

MS. INGE KORTEKAAS KROHN (Orcid ID : 0000-0003-3649-1131)

PROF. CLAUS BACHERT (Orcid ID : 0000-0003-4742-1665)

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PD-1 expression correlates with disease severity and IL-5 in Chronic Rhinosinusitis with Nasal Polyps

Inge Kortekaas Krohn¹, Sonja Bobic¹, James Dooley², Feng Lan³, Nan Zhang³, Claus Bachert³, Brecht Steelant¹, Dominique Bullens^{4, 5}, Adrian Liston², Jan L Ceuppens¹, Sven F Seys¹, Peter W Hellings^{1, 6, 7}

¹ Laboratory of Clinical Immunology, Dpt. Microbiology & Immunology, KU Leuven, Leuven, Belgium

² Translational Immunology Laboratory VIB and KU Leuven, Leuven, Belgium

³ Upper Airway Research Laboratory (URL), Department of Oto-Rhino-Laryngology, University Hospital Ghent, Ghent, Belgium

⁴ Pediatric Immunology, Department of Microbiology and Immunology, KU Leuven, Leuven, Belgium

⁵ Clinical Department of Pediatrics, University Hospitals Leuven, Leuven,

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Belgium

⁶ Clinical division of Otorhinolaryngology, University Hospitals Leuven, Leuven, Belgium

⁷ Academic Medical Centre, Otorhinolaryngology, Amsterdam, The Netherlands

Address for correspondence:

Prof. Dr. Peter W. Hellings

Laboratory of Clinical Immunology

KU Leuven

Herestraat 49, bus 811

Tel.: +32 16 33 23 38

Fax: +32 16 34 60 35

E-mail: Peter.Hellings@med.kuleuven.be

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Abstract

Background: Programmed cell Death 1 (PD-1) is a negative regulator of T cell responses.

Expression of PD-1 and its ligands PD-L1 and PD-L2 in chronic rhinosinusitis with nasal polyps (CRSwNP) is poorly studied.

Methods: Expression of PD-1, PD-L1, PD-L2, TGF- β , IL-5 and IL-10 mRNA was measured by RT-PCR on tissue homogenates of patients with CRSwNP (n=21) and healthy controls (n=21) and on primary epithelial cells. Disease severity was scored using the Lund-Mackay scores of

maxillo-facial CT scans. PD-1 and PD-L1/L2 expression were evaluated at the cellular and tissue level (n=6) by flow cytometry and immunohistochemistry.

Results: PD-1 mRNA expression was increased in tissue homogenates from patients with CRSwNP compared to controls, irrespectively of the atopy status. Importantly, expression of PD-1 correlated with the total CT scan scores ($r=0.5$, $p=0.02$). Additionally, a significant association was found between PD-1 mRNA and expression of IL-5 mRNA in control nasal tissue ($r=0.95$, $p<0.0001$) and in CRSwNP ($r=0.63$, $p=0.002$). PD-1 was expressed on different subsets of T cells and CD11b⁺ dendritic cells. Both PD-1 and its ligands were expressed on primary epithelial cells from control nasal tissue and nasal polyp tissue.

Conclusions: Higher PD-1 expression was found in CRSwNP than in nasal tissue from controls. This was associated with disease severity and tissue IL-5 expression but unrelated to the patients' atopy status.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by inflammation of the nasal mucosa, nasal obstruction and growth of nasal polyps (NP) (1). Nasal polyps are infiltrated with multiple inflammatory cells, such as eosinophils, neutrophils, lymphocytes, innate lymphoid cells (ILC) and mast cells (2,3) There is evidence for a major role of type 2 cytokines in NP disease, as T cell and ILC derived cytokines such as interleukin (IL)-5 are abundant in NP tissue (4). Regulatory T cells (Treg) have potent anti-inflammatory activity via multiple mechanisms, partly via transforming growth factor-beta (TGF- β) production. In CRSwNP, it has been shown that Foxp3 mRNA as well as TGF- β levels are lower compared to

control nasal tissue (5). Decreased infiltration of Tregs and imbalance of the local immune homeostasis might be important for the development of CRSwNP (6). .

Co-stimulatory signaling by the members of the B7 family is required for both T cell activation and inhibition. Signaling through programmed cell death-1 (PD-1 or CD279) via interaction with its ligands B7-H1 (PD-L1) and B7-DC (PD-L2) negatively regulates T lymphocyte activation (7,8). PD-1 is a member of the immunoglobulin superfamily and was first observed in T cells undergoing cell death (9). Several studies have shown that PD-1 plays an important role in immune tolerance and in the regulation of T cell function (10). PD-1 is induced upon T cell activation and PD-1 ligation inhibits T cell receptor (TCR)-initiated events, such as cell proliferation and survival, and cytokine production (7,8,11). PD-L1 and PD-L2 have different expression patterns and different affinity for their receptor (8).

PD-1 is known to be expressed on different immune cells: CD4⁺ and CD8⁺ T cells, B cells, myeloid cells, monocytes (12,13), activated Tregs and mesenchymal stem cells. PD-L1 is mainly expressed on B cells, macrophages and dendritic cells (DC), while PD-L2 is induced upon activation on macrophages and dendritic cells (14).

PD-L1 and PD-L2 are implicated in the modulation of allergic diseases, such as asthma (15,16). PD-1 expression is increased on tissue infiltrating T cells of patients with CRSwNP (17). Local expression of PD-1 and the ligands, PD-L1 and PD-L2 in CRSwNP and how this relates to disease severity remains incompletely understood. Therefore we conducted a study to explore PD-1 expression in CRSwNP and controls at mRNA, tissue and cellular level, and its relation to disease severity and to type 2 and regulatory cytokines.

Materials & methods

Study subjects

Nasal polyp tissue was harvested at the time of endonasal sinus surgery from 21 patients with CRSwNP (median age 51 (40-60)). Diagnosis was confirmed, according to the EPOS criteria (1). Unilateral NP disease, cystic fibrosis, primary ciliary dyskinesia, nasal malignancies, or smoking were exclusion criteria. Control nasal tissue samples were taken from 21 subjects (median age 31 years (25.5-40.5)) who underwent septal or turbinate surgery for functional and/or esthetic reasons. Patient's characteristics are listed in Table 1. Atopy was defined as having at least one positive skin prick test in a panel of 18 most common inhalant allergens (HAL Allergy, Leiden, The Netherlands). Asthma was clinically diagnosed according to GINA guidelines, and based on previous or current proof of reversibility of FEV1 $\geq$ 12% after inhalation of salbutamol or positive histamine provocation test. Aspirin-induced asthma (AIA) syndrome was clinically confirmed asthma with history of aspirin intolerance. The NP size, which is an evaluation of disease severity, was assessed by scoring computed tomography (CT) scans. CT scanning was performed before surgery. All subjects gave written informed consent. The study was approved by the Ethics Committee of the Catholic University Hospitals of Leuven.

Real-time quantitative PCR on tissue homogenates

Tissue homogenates were prepared from turbinates of controls and from polyp tissue of patients with CRSwNP. Total RNA was isolated according to the manufacturers' protocol (RNeasy mini kit, Qiagen, Germantown, Md). cDNA was synthesized using Superscript II RT (Invitrogen, Invitrogen, Carlsbad, Calif) and oligo (dT). The protocols for total RNA and real-

time quantitative PCR (RT-qPCR) have been published previously (18,19). A set of primers and probes was designed and optimized for each of the genes PD-1, PD-L1, PD-L2, and the house keeping gene peptidylprolyl isomerase A (PPIA). Primer sequences can be found in Table 1 in the supplementary Methods. The software used to design the primers and TaqMan probes was Primer Express software (Thermo Fisher Scientific, Waltham, MA USA).

Primer and probe sequences for IL-5 (20) IL-10 and TGF- β (21) were previously reported. RT-qPCR (CFX Connect, Bio-Rad, Hercules, CA USA) was used to run the PCR analysis. A standard curve method was used, and normalization of target gene expression was done by calculating the ratio of gene of interest to the reference gene PPIA.

Immunohistochemistry

For immunohistochemistry, nasal polyp tissues (n=4) and control nasal tissues (n=3) were snap-frozen and proceeded as described (22). Tissue sections (10 μ M) were stained with rabbit polyclonal antibodies against hPD-1 (Novus, Littleton, CO USA), hPD-L1 (E1L3N) (Cell Signaling Technology, Danvers, MA USA) or hPD-L2 (Sigma-Aldrich, Saint Louis, MO USA). Donkey anti-rabbit antibodies labeled with alexa fluor 488 were used as a secondary antibody (Thermo Fisher Scientific, Waltham, MA USA). Nuclei were visualized by DAPI (Thermo Fisher Scientific, Waltham, MA USA). Images were acquired with a Zeiss LSM 510 Meta confocal microscope.

Primary epithelial cell cultures

For investigation of PD-1, PD-L1 and PD-L2 mRNA expression on primary human epithelial cells, additional endonasal sinus surgery tissues were included. Primary epithelial cells were isolated from nasal inferior turbinates (n=8) or nasal polyps (n=5) and cultured on transwell inserts as previously described (23). Briefly, the cells were isolated by pronase treatment (1 mg/ml) overnight at 4°C and two negative selection procedures, and cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco, CA, USA) supplemented with 2% Ultrosor G (Sigma-Aldrich, Saint Louis, MO USA) and penicillin/streptomycin (200 mg/ml) in liquid-liquid interface for 7 days. Then, the culture medium was removed apically, and the cells were cultured in air-liquid interface for another two weeks. The epithelial cells were collected and total RNA was isolated according to the manufacturers' protocol (RNeasy mini kit, Qiagen, Germantown, Md). cDNA was synthesized using the iScript Advanced cDNA-synthesis Kit (Bio-Rad, Hercules, CA USA). The target product was detected by Sso Advanced SYBR Green Super mix (Bio-Rad, Hercules, CA USA) on the LightCycler 480 Instrument II (Roche Applied Science, Penzberg, Germany). The software qBASE was used to design the house keeping genes *ACTB*, *EF-1* and *SDHA* (Supplementary Methods Table 1) and the gene expression was normalized according to a previously described protocol (24).

Flow cytometry immunophenotyping

Multi-color flow cytometry was used to evaluate PD-1, PD-L1 and PD-L2 expression on lymphoid and myeloid cells from nasal polyp tissues (n=3) and control nasal tissues (n=3). Briefly, fresh nasal polyp tissues were cut into smaller pieces and treated with digestion

medium (2.5 mg/mL collagenase D (Roche, Basel, Switzerland) in RPMI medium (Gibco, Thermo Fisher Scientific, Waltham, MA USA) with 1% FCS) at 37°C for 30 minutes. The cell suspension was passed through a cell strainer (70 µm, Greiner Bio-one, Kremsmünster Austria). Conjugated murine monoclonal antibodies were used in two multi-color panels and incubated in the dark at 4°C for 30 minutes. A viability staining was performed using Zombie Yellow (Biolegend, San Diego, CA USA). Data were acquired on a LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA USA) and analyzed by Flowjo V10. The following panels were used:

Panel 1: allophycocyanin eFluor 780 (APCeFl780)-conjugated anti-hCD45 (eBioscience, San Diego, CA USA), fluorescein isothiocyanate (FITC)-conjugated anti-hCD3 (BD, Franklin Lakes, NJ USA), Alexa fluor 700 (AF700)-conjugated anti-hCD4 (eBioscience, San Diego, CA USA), Brilliant violet 650-conjugated anti-hCD25 (BD, Franklin Lakes, NJ USA), Alexa fluor 647 (AF647)-conjugated anti-hCD127 (Biolegend, San Diego, CA USA), phycoerythrin (PE)-conjugated anti-hCCR3 (Biolegend, San Diego, CA USA), peridinin chlorophyll protein/Cyanine5.5 (PerCP/Cy5.5)-conjugated anti-hCCR5 (Biolegend, San Diego, CA USA).

Panel 2: APCeFl780-conjugated anti-hCD45 (eBioscience, San Diego, CA USA), phycoerythrin/Cyanine7-conjugated anti-hHLA-DR (eBioscience, San Diego, CA USA), BV650-conjugated anti-hCD16 (BD, Franklin Lakes, NJ USA), AF700-conjugated anti-hCD14 (eBioscience, San Diego, CA USA), AF647-conjugated anti-hCD11c (Biolegend, San Diego, CA USA), PerCP/Cy5.5-conjugated anti-hCD11b (Biolegend, San Diego, CA USA), FITC-conjugated anti-hCD20 (BD).

For evaluation of PD-1 or PD-L1/L2 expression, Brilliant Violet 421 (BV421)-conjugated anti-hPD-1 (Biolegend, San Diego, CA USA), eFluor 450-conjugated anti-hPD-L1 (Biolegend, San Diego, CA USA), or BV421-conjugated anti-hPD-L2 (BD, Franklin Lakes, NJ USA) were added to both panels.

Radiological evaluation of disease severity

Sinus opacity was scored radiologically by use of the Lund-Mackay score on maxillo-facial CT scans, which enabled to evaluate disease severity and NP size. The Lund-Mackay score is a score system used to quantify the severity of the disease (25).

Statistical analyses

Statistical analysis was performed using Graphpad Prism 5 (La Jolla, CA USA). Differences between 2 groups were analyzed using two-tailed unpaired t-test or Mann–Whitney U test. Data are presented as median with interquartile range (IQR). Spearman's rank correlation coefficient was used to determine correlations when appropriate. Values were considered significantly different when $p < 0.05$.

Results

PD-1, PD-L1 and PD-L2 mRNA expression in nasal polyp tissue

In CRSwNP, PD-1 mRNA expression was significantly higher compared with control nasal tissue ($p < 0.001$, Figure 1). The mRNA expression levels of the ligands PD-L1 and PD-L2 were significantly lower in NP tissue compared with those in control nasal tissue ($p < 0.050$ and $p < 0.01$, respectively) (Figure 1A). The ratios of PD-1/PD-L1 (193.1 ± 60.9) and PD-1/PD-L2 (164.3 ± 50.6) in CRSwNP were significantly higher compared to control nasal tissue (7.1 ± 2.9 , $p < 0.001$ and 6.4 ± 2.9 , $p < 0.001$, respectively).

The use of intranasal steroids by patients with CRSwNP or by control subjects had no effect on the mRNA expression levels of PD-1 ($p = 0.39$ or $p = 0.46$, respectively, data not shown).

Additionally, PD-1 expression was increased in patients with CRSwNP compared to controls, irrespective of the presence of atopy (Supplementary Figure 1A). The presence of asthma (six of eight subjects with asthma were diagnosed with Aspirin-induced asthma (AIA)) tends to be associated with higher PD-1 expression (Supplementary Figure 1B).

Immunohistochemistry

To identify the cells expressing PD-1, PD-L1 and PD-L2, immunohistochemical stainings were performed on freshly frozen NP ($n = 4$) and control nasal tissues ($n = 3$) (Figure 2A). PD-1 expressing cells were found in the submucosa of NP tissue, whereas only few immune cells showed PD-1 expression in control nasal tissue. Additionally, PD-1 expression was found on epithelial cells of both NP tissue and control nasal tissue. Only few PD-L1 and PD-L2

expressing cells were found in the submucosa of NP tissue and control nasal tissue. Besides, PD-L1 and PD-L2 expression was seen in epithelial cells of both NP tissue and control tissue.

The epithelial layer in nasal polyp tissue was disturbed compared to healthy nasal tissue.

mRNA expression in primary epithelial cells

To confirm the expression of PD-1, PD-L1 and PD-L2 on epithelial cells, mRNA was extracted from cultures of primary epithelial cells from healthy nasal inferior turbinate's (n=8) and from nasal polyps (n=5).

mRNA for PD-1 was detected in both cultured epithelial cells from NP tissue and healthy nasal tissue (p=0.5095, Figure 2B). The mRNA levels of PD-L1 and PD-L2 in epithelial cells from NP tissue were increased compared to those of healthy tissue (p=0.0295 and p=0.0186, respectively) (Figure 2B).

Expression of PD-1 and its ligands on different immune cells in NP and control tissue

To further evaluate the PD-1, PD-L1 or PD-L2 expressing cells in the submucosa of NP tissue (n=3) or control nasal tissue (n=3), tissue was homogenized and the suspension of inflammatory cells were prepared and analyzed by multicolor flow cytometry panels. The proportion of T cell, B cell and DC subsets among CD45⁺ cells is shown in Figure 3A. The proportion of PD-1, PD-L1 and PD-L2 expressing cells is shown in Figure 3B. PD-1 was expressed on CD4⁺ T cells and CD4⁻ cytotoxic T cells.

Different T cell subsets were identified based on membrane marker expression: T helper (Th)1 cells (CD45⁺CD4⁺CCR5⁺), Th2 cells (CD45⁺CD4⁺CCR5⁻) and T regulatory cells (Tregs) (CD45⁺CD4⁺CD25⁺CD127^{dim}) (Supplementary Figure 2). PD-1 was expressed on Th1 cells, on Th2 cells, and on Tregs.

We discriminated between B cells (CD45⁺CD20⁺CD11b⁻), CD11b⁺ dendritic cells (CD45⁺CD11b⁺HLADR⁺CD11c⁺CD16⁻) and CD11b⁻ dendritic cells (CD45⁺HLA-DR⁺CD11c⁺CD11b⁻CD16⁻) (Supplementary Figure 2). The percentage of B cells or CD11b⁺ cells expressing PD-1 was low. Interestingly, a higher percentage PD-1 expressing CD11b⁻ DCs were found in NP tissue compared to control tissue (Figure 3B).

TGF- β correlates with PD-L1 and IL-5 correlates with PD-1 mRNA expression

As described in literature (26), we found that the TGF- β mRNA expression was significantly lower in CRSwNP compared to that of controls ($p < 0.05$) (Figure 4A). In CRSwNP, the IL-5 mRNA expression was significantly higher compared to that of controls ($p < 0.01$), and the IL-10 mRNA expression was higher in NP tissue compared to that of controls ($p < 0.0001$, Figure 4A).

We next related the mRNA expression levels of these cytokines to those of PD-1 and its ligands. In NP tissue, TGF- β significantly correlated with PD-L1 mRNA expression (Figure 4B), but not in control tissue. TGF- β and PD-1 mRNA expression may inversely correlate in NP tissue, though our results were not significant ($r = -0.4299$, $p = 0.0518$, data not shown). IL-5 highly correlated with the PD-1 mRNA expression in control nasal tissue and NP tissue (Figure 4B). No correlation was found between IL-10 and PD-1 mRNA expression.

PD-1 mRNA expression correlates with total CT scan scores

Finally, we studied the correlation of PD-1 with disease severity in patients with CRSwNP.

The total CT scan scores significantly correlated with the PD-1 mRNA expression (Figure 5A), but were not associated with TGF- β , IL-5, or IL-10 in nasal polyp tissue (Figure 5B).

Discussion

This study showed the expression of PD-1 and its ligands in NP tissue at the mRNA and the cellular level, and revealed its relevance as a marker for disease severity.

At the mRNA level, we demonstrated a strong up-regulated expression of PD-1 mRNA, and reduced PD-L1 and PD-L2 expressions in NP tissue compared to healthy nasal turbinate tissue (Figure 1). The mRNA expression of PD-1 was increased in almost all CRSwNP patients but to a varying degree, which might be explained by the different endotypes of the disease (27). The increase of PD-1 clearly results from the infiltration of PD-1⁺ T cells in NP tissue.

Using immunohistochemistry we confirmed that PD-1⁺ cells were indeed increased in the submucosa of NP tissue compared to control tissue, although, no quantitative analysis was performed. PD-1 expression by epithelial cells is a new finding, but with this study we cannot explain the biological significance, as the expression is also found in some of the controls. This requires further investigation with increased sample size.

Expression of the ligands is detected on the epithelium (Figure 2), which is in line with literature (17,28,29). There is no clear change in ligand-expressing cells in the submucosa of NP tissue compared to healthy control tissue. On the total tissue, there is a decrease in

mRNA of the ligands, and this may be the result of the higher proportion of total mRNA from cells that do not express the ligand, and of a disturbed epithelial layer in NP tissue.

Previous studies reported that close to 85% of all mononuclear cells in the polyps are CD3⁺CD45⁺ T cells, with minor fractions of CD19⁺ B cells or CD56⁺ NK cells (30), and that CD8⁺ cells are more abundant than CD4⁺ cells in NP (2,17). In single cell suspensions of NP tissue and control nasal tissue we confirmed the expression pattern of PD-1 on different subsets of T cells, B cells and CD11b⁺ dendritic cells (12–14) using multi-color flow cytometry (Figure 3B). A clearly higher percentage of CD11b⁺ DCs expressing PD-1 in NP tissue compared to controls was found and needs further investigation due to the limited number of samples used in this study.

The use of INS or atopy had no effects on the expression levels of PD-1 and on the ligands. Interestingly, the increase in PD-1 expression is more pronounced if the patients have asthma. The combination of CRSwNP with asthma may be an expression of increased disease severity and therefore enhance PD-1 expression.

In the present study, TGF- β mRNA levels were decreased in NP tissue compared to control nasal tissue. This finding is in line with the study of Bachert and coworkers who compared nasal polyps with those of controls and showed significantly lower concentrations of TGF- β in polyp tissue supernatants, whereas IL-5 was increased (4). In another study of patients with CRSwNP, TGF-beta 1 protein concentration, TGF-beta RII and TGF-beta RIII mRNA expression were significantly lower compared to controls (26). Also in a study with CRSsNP and CSRwNP patients, higher concentrations of TGF- β 1 were found in CRSsNP compared with CRSwNP (31). However, in literature the findings of TGF- β concentrations in CRSwNP are contradictory. Go et al. demonstrated increased mRNA expression levels for TGF- β in

nasal polyps compared to the nasal mucosa (32), and Yamin and co-authors showed higher TGF- β 1 and downstream TGF- β 1 signaling in CRSwNP due to TGF- β 1 production by primary nasal epithelial cells (33). The chronic inflammation in CRSwNP may result from dysregulation of the anti-inflammatory mechanisms that normally regulate the immune function. Low levels of TGF- β mRNA in nasal polyps tend to indicate a lower capacity for local induction of Tregs.

Variations in IL-5 mRNA expression levels within the CRSwNP group may be explained by the heterogeneous group of patients included in this study. Evidence for a major role of IL-5 in NP has been shown by the use of anti-IL-5 therapy, which achieved a statistically significant reduction in NP size in 12 of 20 patients (34). A highly positive correlation was found between IL-5 and PD-1 mRNA expression, whereas PD-1 tended to be negatively associated with TGF- β mRNA expression, though the latter results were not significant. . The clinical relevance of PD-1 overexpression in CRSwNP is obvious in view of the correlation with IL-5 and with the total CT scan scores (Figure 4A and 5A).

IL-10 mRNA expression was increased in CRSwNP compared to control tissue (Figure 4A). IL-10 may be implicated in CD8⁺ T cell dysfunction during chronic viral infection. A significant up-regulation of IL-10 by antigen presenting cells is seen in persistent viral infections in mice (35,36). Blockade of IL-10R converted a chronic LCMV infection into a rapidly controlled acute viral infection and prevented the functional exhaustion of memory T cells (37). In chronic infection, effector T cells become 'exhausted' T cells or long-term effector T cells, which are memory-like cells that maintain stable PD-1 expression and produce lower levels of inflammatory cytokines (38). On the other hand, McBerry and co-workers showed that PD-1 deficiency increases IL-10 production during chronic infection in mice (39). In the

present study, we did not record the occurrence of previous viral infection. In future studies, it is therefore of interest to study the role of IL-10 in the PD-1/PD-L pathway in the context of infections.

As far as we know, this is the first study that shows the correlation between the clinical data of nasal polyp size with PD-1 expression at mRNA level (Figure 5A). These data suggest that PD-1 expression could be a useful immunologic marker for disease severity in patients with CRSwNP, but further studies with higher population sizes are needed to confirm this. We do not know whether it is a diagnostic marker as CRS without nasal polyps was not studied.

In conclusion, PD-1/PD-L pathway is considered to play an intriguing role in CRSwNP. We report an increased expression of PD-1 in CRSwNP on T cells and on epithelial cells. In NP tissue, TGF- β correlates with PD-L1 mRNA expression, and the increase of PD-1 correlates with IL-5 mRNA and with CT scan scores. The correlation of PD-1 expression with radiologic assessment of the degree of sinus opacity suggests an important role in the pathophysiology of CRSwNP. The increase is more pronounced if the patients have asthma and could be a marker for disease severity. These data warrant further investigation that may help to understand the immunopathophysiology of CRSwNP in a study with a larger cohort of patients and a wide pattern of cytokine expression.

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Author contributions

IKK, SB, FL and JD performed practical experiments and data analysis. IKK wrote the first draft of the article. DB designed the primers for PD-1, PD-L1 and PD-L2. SS, BS, NZ, CB and JD contributed to the interpretation of the data and statistics. AL, JC and PH designed the study. All authors drafted and revised the manuscript and approved the final version.

Conflict of Interest Statement

In relation to this article, I declare that there are no conflicts of interest.

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Figure legends

Figure 1: The relative mRNA expression of PD-1 and the ligands PD-L1 and PD-L2 in tissue homogenates from patients with CRSwNP (n=21) and controls (n=21) after RT-qPCR analysis normalized to the housekeeping gene PPIA. The data show the median and interquartile range. The Mann-Whitney U test was used for statistical comparison. *p < 0.05, **p < 0.01 versus controls.

Figure 2: A; Immunofluorescence staining (DAPI) on 10 µm slides from fresh frozen nasal polyp tissue (NP) and control nasal mucosa (C) performed with polyclonal antibodies against human (h)PD-1, hPD-L1 or hPD-L2. Scale bars represent 200 µm.

B; Relative mRNA expression of PD-1, PD-L1 and PD-L2 after RT-qPCR analysis on primary epithelial cells isolated and cultured from healthy nasal inferior turbinate's (n=8) and from nasal polyps (n=5). The data show the median and interquartile range. The Mann-Whitney U test was used for statistical comparison. *p < 0.05 versus controls.

Figure 3: A; The percentage of T cell subsets, B cells and dendritic cells in single cell suspensions of nasal polyp tissue (NP, n=3) or control nasal mucosa (C, n=3) analyzed by multi-color flow cytometry.**B;** Percentage of PD-1⁺ T cells, B cells and dendritic cells and of PD-L1 and PD-L2 on B cells and dendritic cells in single cell suspensions of nasal polyp tissue (NP, n=3) or control nasal mucosa (C, n=3) analyzed by multi-color flow cytometry.

Figure 4: A; The relative mRNA expression of TGF-β, IL-5 and IL-10 in patients with CRSwNP (n=20) and controls (n=21). The data show the median and interquartile range. The Mann-Whitney U test

was used for statistical comparison. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ versus controls. **B**; Correlation of TGF- β with PD-L1, and of IL-5 with PD-1 mRNA expression in CRSwNP (n=21) and controls (n=21). The Spearman's rank correlation coefficient was used.

Figure 5: Correlation between the total CT scan score (Lund-Mackay) and PD-1, TGF- β , IL-5, and IL-10 mRNA expression in CRSwNP (n=21).. The Spearman's rank correlation coefficient was used for statistical comparison.

Supplementary Table 1: Sequences of primers and probes used for RT-qPCR

Supplementary Figure 1: A; The effect of atopy in patients with CRSwNP or controls on the mRNA levels of PD-1, PD-L1 or PD-L2. **B**; The effect of asthma in patients with CRSwNP on the PD-1 mRNA expression. The data show the median and interquartile range. The Mann-Whitney U test was used for statistical comparison. ** $p < 0.01$ versus controls (A and B).

Supplementary Figure 2: Representative immune cell analysis in flow cytometry. Leukocytes were first gated on a forward scatter (FSC)/side scatter (SSC) (Size). The single cells were gated on a FSC and on SSC dot plot (Single cells). Viable cells were selected (Viability) using a SSC-A and Zombie Yellow dot plot. CD45-positive cells (CD45) were gated. **A**; Panel 1. Fluorescence minus one (Unstained), PD-1, PD-L1, PD-L2 expression was analyzed on CD4+ T-cells, regulatory T cells, Th1, and Th2 cells. **B**; Panel 2. Fluorescence minus one (Unstained), PD-1, PD-L1, PD-L2 expression was analyzed on B cells using the CD20+CD11b- gate, and on dendritic cells using the CD11c+HLA-DR+ gate including gating on CD11b+ and CD11b- cells.

Table 1: Patient characteristics*

	CRSwNP (n=21)	Controls (n=21)
Age (years)	51 (40-60)	33 (27-41)
Gender (M/F)	16/5	10/11
Atopy (yes/no)	6/15	7/14
Asthma (yes/no)	9/12	3/18
AIA (yes/no)	6/15	0/21
Nasal steroids (yes/no)	18/3	11/10
Smoking (yes/no)	0/21	0/21

* Data are given as median and interquartile range

AIA = aspirin intolerant asthma





