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High frequency of macrophages expressing elevated level of CD80, PD-Ls and TLR1 in nasal polyps of CRS patients

Mariusz Kaczmarek[a], Jacek Banaszewski[b], Małgorzata Leszczyńska[b], Małgorzata Łagiedo-Żelazowska[b,*], Aneta Nowicka[b], Angelika Romańska[a], Małgorzata Wierzbicka[b], Grzegorz Dworacki[a]

[a] Department of Immunology, University of Medical Sciences in Poznan, Poland
[b] Department of Otolaryngology and Laryngological Oncology, University of Medical Sciences in Poznan, Poland

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ABSTRACT

Identification of the association between tissue biomarkers, their surrogates in blood and clinical features, could provide new diagnostic tools and facilitate adequate choices of therapeutic interventions for selected patients suffering from CRS. The aim of present study was the assessment of macrophages in the polyp tissue and monocytes in the peripheral blood in the course of CRSwNP, and their functional immunophenotype. We analyzed 31 patients with CRSwNP. Nasal mucosa tissue was obtained via functional endoscopic sinus surgery (FESS). The control group included 10 patients with deviated nasal septum (DNS). Fluorochrome stained cells were proceed to acquisition using FACS Canto flow cytometer, and the results were analyzed using the software FACS Diva. In our study, we observed a significantly higher level of CD80, CD274, CD273 and TLR1 in nasal polyps compared to blood samples from patients with CRSwNP. This finding may suggest the importance of the PD-1 pathway as a therapeutic target in CRS and an important role for TLR1 in nasal polyp formation and maintenance. Our results may provide some insight into potential future targets of recurrent nasal polyp treatment and contribute to a better understanding of the inflammatory process in Chronic Rhinosinusitis.

1. Introduction

Chronic rhinosinusitis (CRS) is defined by an inflammatory process of the mucosa of the nasal cavity and paranasal sinuses, which manifests following clinical symptoms: nasal obstruction/congestion, nasal discharge, facial pain/pressure and reduction or loss of smell lasting for at least 3 months. Endoscopy or computed tomography is necessary for confirmation of diagnosis (Desrosiers et al., 2011). The worldwide prevalence of CRS significantly increasing. Disease impairs patients' quality of life, also is associated with a high socioeconomic burden (Hastan et al., 2011; Bhattacharyya, 2011; Kim et al., 2011a). CRS term includes the physiologically heterogeneous group of disorders. However, the pathogenesis of CRS remains still unclear. The main classification of CRS is principally based on the presence (CRSwNP) or absence of nasal polyps (CRS without nasal polyps, CRSsNP). These two CRS subtypes also show differences in remodeling and inflammation profiles. The classification of other CRS sub-groups based on particular links with concomitant conditions such as asthma, atopy, aspirin hypersensivity, allergic fungal rhinosinusitis or cystic fibrosis, is necessary to optimize effective treatment. CRS is known to affect patients with co-morbid conditions such as asthma and atopic diseases more frequently than the general population (Hamilos, 2011). Aspirin–exacerbated respiratory disease, known as Samter's triad, which is described as a collection of findings including asthma, aspirin hypersensitivity and nasal polyposis, occurs with higher prevalence in the group of patients with CRS with nasal polyps (CRSwNP) and affects the severity of clinical manifestations (Berges-Gimeno et al., 2002). Published data indicates a variety environmental, genetic and host factors, which have been taken into consideration as a possible CRS reason. Colonization of the mucous membrane covering paranasal sinuses by fungi and bacteria connected with biofilms and superantigens, as well as alterations in mucociliary clearance or defects in the barrier function of mucosa could also play a significant role in nasal polyps formation. It still remains unclear whether they are two different problems, whether subsequent stages of the same disease. Among various etiological aspects, the issue of innate and adaptive immune response seems to be

* Corresponding author at: Department of Otolaryngology and Laryngological Oncology, University of Medical Sciences, Przybyszewskiego Street 49, 60-355 Poznan, Poland.
E-mail address: mlagiedo@ump.edu.pl (M. Łagiedo-Żelazowska).

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significant for nasal polyps formation. The complexity of inflammatory chemokines and cytokines is apparent from the different pathophysiological mechanisms contributing to other CRS subtypes. Moreover, within each subset there are many differences in immune phenotypes proving multifactorial background of disease. Therefore, such diversity results in distinct clinical presentations as well as variability in the degree of recalcitrance to medical and surgical therapy. Several studies have attempted to classify CRS based on inflammatory and immunohistochemical markers. Edematous stromal tissue, albumin accumulation and the formation of pseudocysts are typical features of nasal polyps, whereas CRSsNP is marked by fibrosis chatacterized by excessive collagen deposition and basement membrane thickening (Van Bruaene et al., 2009). With the recent progress in research methods, some paradigms of inflammatory phenotypes in CRS, such as T-Helper polarization, have been established. As one of the major cell types involved in the innate immune response, macrophages play a crucial role as the first-line of defense through the elimination foreign pathogens, while also coordination of the adaptive immune response. These phagocytic cells are essential in maintaining homeostasis by supporting tissue development, promoting the inhibition of inflammation through clearing apoptotic cells or necrotic debris, and supporting wound healing and repairing damaged tissue (Gordon, 2003). Under specific physiological and pathological signals, macrophages can exert different immunological processes thanks to their functional plasticity. According to their phenotypes and cytokine production patterns, macrophages can alter their polarization profile towards two opposite states: classically activated macrophages (M1) and alternatively activated macrophages (M2) (Liu et al., 2006). Being involved in the outcome of many diseases, including allergic disorders, chronic inflammation, cancer and infections, macrophages can exert protective and pathogenic activity as well. Therefore, their functional status can influence disease duration and severity. Since the discovery of the ability of macrophages to change their activation states in response to numerous modulators, therapeutic strategies targeting macrophage polarization have emerged as important research aims (Tarique et al., 2015). In light of the current scientific advances including cell therapy, understanding of macrophage biology and phenotypic characterization of the immune microenvironment seems to be necessary now to applicate appropriate treatment in the future. The aim of present study was the assessment of macrophages in the polyp tissue and peripheral blood in the course of CRSwNP, and their functional immunophenotype.

2. Material and methods

2.1. Patients

Patients were recruited from the Department of Otolaryngology – Head and Neck Surgery of Poznan University of Medical Sciences. The local Ethics Committee of Poznan University of Medical Sciences approved the study, and informed consent was obtained from all participants prior to inclusion in the study. We analyzed 31 patients with CRSwNP. Nasal mucosa tissue was obtained via functional endoscopic sinus surgery (FESS) (Fig. 1). The control group included 10 patients with deviated nasal septum (DNS). Sinus mucosal biopsies were taken from middle meatus of patients with CRSwNP and DNS. Patients who were treated with oral steroids during last 30 days, antifungal medications or immunotherapy, were excluded from the study.

2.2. Immunophenotypic assessment of monocytes/macrophages

Tissue material and peripheral blood (EDTA) samples were transported to the Department of Immunology, Poznan University of Medical Sciences in sealed containers at 2–8 °C within 24 h of collection. Blood samples were analyzed as received, while the nasal tissue samples were mechanically triturated, washed 3 times with saline, and centrifuged, then the supernatant was removed and the cells were resuspended in 1 ml of saline. Materials were incubated with antibodies for 15 min in the dark. The antibodies used are listed in Table 1. Samples without added antibodies were used as negative controls. Next, erythrocyte residues were lysed using lysing solution (BD Bioscence). Finally, all lysed residues of erythrocytes, soluble proteins, and unbound antibodies were washed-out by centrifugation. Fluorochrome stained cells were proceed to acquisition using FACS Canto flow cytometer (BD Biosciences), and the results were analyzed using the software FACS Diva (BD Biosciences). The percentage of positive cells and mean fluorescent intensity (MFI) were assessed.

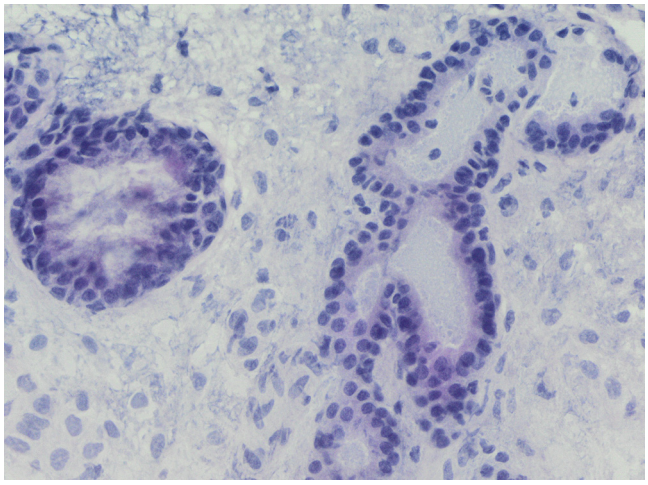

Fig. 1. Nasal polyp tissue. H + E, 400x.

Table 1
The antibodies used in flow cytometric assessment.

Flow cytometry panel for immunophenotyping of monocytes/macrophages population					
Fluorochromes and Antibodies					
	PerCP	**APC**	**APC-Cy7**	**PE**	**FITC**
1	HLA-DR	CD14	CD45	CD80	CD86
2	HLA-DR	CD14	CD45	CD273	CD274
3	HLA-DR	CD14	CD45	TLR1	–
4	HLA-DR	CD14	CD45	TLR2	–
5	HLA-DR	CD14	CD45	TLR4	–

2.3. Statistical analysis

For parametric data, differences between the values were determined using a Student's *t*-test. Grouped data were analyzed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. When the equal variance test failed, a non-parametric Mann– Whitney test was used to evaluate statistical significance. The Wilcoxon matched pairs test was used to analyze differences between blood and nasal tissue. Correlation analysis was performed using Spearman's rank correlation for nonparametric data. Significance was accepted at $P < 0.05$.

3. Results

3.1. Monocytes/Macrophages

The overall percentage of monocyte/macrophage cells was significantly higher in patient's blood samples than in patient's nasal polyps respectively (4.86 ± 2.20, 2.54 ± 1.60, $P < 0.05$). In the blood samples, no statistically significant differences were found between controls (5.06 ± 1.02) and CRS patients (4.86 ± 2.20),

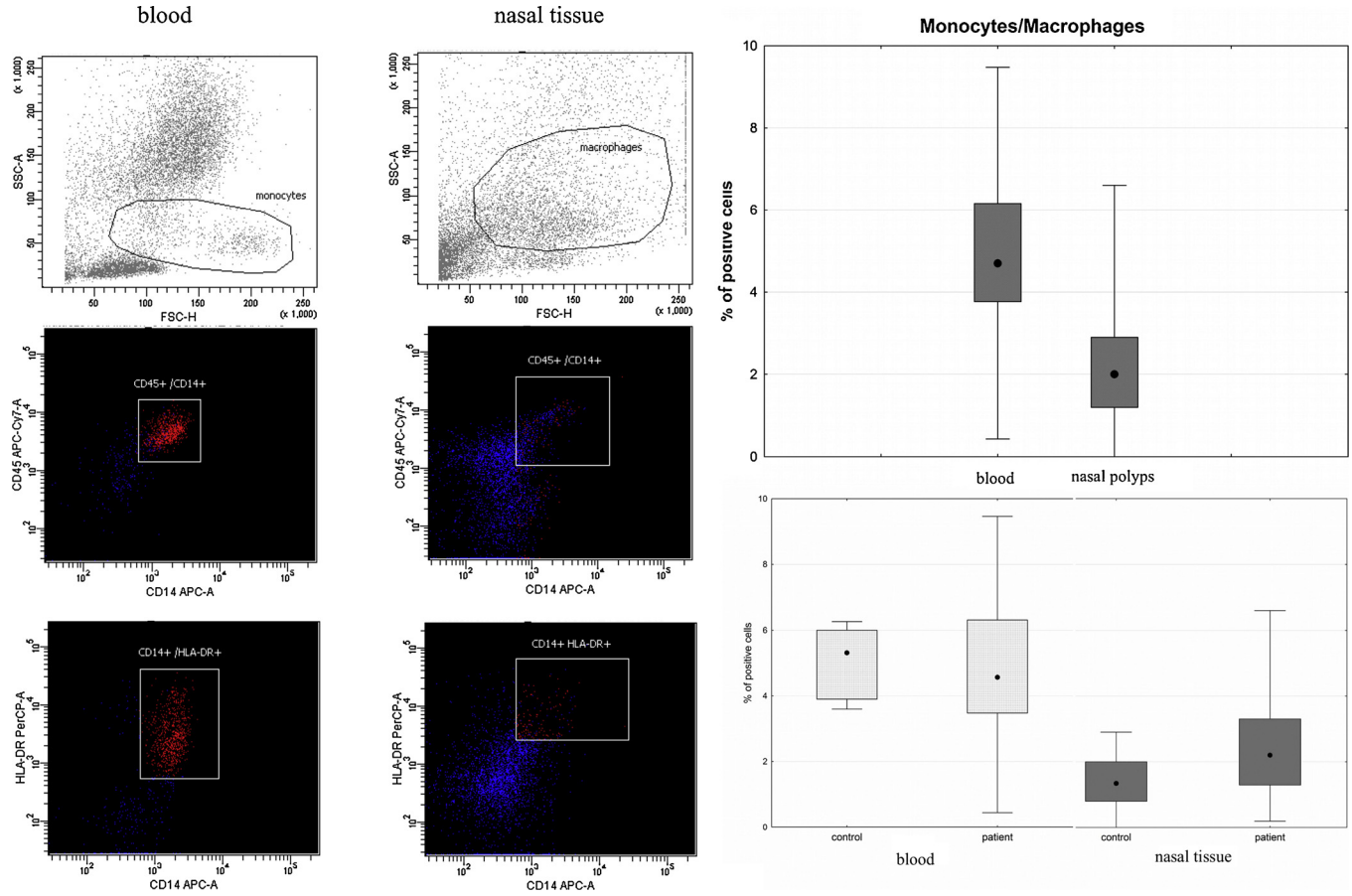

Fig. 2. Flow cytometric assessment of monocytes and macrophages in blood and nasal tissue from CRS patients.

P = .514. In the nasal tissue, the percentage of monocytes/macrophages was significantly higher in CRS patients (1.44 ± 0.87) as compared to controls groups (2.54 ± 1.60), P = .046; Fig. 2.

3.2. CD80/CD86

We observed a significantly higher level of monocytes with CD86 expression than CD80 in patient's blood respectively (91.65 ± 10.45, 9.70 ± 14.20, P < 0.05) and patient's nasal polyps respectively (85.03 ± 11.48, 51.05 ± 27.74, P < 0.05). No statistically significant differences in the CD80/CD86 levels were found between the control and CRS patient groups (P > 0.05). CD80 showed a significantly higher expression in patient's nasal polyps than in patient's blood respectively (51.05 ± 27.74, 9.70 ± 14.20, P < 0.05; Fig. 3.

3.3. CD274/CD273

CD274 (PD-L1) expression was significantly higher in the patient's nasal polyp tissue than in patient's blood respectively (72.18 ± 20.08, 10.24 ± 11.83, P < 0.05), but there was no significant difference between the control (P = .186) and patient groups (P = .638). CD273 (PD-L2) expression was also significantly higher in the patient's nasal polyp tissue than in patient's blood respectively (63.35 ± 25.88, 17.26 ± 24.61 P < 0.05) but the difference between the control (P = .439) and patient groups was not significant (P = .456) Fig. 4.

3.4. TLR 1, TLR2, TLR4

We detected significant differences in the expression of TLR1 between patient's blood and patient's nasal polyps respectively (61.80 ± 14.00, 80.92 ± 12.40, P < 0.05). However, there were no significant differences between the control (P = .632) and patient groups (P = .774). In contrast, the expression of TLR2 (84.69 ± 8.43) and TLR4 (67.73 ± 19.54) was lower in blood samples from the patients group as compared to controls (TLR2: 92.42 ± 7.51, P = .009; TLR4: 82.88 ± 14.28, P = .020). No significant differences between TLR2 and TLR4 expression were observed in nasal polyps samples respectively (P = .230, P = .187) Fig. 5.

4. Discussion

The search for risk factors that contribute to CRS development has attracted the attention of an increasing number of scientists. The influence of occupational exposure to dust or poisonous gas, a pet (Wen-Xiang et al., 2016), smoking, obesity (Ahn et al., 2016), male sex, influenza vaccination, septal deviation and persistent allergic rhinitis (Kim et al., 2011b) has been discussed, but no single risk factor has been identified that can entirely explain the origins of the syndrome. Approximately 80% of Caucasian patients (Fokkens et al., 2012) and 50% of East Asian patients (Cao et al., 2009) suffering from CRSwNP present eosinophil infiltration along with type 2 inflammation and elevated levels of type 2 cytokines, such as IL-5 and IL-13 (Min et al., 1999). Increased levels of mast cells and basophils have also been reported in patients with CRSwNP compared to healthy controls (Mahdavinia et al., 2014; Takabayashi et al., 2012). In Asians, nasal polyps express a predominant TH1/TH17 polarization with a more neutrophilic inflammation (Zhang et al., 2008). In CRSsNP, T cell patterns are similar in Caucasian and Asian patients and the vast majority of patients show a TH1–skewed inflammatory response with active TGF-β1 signaling (Li et al., 2010). The identification of specific inflammatory cell profiles is a challenging task in the comprehensive understanding of the etiology of CRS. Novel evidence can be used to

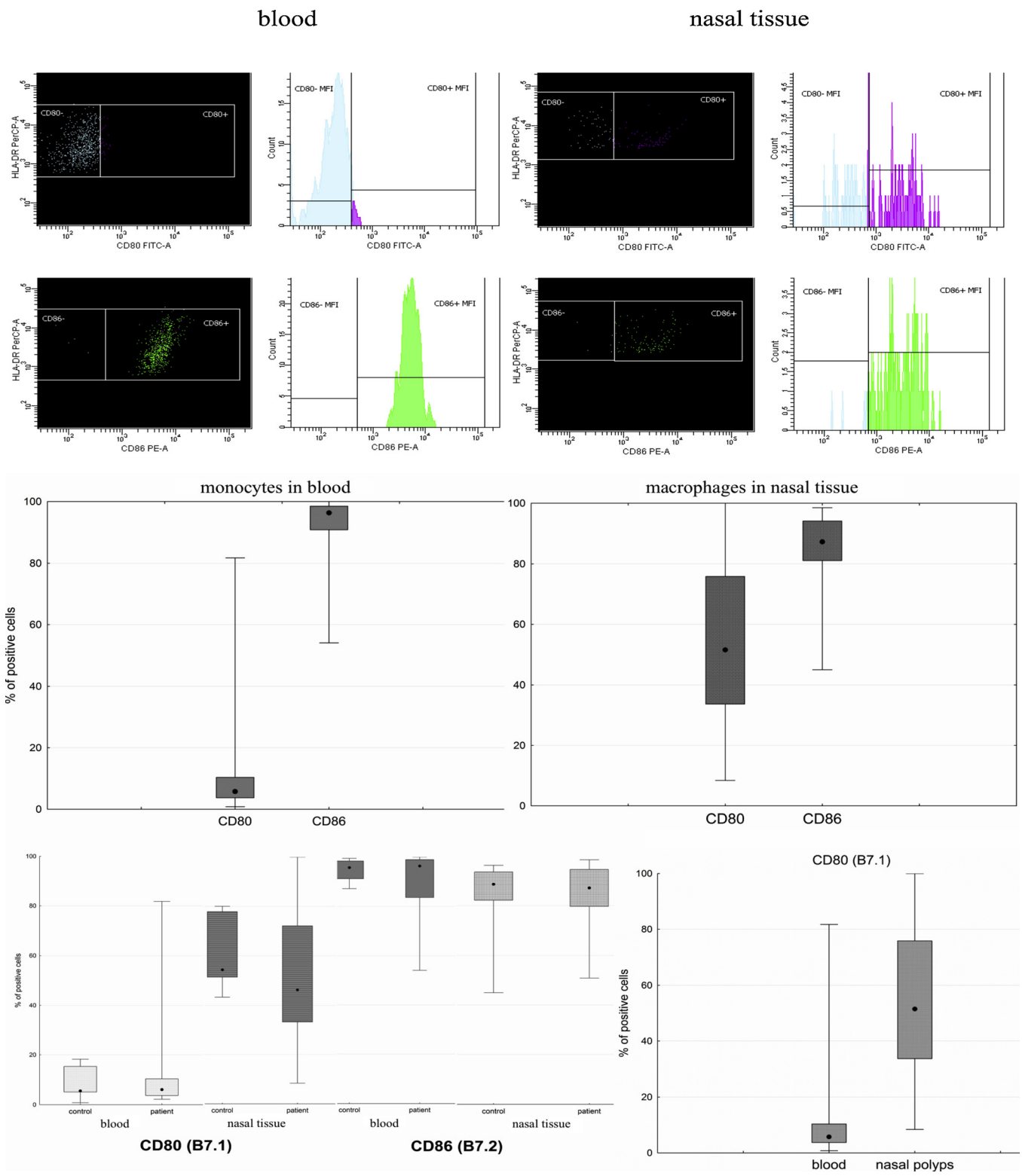

Fig. 3. Flow cytometric assessment of CD80 and CD86 in blood and nasal tissue from CRS patients.

enhance diagnosis and the application of individualized therapy. In recent years, many studies have examined the relationship between macrophages and CRS. In particular, M2 type macrophages are responsible for the induction and perpetuation of nasal polyps (NPs) (Krysko et al., 2011), probably through factor XIIIA production (Takabayashi et al., 2013). M2 macrophages are thought to play a role in the promotion of tissue remodeling by producing extracellular matrix (ECM) components and angiogenic factors, promoting wound healing participating in tumor progression (Rigamonti et al., 2014). Factor XIIIA, which is responsible for forming cross-links between fibrin molecules, enhances the main component of the NP. It also plays an essential role in angiogenesis (Lauer et al., 2002), as well as phagocytosis (Törőcsik et al., 2005). Another important function of macrophages is their ability to regulate the immune environment through the secretion

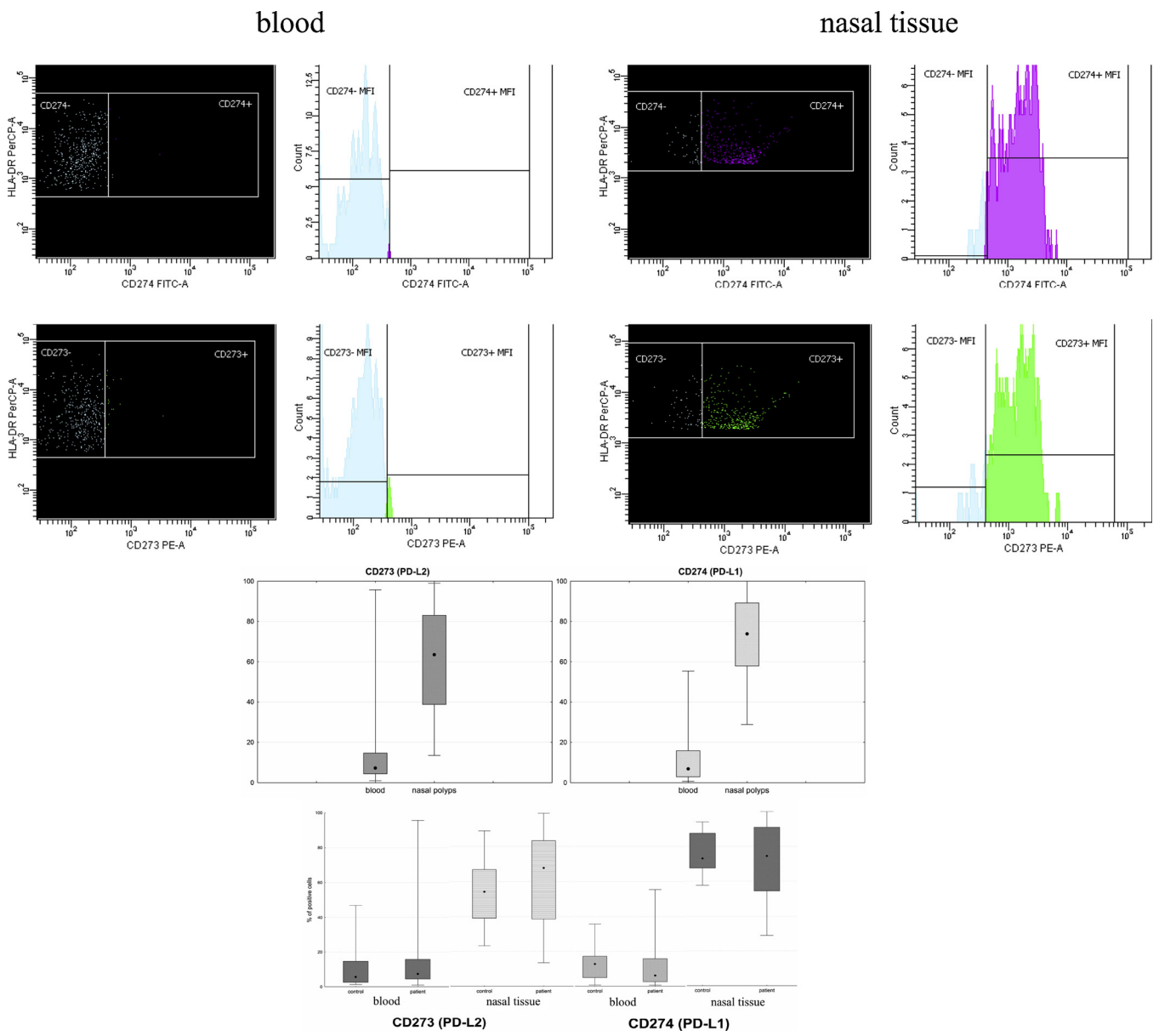

Fig. 4. Flow cytometric assessment of CD273 and CD274 in blood and nasal tissue from CRS patients.

of cytokines and chemokine mediators. Furthermore, some theories suggest that a positive feedback loop may exist for eotaxin production, resulting in the development of a Th2 response. Macrophages may influence the inflammatory response in many ways and contribute to its chronic course. M1 macrophages are induced to differentiate by intracellular pathogens, bacterial cell wall components and products like lipoproteins and inflammatory cytokines such as interferon gamma (IFN-γ) or tumor necrosis factor alpha (TNF-α) (Biswas and Mantovani, 2010). As potent effector cells against microorganisms with tumoricidal activity, M1 macrophages have a pro-inflammatory phenotype and induce the T helper 1 (Th1) immune response. M1 macrophages express opsonic receptors, most TLRs and inducible NO synthase (iNOS) and secrete IL-1β, IL-6, IL-12, TNF-α. Fungal cells, parasites, agonists of TLRs or IL-1 receptors, complements, immune complexes, apoptotic cells, macrophage colony stimulating factor (MCSF), IL-4, IL-13, IL-21, IL-10, tumor growth factor beta (TGF) or glucocorticoids are thought to be involved in M2 subpopulation polarization (Martinez et al., 2009; Murray et al., 2014). In the current literature, M2 macrophages are widely considered to be anti-inflammatory, trophic or regulatory cells. High phenotypic heterogeneity among M2 macrophages, which results from a wide spectrum of stimuli, causes that classification scheme for non-classically activation has been proposed (Martinez and Gordon, 2014). High levels of scavenger, mannose and galactose receptors characterize M2 cells. In contrast to M1 macrophages, arginine metabolism is shifted to the generation of polyamines and proline in M2 macrophages (Martinez et al., 2006). As the M1–M2 paradigm mirrors the polarization of T helper cells, alternatively activated macrophages propagate a Th2 profile and contribute to the recruitment of eosinophils (Mantovani et al., 2004). These cells are associated with low IL-12 and high IL-10 expression (Mantovani et al., 2002). One of the possible cause of CRS seems to be impaired elimination of Staphylococcus aureus or Alternaria from the nasal mucosa membrane, which may be the result of anti-inflammatory M2 macrophages effect by IL-4 (Varin et al., 2010) or the low-level of IFNγ in CRSwNP, which contributes to the survival of these pathogens (Kubica et al., 2008). Moreover, the fibronectin released by macrophages may be involved in bacterial adhesion to nasal mucosa membrane (Schwarz-Linek et al., 2004). In our study, we observed a statistically significant difference between the numbers of macrophages in nasal polyps compared to control nasal tissue. Increased numbers of macrophages in the polyp area may

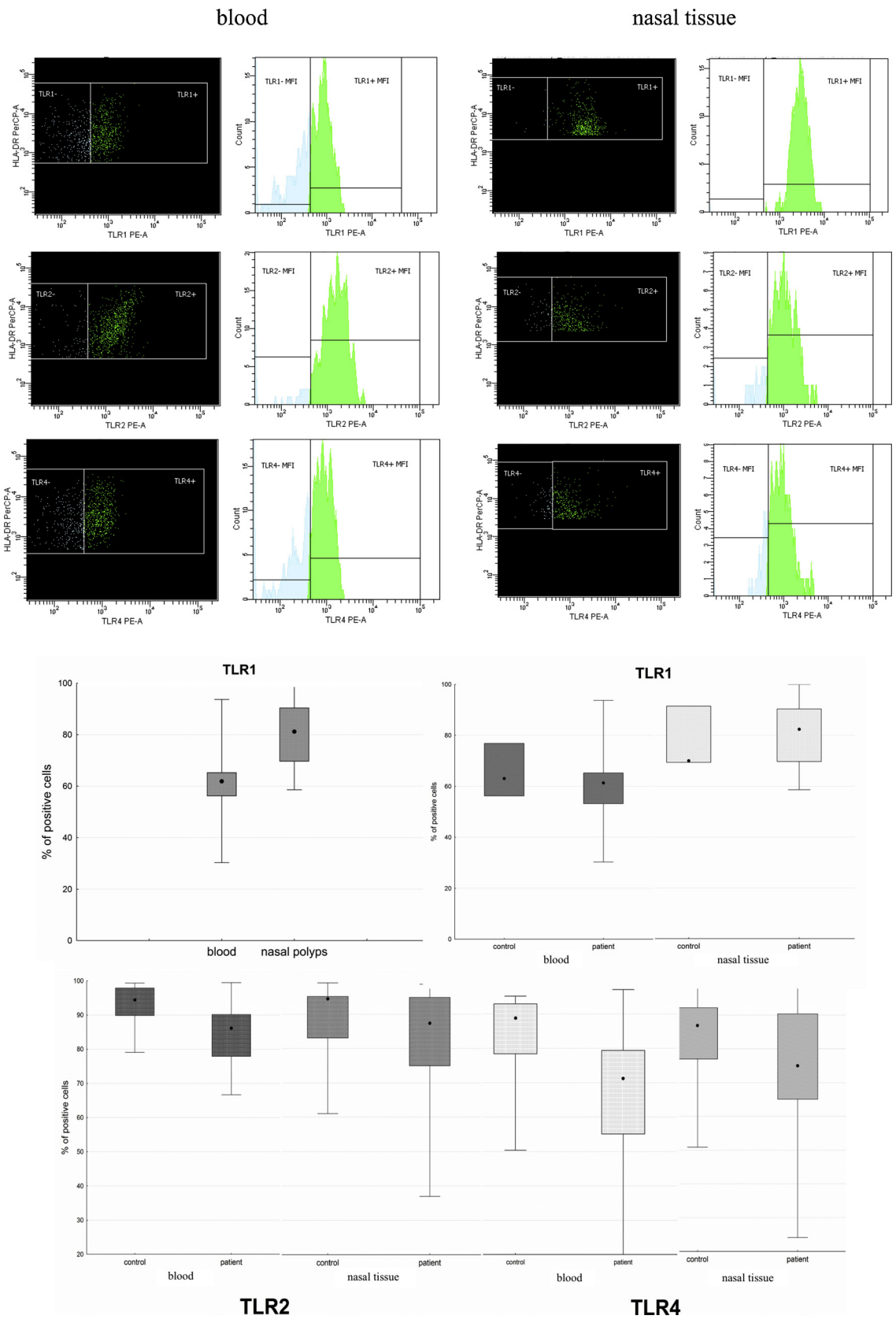

Fig. 5. Flow cytometric assessment of TLR1, TLR2 and TLR4 in blood and nasal tissue from CRS patients.

Table 2
Expression and Mean Fluorescence Intensity of analyzed particles in blood and nasal tissue of CRS patients.

Parameter	Blood			Nasal polyps		
	Patient n = 31 X ± SD (*M*)	Control n = 10 X ± SD (*M*)	p value	Patient n = 31 X ± SD (*M*)	Control n = 10 X ± SD (*M*)	p value
Monocytes/Macrophages (HLADR/CD14/CD45) – proportion of cells (%)	4.86 ± 2.20 (4.56)	5.06 ± 1.02 (5.31)	0.514	2.54 ± 1.60 (2.20)	1.44 ± 0.87 (1.35)	0.047
CD80 expression – proportion of cells (%)	9.70 ± 14.20 (6.04)	8.16 ± 6.13 (5.55)	0.940	51.05 ± 24.74 (46.20)	61.94 ± 15.59 (54.40)	0.145
CD80 MFI	748.55 ± 377.41 (639.00)	623.50 ± 100.28 (598.00)	0.439	2418.84 ± 1072.46 (2132.00)	2195.33 ± 462.86 (2127.00)	0.871
CD86 expression – proportion of cells (%)	91.65 ± 10.45 (96.34)	94.65 ± 4.16 (95.69)	0.867	85.03 ± 11.48 (87.10)	84.47 ± 15.83 (88.60)	0.821
CD86 MFI	6062.48 ± 7927.40 (4580.00)	4708.50 ± 1325.25 (4892.00)	0.820	3869.42 ± 1694.48 (3487.00)	3547.56 ± 1433.14 (3604.00)	0.538
CD273 expression – proportion of cells (%)	17.26 ± 24.61 (7.44)	13.67 ± 16.75 (5.67)	0.439	63.35 ± 25.88 (67.80)	54.28 ± 23.60 (54.20)	0.456
CD273 MFI	819.55 ± 588.00 (730.00)	675.60 ± 479.00 (569.50)	0.295	2422.42 ± 1061.11 (2164.00)	2080.56 ± 847.59 (1747.00)	0.400
CD274 expression – proportion of cells (%)	10.24 ± 11.83 (6.21)	13.50 ± 10.07 (12.78)	0.187	72.18 ± 20.08 (74.50)	75.92 ± 12.62 (73.10)	0.639
CD274 MFI	829.52 ± 498.40 (653.00)	571.60 ± 200.30 (617.00)	0.154	2241.26 ± 1298.47 (1901.00)	1880.44 ± 342.64 (1738.00)	0.910
TLR1 expression – proportion of cells (%)	61.80 ± 14.00 (61.35)	65.37 ± 10.48 (63.05)	0.632	80.92 ± 12.40 (82.40)	76.97 ± 12.59 (70.00)	0.774
TLR1 MFI	1357.42 ± 578.67 (1216.00)	1142.00 ± 163.34 (1177.00)	0.774	3324.00 ± 1644.13 (2995.00)	2232.33 ± 297.31 (2275.00)	0.251
TLR2 expression – proportion of cells (%)	84.69 ± 8.43 (86.09)	92.42 ± 7.51 (94.40)	0.009	82.07 ± 16.36 (87.40)	88.52 ± 11.90 (94.50)	0.230
TLR2 MFI	2324.94 ± 1279.96 (1813.00)	3306.70 ± 1526.72 (3353.50)	0.054	3015.94 ± 1424.43 (2610.00)	3067.40 ± 1639.96 (2717.00)	0.940
TLR4 expression – proportion of cells (%)	67.73 ± 19.54 (71.24)	82.88 ± 14.28 (88.84)	0.020	74.14 ± 18.19 (75.00)	82.08 ± 14.88 (86.90)	0.187
TLR4 MFI	1364.19 ± 518.31 (1216.00)	1723.60 ± 618.90 (1602.50)	0.078	2919.29 ± 1392.08 (2566.00)	3284.20 ± 2403.40 (2366.50)	0.844

X mean, SD standard deviation, M median.
P < 0,05.

suggest a defective host defense mechanism in the early stage of the disease. The chronological order and relationship between inflammation and tissue remodeling in polyp development is still not clear, but these findings suggest that macrophages are mightily involved in processes of the nasal polyp formation. CD80 and CD86 are proteins found on monocytes and activated B cells that provide a co-stimulatory signal necessary for T cell activation and survival. Both CD80 and CD86 can provide co-stimulation leading to T cell proliferation and IL-2 production (Levine et al., 1995). CD80 and/or CD86 are also important in allergen–induced secretion of interleukin (IL)-5 and IL13 (Jaffar et al., 1999). CD80 and CD86 are able to co-stimulate IL-4, IL-5 and IFN-γ production in a murine model of allergic pulmonary inflammation (Mark et al., 1998). Regulation of the expression of CD80 and CD86 on monocytes/macrophages may be an important feature of the biology of these molecules with potential implications in self/non-self discrimination. Identification of the mechanisms by which molecular events induce the expression of CD80 and up-regulate the expression of CD86 during the interaction of pathogens with macrophages may explain the development of nasal polyps. In our study, we observed a significantly higher level of CD80 in nasal polyps compared to blood samples from patients with CRS. It is well recognized that cognate signals and certain cytokines can induce or up-regulate the expression of CD80 and CD86 on macrophages. Some studies have demonstrated that the expression of CD80 and up- regulation of the expression of CD86 on human monocytes induced by pathogens, occurred in the absence of significant concentrations of lymphocytes (Subauste et al., 1998). Such high expression of CD80 in nasal polyps could have important implications for the efforts to establish a vaccine against recurrent nasal polyp formation. PD-L1 (B7-H1, CD274) and PD-L2 (B7-H2, B7-DC, CD273) belong to the B7:CD28 proteins family and regulate T cell activation and peripheral tolerance (Keir et al., 2008). The interaction of CD274 and CD273 with their receptor, PD-1, delivers an inhibitory signal to T cells, inhibiting cell proliferation and cytokine production, particularly the production of IL-10 (Sharpe et al., 2007). During chronic viral infection, an impairment of immune function occurs, consisting of a progressive loss of effector T cell responses known as “T cell exhaustion”. The pathway consisting of CD273 and CD274 (PD-1 pathway) has been suggested to play a key role in this T cell exhaustion, as a result of chronic viral infection, and under some conditions, blockade of this pathway is able to restore many T cell functions (Freeman et al., 2006). In our study we observed statistically higher levels of CD274 and CD273 in nasal polyps than in the blood of CRS patients. This finding may suggest the importance of the PD-1 pathway as a therapeutic target in CRS, and such patients are likely to be screened for the existence of viruses in their body tissues. However, further studies are necessary to confirm the functions of PD-L1 and PD-L2 in human macrophages during the development of CRS. Toll-like receptors (TLR) are the most important pattern recognition receptors (PPR), which mediate immune response between innate and adaptive immunology system, and hence their potential role in the development of CRS should not be overlooked. The human TLRs include a family of ten related proteins. TLR deficiency is thought to lead to susceptibility to infection, while abnormal TLR regulation may cause proinflammatory hyperactivation. All of TLRs can be expressed by macrophages. Studies examining the immunological role of TLRs may shed some light on the pathogenesis of CRS. Recent studies have shown that patients with CRSwNP have increased TLR2, TLR3 and TLR4 expression in macrophages (Hirschberg et al., 2016). However, the expression of TLR2 in CRSwNP is somewhat controversial, since one study showed elevated TLR2 expression in patients with CRSwNP (Zhang et al., 2013a) while another study demonstrated lower TLR2 levels in nasal tissue from these patients (Wei et al., 2014). The levels of TLR7 have also been shown to be elevated in CRSwNP (Zhang et al., 2013b). In our study, we observed enhanced expression of TLR1 on the macrophages present in nasal polyps from CRS patients compared to monocytes isolated from blood. In contrast, no differences were observed in the

expression of TLR2 and TLR4 between these two tissues. This may suggest an important role for TLR1 in nasal polyp formation and maintenance. However, we also observed a significantly lower expression of TLR2 and TLR4 on monocytes in the blood of CRS patients as compared to control blood. This difference could contribute to reduced innate immune responses in CRS patients, resulting in the formation of nasal polyps (Table 2).

5. Conclusions

Identification of the association between tissue biomarkers, their surrogates in blood and clinical features could provide new diagnostic tools and facilitate adequate choices of therapeutic interventions for selected patients suffering from CRS. Our results may provide some insight into potential future targets of recurrent nasal polyp treatment and contribute to a better understanding of the inflammatory process in CRS.

Disclosure statement

All authors declare no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years and no other relationships or activities that could appear to have influenced the submitted work.

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Authors declare none conflict of interest.

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