). Sections were then counterstained with hematoxylin. Following immunostaining, the samples were examined and compared to the corresponding H&E slides in order to ensure the integrity of the tissue architecture following staining. All samples were analyzed manually by 2 observers using an Olympus light microscope. Images were captured using Nikon TL and Japan Light microscope and viewed via NIS elements software as well. The extent of mast cell infiltration in tryptase-mAb stained NP specimens was quantified as the average of positively stained cells corresponding to mast cell infiltration in 5 randomly chosen 400 high-power fields (hpf). Similarly, the extent of eosinophil infiltration in EPX-mAb stained NP specimens was quantified as the average score corresponding to eosinophil infiltration in 5 randomly chosen 400 hpf: score 1 for <10 cells/hpf; score 2 for 10–24 cells/hpf; score 3 for 25–39 cells/hpf; score 4 for 40–54 cells/hpf; score 5 for 55–69 cells/hpf; score 6 for 70–84 cells/hpf; and score 7 for >85 cells/hpf [17] (Table 2 in online suppl. material). For CD48 staining, slides were stained by Dako autostainer after each tissue section was deparaffinized by warming up to 75°C, then incubated in 95°C with Ultra Cell Conditioner for 8 min. Slides were then incubated for 40 min in 37°C with 36 μ g/mL anti-CD48 antibody (Rabbit monoclonal anti-human CD48 antibody; ab134049; Abcam) or 20 μ g/mL isotype control (polyclonal rabbit IgG [AB-105-C; R&D systems]), washed, and counterstained with hematoxylin (Gill II). Stained sections were analyzed manually by 2 independent observers. %CD48⁺ eosinophils were calculated by dividing the number of CD48⁺ eosinophils (in CD48-mAb stained sections) to the number of total infiltrating eosinophils in the H&E consecutive sections using Nikon upright microscope. Three consecutive $\times 400$ hpf were analyzed in each of the specimens: allergy⁻ asthma⁻ (group 1; $n = 4$; 4 M/0 F; 34.7 ± 21.6 years), allergy⁻ asthma⁺ (group 2; $n = 5$; 3 M/2 F; 45.2 ± 18.7 years), allergy⁺ asthma⁻ (group 3; $n = 5$; 3 M/2 F; 41.2 ± 12.9 years), and allergy⁺ asthma⁺ (group 4; $n = 7$; 3 M/4 F; 49.7 ± 10 years).

Isolation of NP Cells and Eosinophil CD48 Surface Receptor Analysis by Flow Cytometry

Cell suspensions from NP tissue were obtained after enzymatic digestion as described previously [21]. Collected cells were resuspended in 10 mL medium (RPMI, 10% FCS), and viability was assessed with trypan blue exclusion test (Trypan Blue, Sigma). Cell suspension was stained with anti-CD48 mAb (BD Pharmingen) or an irrelevant isotype-matched control mAb (DakoCytomation) (1 μ g/mL) or with anti-2B4 (PP35, eBioscience, San Diego, CA, USA) or irrelevant isotype-matched control Ab (1–5 μ g/mL). Eosinophils in the cell suspension were identified with Kimura's staining and as CCR3⁺, SSC high cells using a flow cytometer (BD Biosciences FACSCalibur and CellQuest software) as previously described [13].

Peripheral Blood Eosinophils CD48 Membrane Expression and Serum Soluble CD48 Levels Evaluation

Eosinophils of healthy controls (allergy⁻ asthma⁻) and AR (allergy⁺ asthma⁻) patients were identified in peripheral blood cells by staining with PE anti-human CCR3 (R&D Systems, Minneapolis, MN, USA), or the appropriate isotype FITC mouse IgG1 control (MOPC-21; BioLegend, San Diego, CA, USA), and stained with anti-CD48 mAbs or the relevant isotype control and analyzed by flow cytometry. sCD48 was evaluated in the serum of the same volunteers by a specific ELISA kit [23].

Fig. 1. Immunohistochemical staining of mast cells and eosinophils in NPs. **A** Mast cells were identified by anti-tryptase-mAb staining. Mast cells in allergy⁺ asthma⁺ NPs (group 4); $\times 100$ magnification (**a**); Enlargement of the selected field in (**a**) (blue square); $\times 400$ magnification (**b**). Mast cells in allergy⁻ asthma⁻ NPs (group 1); $\times 100$ magnification (**c**); Enlargement of the selected field in (**c**) (blue square); $\times 400$ magnification (**d**). Yellow arrows show mast

cells. **B** Eosinophils were identified by anti-EPX-mAb staining. Eosinophils in allergy⁺ asthma⁺ NPs (group 4); $\times 100$ magnification (**e**); Enlargement of the selected field in (**e**) (blue square); $\times 400$ magnification (**f**). Eosinophils in allergy⁺ asthma⁻ NPs (group 3); $\times 100$ magnification (**g**); Enlargement of the selected field in (**g**) (blue square); $\times 400$ magnification (**h**). Yellow arrows show eosinophils. H&E, hematoxylin and eosin; NPs, nasal polyps.

Statistical Analyses

Histochemistry and Immunohistochemistry for Mast Cells, Eosinophils, and CD48

All variables in the study are quantitative. The nonparametric Kruskal-Wallis and Mann-Whitney tests were used to detect differences among the study groups. Pearson's correlation was used to examine relations between 2 different variables. Two-way ANOVA analysis was carried out after proving normal distribution using the Kolmogorov-Smirnov test for the relevant variables. SPSS software was used to analyze eosinophils and mast cell infiltration in the NP tissue specimens. Prism V software was used to analyze the % of CD48⁺ eosinophils in the specimens. *p* value of <0.05 was considered statistically significant in all studies.

Isolation of NP Cells and Eosinophil CD48 Surface Receptor Analysis by Flow Cytometry

Using the Prism software, the nonparametric Mann-Whitney test was used to detect differences in surface receptor expression among the study groups. All results are expressed as mean $\pm$ SD.

Peripheral Blood Eosinophils CD48 Membrane Expression and Serum Soluble CD48 Levels Evaluation

Using the Prism software, statistical analyses were performed using the student's paired *t* test. All results are expressed as mean $\pm$ SD for both mCD48 expression analyses on peripheral blood eosinophils and sCD48 levels in the plasma.

Results

NPs Are Associated with Mast Cells and Eosinophils Infiltration

NPs of all the study groups were characterized by mast cell and eosinophil infiltration, particularly evident in the stroma and connective tissue (Fig. 1). Most of the cells looked intact. Interestingly, both mast cells and eosinophils were more commonly observed adjacent to blood vessels in allergy⁺ asthma⁺ and allergy⁺ asthma⁻ NPs in comparison to the other NP patients. Differences among the 4 study groups regarding mast cell infiltration are presented in Figure 2A, a. Kruskal-Wallis test comparing mast cell infiltration among the 4 study groups was significant ($p < 0.001$). Mast cell infiltration in the tissue did not correlate to the total peripheral blood IgE levels obtained before surgery for all the groups ($n = 16$, $p = 0.492$, nonsignificant, data not shown). Asthmatic (groups 2; 4) and nonasthmatic groups (groups 1; 3) (post hoc analyses, Mann-Whitney test) showed no significant differences in the mast cell infiltration between them (Fig. 2A, b; $p = 0.960$). However, significantly elevated mast cell infiltration was found in the allergic groups (groups 3; 4) in

Fig. 2. Comparison of eosinophils and mast cell infiltration in NPs among the study groups. **A** Numbers of mast cells among the 4 study groups. **a** Mast cell infiltration was significantly higher in the NPs of *allergy⁺asthma⁺* patients (group 4) than in the *allergy⁻asthma⁻* patients (group 1) and the *allergy⁻asthma⁺* group (group 2). In addition, mast cells infiltration was significantly higher in the *allergy⁺asthma⁻* group (group 3) than in groups 1 and 2. **b** Numbers of mast cells among asthmatic (groups 2 and 4) and nonasthmatic (groups 1 and 3) patients. **c** Number of mast cells

among allergic (groups 3 and 4) and nonallergic (groups 1 and 2) patients. **B** Number of eosinophils among the 4 study groups. **a** Eosinophils infiltration was significantly higher in the NPs of *allergy⁺asthma⁺* patients (Group 4) than in the *allergy⁻asthma⁻* patients (Group 1). **b** Distribution of eosinophils among asthmatic (Groups 2 and 4) and nonasthmatic (groups 1 and 3) patients in the study. **c** Distribution of eosinophils among allergic (groups 3 and 4) and nonallergic (groups 1 and 2) patients in the study. * $p < 0.05$. H&E, hematoxylin and eosin; NPs, nasal polyps.

comparison to the nonallergic groups (groups 1; 2) as shown in Figure 2A, c (Mann-Whitney test; $p = 0.001$). When the 4 study groups were evaluated in terms of eosinophil infiltration (Fig. 2B, a), significant differences were observed among the groups ($p = 0.044$, Kruskal-Wallis test). Post hoc analyses (Mann-Whitney test) showed significant increase in eosinophil infiltration only in the *allergy⁺asthma⁺* group in comparison to the *control* group ($p = 0.035$). Interestingly, eosinophil infiltration in the tissue did not correlate to the % of peripheral blood eosinophils obtained before surgery ($n = 22$, $p = 0.513$,

data not shown). As found for the mast cells also eosinophils infiltration in the tissue did not correlate to the total peripheral blood IgE levels obtained before surgery ($n = 16$, $p = 0.510$, nonsignificant, data not shown). Eosinophil infiltration in asthmatics groups (groups 2; 4) was significantly higher than nonasthmatic groups (groups 1; 3) (Fig. 2B, b; $p = 0.04$). Similarly, when the patients were divided into allergic and nonallergic groups, a higher eosinophil infiltration was observed in the allergic group (Fig. 2B, c; $p = 0.013$).

Fig. 3. Immunohistochemical analysis of CD48 positive eosinophils in NPs. Eosinophils in *allergy⁻asthma⁻* NP (group 1) were identified by H&E staining; $\times 100$ magnification (**a**); Enlargement of the selected field in (**a**) (blue square); $\times 400$ magnification (**b**); NPs stained with anti-CD48 mAb; $\times 100$ magnification (**c**); Enlargement of the selected field in (**c**) (blue square); $\times 400$ magnification (**d**). Red arrows in (**b**) show CD48 negative eosinophils. Yellow arrows show CD48 positive eosinophils; stained with H&E in

(**b**) and stained with anti-CD48 mAb in (**d**); Comparison of the percentage of CD48 expressing eosinophils among the different groups (**e**). % of CD48 positive eosinophils was calculated by dividing the number of CD48 positive eosinophils (in CD48 – mAb stained sections) to the number of total infiltrating eosinophils in the consecutive H&E sections. For each sample, the average of 3 fields was calculated. * $p < 0.05$; ** $p < 0.01$. H&E, hematoxylin and eosin; NPs, nasal polyps; EOS, eosinophils.

CD48 Is Expressed on Eosinophil Infiltrating NPs, and Its Expression Is Higher in Nonallergic Asthmatic Patients

First, we analyzed CD48 positive staining in slides of NPs belonging to the 4 different groups. Several infiltrating cells were stained by anti-CD48 mAbs in all the study groups. Analysis of CD48 stained sections and the consecutive H&E sections (Fig. 3) allowed us to identify CD48 stained eosinophils out of total eosinophils number. Differences in the percentages of CD48 stained eosinophils among the 4 study groups were found. The percentage of CD48 positive eosinophils out of total eosinophils number was significantly higher in the *allergy⁻asthma⁺* group (group 2; 71.3%) than in the control group (group 1; 50.2%) (Mann-Whitney test, $p = 0.0159$), to the *allergy⁺asthma⁻* group (group 3) (42.2%) (Mann-Whitney test, $p = 0.0079$) and, interestingly, to the *allergy⁺asthma⁺* group (group 4; 40.1%) (Mann-Whitney test, $p = 0.0152$). Of the overall CD48 positive cells, 40.1% were eosinophils from group 1; 71.3% from group 2; 42.2% from group 3; and 50.2% from group 4. As shown in Figure 3e, Kruskal-Wallis was significant

among all the groups ($p = 0.0088$). Examining mCD48 expression on peripheral blood eosinophil in a cohort of allergic rhinitis patients (*allergy⁺asthma⁻*), we interestingly found that they expressed significantly lower levels of mCD48 as compared to healthy donors (*allergy⁻asthma⁻*) (Fig. 1a in online suppl. material). Nevertheless, sCD48 expression in the plasma of *allergy⁺asthma⁻* patients was increased, although not significantly, in comparison to healthy controls ($p > 0.05$) (Fig. 1b in online suppl. material).

To further reconfirm the data that CD48 is prominently expressed on eosinophils of *allergy⁻asthma⁺* in comparison to *allergy⁻asthma⁻* patients, we evaluated mCD48 and its high-affinity ligand m2B4 expression on eosinophils from enzymatically digested NP tissue from these 2 groups of patients (online suppl. Fig. 2). Also, by this methodology, mCD48 was found to be significantly enhanced on eosinophils in *allergy⁻asthma⁺* patients. In addition, and interestingly, we also found that 2B4 expression was significantly increased on the same eosinophils (Fig. 4).

Fig. 4. CD48 and 2B4 receptors expression in NP eosinophils. Flow cytometry analysis of CD48 and 2B4 receptors expression in enzymatically disrupted NP eosinophils from *allergy⁺ asthma⁺* ($n = 5$) and *allergy⁻ asthma⁻* donors ($n = 4$). Cell suspensions were incubated with specific anti-CD48 mAb and anti-2B4 mAb and the appropriate isotype control. Data are shown as the mean fluorescence intensity fold over isotype control $\pm$ SD. NPs, nasal polyps.

Discussion

While the pathogenesis of CRSwNPs is not completely understood, it is evident that mast cell and eosinophil infiltration in NPs plays an important role. This is the first study that investigated the interplay of asthma and allergy etiopathology in CRSwNP patients by specifically studying infiltrating mast cells and eosinophils and the pro-inflammatory marker CD48.

CD48 is a member of the *Signaling Lymphocytic Activation Molecule Family* (SLAMF). More specifically, it is a SLAMF2 member being a glycosylphosphatidylinositol-anchored receptor (and not a transmembrane receptor as in the SLAMF1). In human eosinophils, it has been demonstrated by our group that mCD48 cross-linked with specific antibodies induced Fyn-kinase phosphorylation, which was responsible for the subsequent cell activation. In addition, *S. aureus* exotoxins (SEB, PtA, and PGN) incubation with human eosinophils increases the phosphorylation levels of Fyn in a CD48-dependent manner. In addition, SEB induces a time-dependent release of

sCD48 from human eosinophils due to cleavage of mCD48 by intrinsic phospholipase C and D with no significant change in mCD48 levels because of CD48 de-novo synthesis and the presence of intracellular reservoirs.

2B4, the high-affinity ligand for CD48, is a transcellular receptor composed of an extracellular domain, transmembrane domain, and an intracellular domain containing intracellular tyrosine-based switch motifs, responsible for signal transduction. In human eosinophils, 2B4 binding to its high-affinity ligand CD48, initiates the phosphorylation of the intracellular tyrosine-based switch motifs regions in the 2B4 receptor intracellular portion, leading to SAP recruitment of Fyn, and resulting cell activation [19, 24].

Although our study has a relatively small number of patients in each cohort, by dividing the 27 CRSwNP patients into 4 groups, based on the clinical status of asthma and allergy, we were able to examine closely the correlations of these 2 diseases with mast cell and eosinophil infiltration in NPs, thus gaining further insight into the pathogenesis of CRSwNPs, and eventually, potentially leading to the design of better treatments.

The results of our study show a significant infiltration of mast cells in NPs of allergic patients in comparison to nonallergic patients, with or without asthma. Since asthma does not appear to have an add-on effect on mast cell infiltration in NPs, the primary factor affecting mast cell infiltration in NPs appears to be the presence of clinical allergy. This is highly important for choosing the proper patients for treatments targeting specific components or related components of mast cells.

Eosinophils in NPs were significantly elevated comparing asthmatic (groups 2 and 4) to nonasthmatic (groups 1 and 3) patients and allergic (groups 3 and 4) to nonallergic (groups 1 and 2) patients. However, comparing the 4 study groups, eosinophils were significantly elevated only in patients who were both asthmatic and allergic (*allergy⁺ asthma⁺*) in comparison to the control group (*allergy⁻ asthma⁻*). This suggests that the effect of elevated eosinophils in allergic and in asthmatic patients may be due to a synergistic effect of these diseases on eosinophil infiltration into the NPs.

No correlation was found between the level of mast cell infiltration into NPs to the levels of total IgE in peripheral blood (obtained before surgery), suggesting that the clinical status plays a more powerful role than the levels of blood IgE. Similarly, no correlation was found between the percentage of eosinophils in the peripheral blood (also obtained before surgery) and the levels of eosinophil infiltration into NPs. Moreover, the percentage of eosino-

phils in the peripheral blood (% eosinophils) was not significantly different among the groups, although it was close to reaching significance, with $p = 0.071$, when comparing asthmatic patients to nonasthmatic patients. Similarly, significant differences were not found between total IgE levels in the peripheral blood from the different subgroups. Therefore, total IgE levels and % eosinophils in the peripheral blood do not appear to be reliable biomarkers to differentiate endotypes of CRSwNPs. On the other hand, mast cells and eosinophils infiltration into NPs may be more reliable biomarkers.

Our results suggest that potential preferentially topical treatments targeting mast cells and eosinophils in relevant patients, chosen by clinical characteristics of asthma and allergy and by integrating histopathological studies of tissue infiltration of mast cells and eosinophils in the process of decision-making, may be effective in preventing the development of NPs. Additionally, these treatments may prevent the recurrence of NPs in CRSwNP patients who have already undergone nasal polypectomy. The current study is the first to group and analyze patients according to their clinical asthma and allergy status, thus providing a better way to dissect the effects of asthma and allergy and to study the interactions between them without common confounding factors such as systemic comorbidities and smoking.

We have previously described that CD48 expression on blood and NP-derived eosinophils are increased in asthmatic patients [25]. Our results show that CD48 expression on eosinophils from nonallergic asthmatic patients (*allergy⁻asthma⁺*) is significantly higher than in the other subgroups. In general, CD48 expression on eosinophils was found to be significantly higher in asthmatic patients in comparison to nonasthmatic patients. Moreover, in allergic rhinitis nonasthmatic patients, mCD48 on peripheral blood leukocytes was significantly decreased in comparison to healthy donors as previously described on peripheral blood eosinophils from atopic dermatitis patients in comparison to healthy donors [24]. This indicates that allergy mediators, cytokines, and other factors might decrease CD48 expression. We have previously shown that IL3 can increase CD48 expression on eosinophils but not IL-5 or GM-CSF [25]. Importantly, sCD48 in the serum of these allergic rhinitis nonasthmatic patients was slightly but not significantly elevated in allergic rhinitis patients. On the other hand, we have previously shown that in nonallergic asthmatic patients sCD48 in plasma and mCD48 on peripheral blood eosinophils were both significantly elevated [21]. In light of these findings, we may consider mCD48's expression on peripheral blood eosinophils, and

sCD48 levels in the plasma, as specific markers of asthma and of lung inflammatory diseases. CD48 has also been shown previously to be associated with OMRDL3, a candidate gene for susceptibility in asthma [26, 27]. The increase of CD48 in nonallergic asthmatic NPs was further corroborated with what we found by analyzing flow cytometry membrane CD48 expression on eosinophils in enzymatically digested NPs. Indeed, eosinophils obtained from nonallergic asthmatics expressed higher levels of CD48 and also its high-affinity receptor/ligand 2B4 demonstrating a hyperactive eosinophil phenotype. Finding that eosinophils obtained from nonallergic asthmatics express higher levels of CD48 and 2B4 may suggest a significant role for CD48-2B4 interactions in NPs and asthma. Eosinophils from asthmatics may be, therefore, more prone to activation and thus, may be associated with the bad prognosis seen in asthmatics versus nonasthmatics.

Study limitations include a retrospective design of all the 3 sub-studies. Another limitation is the small sample size of our cohorts. Moreover, assessment of CD48 positive eosinophils was not made in the same tissue section or at the exact tissue level, due to technical limitations. This might have led to bias and less sensitivity in detecting the CD48 positive eosinophils using manual visual quantification of consecutive sections. Another limitation is that we had 3 different cohorts for each of the sub-studies since they were carried out at different time points.

A future study should have a larger sample size and a prospective design. Immunofluorescence should be optimized for NP tissue to co-localize CD48 and eosinophils in the same tissue section for a more sensitive and specific assessment. Histopathological, peripheral blood eosinophils and digested NP eosinophils should be analyzed for each patient.

In conclusion, this study suggests CD48 as a potential target in asthmatic patients suffering from CRSwNPs. It could be regarded as a foundation/starting point for future studies in order to establish a personalized therapy for CRSwNPs.

Acknowledgements

We would like to thank Dr. Elizabeth Jacobsen for providing the anti-EPX antibody and technical support, Dr. Anna Lazutkin for obtaining informed consents from patients, Dr. Nir Pillar for his help in the assessment of the immunohistochemical stains, Prof. Ariel Munitz for his work on digested NPs and analysis, Mrs. Tali Abdulah-Avraham for aiding in the statistical analyses, Dr. Yael Feinstein-Rotkopf and Dr. Zakhariya Manevich for assistance in the light microscopy imaging and analyses, and to Ms. Madelyn Segev for her help in proofreading the manuscript.

Statement of Ethics

The Internal Review Board (Helsinki Committee) of Hadassah Hospital approved the studies, and informed consent was obtained from all patients (HMO-0591-17; HMO-0410-14).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported in part by the Aimwell Charitable Trust (UK), Emalie Gutterman. Memorial Endowed Fund (USA), Israel Science Foundation (Grant No. 442/18), Israel Ministry of Science & Technology, and Rosetrees Trust (UK). F.L.S. is affiliated with the Adolph and Klara Brettler Medical Research Center at the School of Pharmacy of The Hebrew University of Jerusalem.

Author Contributions

Francesca Levi-Schaffer, Ron Eliashar, and Yara Zoabi have conceived and designed the study. Yara Zoabi, Fidan Rahimli, and Yael Minal Fleminger have collected the data, performed the experiments, and performed the analysis. Yara Zoabi, Francesca Levi-Schaffer, Ron Eliashar, and Fidan Rahimli have written the paper.

References

- 1 Fokkens WJ, Lund VJ, Hopkins C, Hellings PW, Kern R, Reitsma S, et al. European position paper on rhinosinusitis and nasal polyps 2020. *Rhinology*. 2020 Feb;58(Suppl S29):1–464.
- 2 Bachert C, Zhang L, Gevaert P. Current and future treatment options for adult chronic rhinosinusitis: focus on nasal polyposis. *J Allergy Clin Immunol*. 2015 Dec;136(6):1431–40.
- 3 Bachert C, Zhang N, Hellings PW, Bousquet J. Endotype-driven care pathways in patients with chronic rhinosinusitis. *J Allergy Clin Immunol*. 2018 May;141(5):1543–51.
- 4 Minai-Fleminger Y, Levi-Schaffer F. Mast cells and eosinophils: the two key effector cells in allergic inflammation. *Inflamm Res*. 2009 Oct;58(10):631–8.
- 5 Hulse KE, Stevens WW, Tan BK, Schleimer RP. Pathogenesis of nasal polyposis. *Clin Exp Allergy*. 2015 Feb;45(2):328–46.
- 6 ICON: chronic rhinosinusitis [World Allergy Organization Journal] Full Text [Internet]. [cited 2021 Feb 21]. Available from: <https://waojournal.biomedcentral.com/articles/10.1186/1939-4551-7-25>.
- 7 Wang X, Zhang N, Bo M, Holtappels G, Zheng M, Lou H, et al. Diversity of TH cytokine profiles in patients with chronic rhinosinusitis: a multicenter study in Europe, Asia, and Oceania. *J Allergy Clin Immunol*. 2016 Nov;138(5):1344–53.
- 8 Wang K, Deng J, Yang M, Chen Y, Chen F, Gao WX, et al. Concordant systemic and local eosinophilia relates to poorer disease control in patients with nasal polyps. *World Allergy Organ J*. 2019 Aug;12(8):100052.
- 9 Sun C, Ouyang H, Luo R. Distinct characteristics of nasal polyps with and without eosinophilia. *Braz J Otorhinolaryngol*. 2017 Feb;83(1):66–72.
- 10 Takabayashi T, Kato A, Peters AT, Suh LA, Carter R, Norton J, et al. Glandular mast cells with distinct phenotype are highly elevated in chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol*. 2012 Aug;130(2):410–e5.
- 11 Hua X, Naselsky WC, Jania CM, Chason KD, Huang JJ, Doerschuk CM, et al. Mast cell deficiency limits the development of chronic rhinosinusitis in mice. *Ann Otol Rhinol Laryngol*. 2016 Apr;125(4):290–6.
- 12 Wang C, Zhang L. Use of biologics in chronic sinusitis with nasal polyps. *Curr Opin Allergy Clin Immunol*. 2019 Aug;19(4):365–72.
- 13 Breuer O, Gangwar RS, Seaf M, Barhoum A, Kerem E, Levi-Schaffer F. Evaluation of soluble CD48 levels in patients with allergic and nonallergic asthma in relation to markers of type 2 and non-type 2 immunity: an Observational Study. *J Immunol Res*. 2018 Sep;2018:e4236263.
- 14 Migalovich-Sheikhet H, Friedman S, Mankuta D, Levi-Schaffer F. Novel identified receptors on mast cells. *Front Immunol*. 2012;3:238.
- 15 Ashqar F, Levi-Schaffer F, Eliashar R. The role of the co-receptors 2B4/CD48 in allergic eosinophilic airway inflammation. *Adv Cell Mol Otolaryngol*. 2015 Jan;3(1):27169.
- 16 Munitz A, Bachelet I, Fraenkel S, Katz G, Mandelboim O, Simon HU, et al. 2B4 (CD244) is expressed and functional on human eosinophils. *J Immunol*. 2005 Jan;174(1):110–8.
- 17 Elishmereni M, Fyhrquist N, Singh Gangwar R, Lehtimäki S, Alenius H, Levi-Schaffer F. Complex 2B4 regulation of mast cells and eosinophils in murine allergic inflammation. *J Invest Dermatol*. 2014 Dec;134(12):2928–37.
- 18 Landolina N, Gangwar RS, Levi-Schaffer F. Mast cells' integrated actions with eosinophils and fibroblasts in allergic inflammation: implications for therapy. *Adv Immunol*. 2015;125:41–85.
- 19 Pahima H, Puzzovio PG, Levi-Schaffer F. 2B4 and CD48: a powerful couple of the immune system. *Clin Immunol*. 2019 Jul;204:64–8.
- 20 Munitz A, Bachelet I, Finkelman FD, Rothenberg ME, Levi-Schaffer F. CD48 is critically involved in allergic eosinophilic airway inflammation. *Am J Respir Crit Care Med*. 2007 May;175(9):911–8.
- 21 Gangwar RS, Minai-Fleminger Y, Seaf M, Gutgold A, Shikotra A, Barber C, et al. CD48 on blood leukocytes and in serum of asthma patients varies with severity. *Allergy*. 2017 Jun;72(6):888–95.
- 22 Stevens WW, Schleimer RP, Kern RC. Chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol Pract*. 2016 Jul–Aug;4(4):565–72.
- 23 Biggs TC, Hayes SM, Harries PG, Allan RN, Walls AF, Pender SLF, et al. Immunological profiling of key inflammatory drivers of nasal polyp formation and growth in chronic rhinosinusitis. *Rhinology*. 2019 Oct;57(5):336–42.
- 24 Minai-Fleminger Y, Gangwar RS, Migalovich-Sheikhet H, Seaf M, Leibovici V, Hollander N, et al. The CD48 receptor mediates *Staphylococcus aureus* human and murine eosinophil activation. *Clin Exp Allergy*. 2014 Nov;44(11):1335–46.
- 25 Munitz A, Bachelet I, Eliashar R, Khodoun M, Finkelman FD, Rothenberg ME, et al. CD48 is an allergen and IL-3-induced activation molecule on eosinophils. *J Immunol*. 2006 Jul;177(1):77–83.
- 26 Schedel M, Michel S, Gaertner VD, Toncheva AA, Depner M, Binia A, et al. Polymorphisms related to ORMDL3 are associated with asthma susceptibility, alterations in transcriptional regulation of ORMDL3, and changes in TH2 cytokine levels. *J Allergy Clin Immunol*. 2015 Oct;136(4):893–e14.
- 27 Ha SG, Ge XN, Bahaie NS, Kang BN, Rao A, Rao SP, et al. ORMDL3 promotes eosinophil trafficking and activation via regulation of integrins and CD48. *Nat Commun*. 2013;4:2479.