Alteration of blood monocyte subsets in chronic rhinosinusitis with regard to anti-inflammatory 1,8-Cineol treatment*

Christina Polasky, Kristin Loyal, Christian Idel, David Wetterauer, Mathias Heidemann, Karl-Ludwig Bruchhage*, Ralph Pries‡

Department of Otorhinolaryngology, University Hospital of Schleswig-Holstein, Lübeck, Germany

Abstract

Background: Chronic rhinosinusitis (CRS) affects about 10% of the European population causing considerable disease burden. The inflammatory microenvironment is mainly Th2 driven, but the impact of monocytes is still poorly understood. Aim of this study was to comprehensively investigate the composition of circulating monocytes and T cells in CRSwNP and CRSSNP patients, particularly with regard to the therapeutic herbal monoterpene 1,8-Cineol.

Methodology: We analyzed the distribution of CD14 and CD16 classified monocyte subsets and the T-cell subset composition with respect to their PD-1 and PD-L1 expression in the peripheral blood of CRS patients using flow cytometry. Additionally, the M1/M2 like macrophage infiltration in nasal tissue and polyps was examined by immunofluorescence staining.

Results: Data revealed a decrease of classical monocytes accompanied by a significant increase of intermediate CD16+ monocytes in CRSwNP and CRSSNP patients compared to healthy donors. PD-L1 expression on overall monocytes was also significantly increased in CRSwNP and CRSSNP patients. CRS patients with a severe drop of the proportion of classical monocytes showed a significant restoration of this subset in response to two-week 1,8-Cineol treatment.

Conclusions: Our data indicate a CRS-induced shift of peripheral monocyte subsets to more inflammatory phenotypes that might be reversed by the herbal drug 1,8-Cineol.

Key words: chronic rhinosinusitis, monocytes, 1,8-cineol

Introduction

Chronic Rhinosinusitis (CRS) is a common disease worldwide and affecting about 10% of the European population (1, 2). On epidemiologic grounds, different factors have been associated with CRS prevalence, such as air pollution, active cigarette smoking, perennial allergic rhinitis, individual immune barrier dysfunctions or alterations in the eicosanoid pathway (3-5). In addition, various fungi and microbial pathogens such as Staphylococcus aureus are supposed to trigger CRS emergence (6, 7). Chronic rhinosinusitis can occur without (CRSSNP) or with a manifestation of nasal polyps (CRSwNP).

Although our knowledge of the pathogenesis, inflammatory processes as well as the cellular progression of this disease grows permanently, the exact multifactorial mechanisms still remain elusive (8). In fact, the inflammatory milieu of the sinonasal mucosa in CRSSNP patients is characterized by a mixed T helper (Th) Th1/Th2 or a Th1 phenotype, whereas in CRSwNP a Th2 polarization is dominant (8, 9). Th2 cytokines promote rhinorhoea, mucus production and hyperplasia. Monocyte-derived macrophages in the sinunasal tissue can directly and indirectly...
Monocyte alteration in CRS

induce a Th2 cell response, but mechanisms in CRS are still poorly understood (11). Moreover, nasal polyps are also known to be infiltrated by eosinophilic granulocytes (12). Most common therapies of chronic rhinosinusitis comprise administration of corticosteroids or antibiotics and surgery (13). Furthermore, novel biologicals targeting IgE, IL-5 or IL-4 and IL-13 have been introduced recently (14, 15). The anti-inflammatory monoterpene 1,8-Cineol is a natural plant-based therapeutic agent that is commonly applied to treat various chronic and acute airway diseases as well as patients with CRS. It has been shown that 1,8-Cineol is able to significantly reduce the production of proinflammatory mediators such as TNF-α, IL-1β, and IL-6 from monocytes (16, 17) as well as the IL-4 and IL-5 production from lymphocytes (18). There are only few studies on systemic changes in monocyte or T-cell composition in peripheral blood from CRS patients. Circulating monocytes can be subdivided into three subsets with regard to their CD14 and CD16 expression pattern (19-21). CD14++CD16- are referred to as ‘classical’ monocytes and comprise the biggest compartment with about 85% of monocytes. Classical monocytes are the most migrative and phagocytotic cytokine producers among monocyte subsets. CD14+CD16+ “intermediate” monocytes are with 5-10% considered as the superior antigen presenting cells. The remaining 5-10% constitute CD14dim++CD16-“non-classical” monocytes which exert a marked patrolling behaviour. Some studies hypothesised that both CD16+ subsets emerge from the classical monocyte population and thus are more closely related to each other (19), whereas others assume that the non-classical subset is solely different regarding the gene expression profile (22). Although classical monocytes constitute always the major compartment of monocytes in the periphery, an increase of CD16+ monocytes has been described in many chronic inflammatory conditions as persistent viral infections (23), rheumatoid arthritis (24), asthma (25), and obstructive sleep apnea syndrome (26). All monocyte subsets are able to acquire macrophage morphology and characteristics, but the exact differentiation potential of the different subsets to macrophages remains incomplete (27). The polarization of monocytes/macrophages to the classic pro-inflammatory type (M1-like) or the alternative protective type (M2-like) resembles an important factor in inflammatory and neoplastic diseases (28). It remains to be determined comprehensively whether and how the function and phenotypes (M1 vs. M2) of macrophages participate in the CRSwNP pathogenesis. It has been shown that CRS is associated with M2 polarization which propagate a Th2 dominant response (11).

T cells infiltrate and differentiate in inflammatory tissue to competent effector cell subsets depending on the inflammatory milieu and stimuli provided by innate immune cells such as monocytes. Nevertheless, effector, memory and specific cytokine producing helper cell subtypes can be found in blood as well. Following encounter to foreign antigens a single naive T cells is able to generate multiple subsets of effector and memory cells with distinct differentiation states and functionality. Analysis of peripheral T-cell subsets can provide comprehensive information on an individual’s health and disease status, respectively or any immune disturbances (29-31).

Only a few studies have investigated the peripheral changes of monocyte subsets or T-cell composition in chronic rhinosinusitis, not to speak of the therapeutic impact of 1,8-cineol on these cells. Therefore, the aim of this study was to understand the differentiation patterns of circulating monocyte subsets in the peripheral blood of CRS patients, also with regard to the therapeutic administration of 1,8-Cineol. Furthermore, a detailed analysis of the circulating CD4/CD8 T-cell subset composition and expression of activation markers programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) was carried out, since recent studies corroborated alterations of T-cell differentiation within the microenvironment of nasal polyps (32). The study aimed to increase our understanding on immunological changes in these cells and the possible therapeutic benefit of 1,8-Cineol on cellular level in response to treatment of chronic rhinosinusitis.

Materials and methods

Ethics statement

Medical examinations and surgical treatments were carried out at the Department of Otorhinolaryngology, University Hospital Schleswig-Holstein, Campus Lübeck. All patients have given their written informed consent. The study was approved by the local ethics committee of the University of Lübeck (approval number 18-322) and conducted in accordance with the ethical principles for medical research formulated in the WMA Declaration of Helsinki.

Blood collection

Throughout, blood samples were collected from healthy donors (n = 29), CRSwNP patients (n = 57) as well as from CRSSNP patients (n = 19). Subjects were recruited into the study based on the diagnostic criteria of CRS including medical history, physical examination, nasal endoscopy, and computed tomography (CT) scan of the sinuses. In addition, blood samples were collected from patients (n = 26) after 14 days of 1,8-Cineol (CNL-1976) administration. Blood was drawn by venipuncture into a sodium citrate containing S-Monovette (Sarstedt; Nümbrecht, Germany). 1,8-Cineol was used in terms of the clinically approved drug Soledum Kapselforte® (capsules) (Cassella-med GmbH & Co. KG, Cologne, Germany). For therapeutic use patients have been prescribed Soledum capsules (3 x 200 mg/day) over 14 days. All analysed CRS patients had a mean age of 53 (± 15), and all healthy donors had a mean age of 44 (± 15) years.
Staining of monocyte subsets in whole blood
Within 4 h after blood collection, 20 µl of citrate blood was diluted in 80 µl PBS. Blood cells were stained with following antibodies: CD45-PE, CD14-FITC, CD16-BV-510 and HLA-DR-APC-Cy7 (all from Biolegend, San Diego, CA, USA). After 25 min staining in the dark, 650 µl RBC Lysis Buffer (Biolegend) were added to the samples and incubated for another 20 min. Subsequently, suspension was centrifuged at 400 x g for 5 min and supernatant was discarded. Cell pellet was resuspended in 100 µl fresh PBS and ready for FACS analysis.

Staining of T-cell subsets in isolated PBMC
PBMC were isolated from the remaining blood by density gradient centrifugation in Biocoll (Biochrom GmbH, Berlin, Germany) at 400 x g for 20 minutes. The upper PBS/plasma layer was removed and discarded. The PBMC layer was carefully harvested and transferred to a new 15 ml tube and washed once with PBS. The supernatant was discarded again and the PBMC pellet was resuspended in 1 ml PBS. For analysis of T-cell subsets, 100 µl of the cell suspension were incubated with 2 different antibody cocktails: A) CD3-PerCP, CD4-PE-Cy7, CD8-BV-510, PD-1-PE, PD-L1-APC, CD45RA-APC-Cy7 and CCR7-BV421. B) CD3-PerCP, CD4-PE-Cy7, CD8-BV-510, PD-L1-APC, CCR6-APC-Cy7 and CXCR3-BV421. After 25 min staining in the dark, cells were washed with PBS and centrifuged at 400 x g for 5 min. Cell pellets were resuspended in 200 µl PBS and ready for FACS analysis.

FACS analysis
Flow cytometry was performed with a MACSQuant 10 flow cytometer (Miltenyi Biotec, Bergisch-Gladbach, Germany) and data was analysed using the FlowJo software version 10.0 (FlowJo, LLC, Ashland, OR, USA). All antibody titrations and compensations were performed in beforehand. For whole blood measurements, at least 100,000 CD45+ leukocytes were analysed. For T-cell analysis, 100,000 events within the PBMC gate were measured. Gating of monocyte subsets as well as T-cell subsets was performed as described before [26]. In brief, CD45 was used as a pan leukocyte marker to facilitate whole blood measurement and monocytes were first roughly gated by their FSC/SSC characteristics and the positivity for CD14 and CD16. Remaining neutrophil granulocytes, NK cells and B cells were excluded by help of HLA-DR expression, which is specific for monocytes. Remaining real monocytes were then subgated into CD14+CD16- (classical), CD14+CD16+ (intermediate) and CD14-CD16+ (non-classical) monocytes. Lymphocytes were also gated by their FSC/SSC characteristics and T cells were...
Monocyte alteration in CRS

Monocyte alteration in CRS firstly separated by their CD3 and CD4 or CD8 expression, respectively. T-cell differentiation states were analysed using CCR7 and CD45RA isoform. CD4+ T helper Cell subsets were distinguished by help of CXCR3 and CCR6. Additionally, PD-1 and PD-L1 expression was analysed for each subset and presented as Mean Fluorescence Intensity (MFI). Monocytic PD-L1 expression was also assessed by gating the CD4dim+CD3− bigger cells in the PBMC fraction, which resemble monocytes.

Immunofluorescence

For cryostat sectioning, several fresh tissue probes (n = 25) were resected and directly stored at -80°C prior to use. Nasal polyp or inferior turbinate tissues were cryosectioned at 6 µm with Cryostat CM 3050S (Leica Microsystems). Frozen sections were thawed for 10 minutes at room temperature, fixed in cold acetone and washed with PBS and TBS. Sections were blocked (7.5% mixed normal goat- and rabbit-serum in PBS) for 2 hours at room temperature. CD163 (mAb, mouse anti-human, 10D6, Invitrogen) was diluted and slides were incubated overnight at 4°C. Slides were then washed in PBS and secondary antibody (goat F(ab)2 anti-mouse IgG, DyLight® 488, abcam) was incubated for 45 minutes at room temperature. After washing with PBS and TBS, CD68 (XP® rabbit mAb anti-human, D4B9C, labelled with AlexaFluor® 555 conjugate, Cell Signaling) was diluted and incubated overnight at 4°C. To quench autofluorescence Vector® TrueView® Kit (Vector Laboratories) was used according to the protocol on every section. Slides were then coverslipped with DAPI (Vectashield®, Vector Laboratories).

For the exclusion of unspecific binding, control sections were stained only with the polylink secondary antibody. Isotype-matched control antibodies (eBiosciences) were used as negative controls. For semiquantification, positive signals in at least 5 random high-power fields (HPF, 400x) were visualized with Axiosvert 200 M (Zeiss), counted, and expressed as mean percentage of CD68+ or CD163+ macrophages against total macrophages.

Statistical analysis

Statistical analyses were performed with GraphPad Prism Version 7.0f. The mean and standard error (SEM) are presented. The differences between groups were determined after testing for Gaussian distribution (normality tests), and applying parametric (student’s t-Test), or non-parametric 1-way Anova with Bonferroni post hoc test. The correlation between parameters was calculated using multivariate regression with the Pearson correlation coefficient. p < 0.05 (*), p < 0.01 (**), and p < 0.001 (**). Number of samples and additional statistical details are given in the respective figure legends, when appropriate.

Results

Alteration of monocyte subsets in CRS patients

Our data revealed alterations of the abundance of monocyte subsets in CRSwNP and CRSsNP patients compared to healthy donors (HD). Classical monocytes were significantly decreased in CRSwNP as well as CRSsNP patients compared to healthy donors (Figure 2A). Intermediate monocytes showed a significant increase in both patient groups compared to healthy donors (Figure 1A). Furthermore, percentage of non-classical monocytes was positively correlated with the serum levels of IgE in CRSwNP patients (Figure 1B) (r = 0.358; p = 0.048).

The PD-L1 expression of monocytes was also analysed during FACS analysis of PBMCs. Monocytes were gated by their FSC/SSC characteristics and further on by help of their low positivity for CD4 and missing CD3 expression. Our data revealed a significantly higher PD-L1 expression on monocytes of CRSwNP as well as in CRSsNP patients compared to healthy donors (Figure 1C).

Alteration of T-cell subsets in chronic rhinosinusitis patients

The T-cell subset distribution as well as their PD-1 and PD-L1 expression were also analysed in CRSwNP as well as CRSsNP patients compared to healthy donors. The T-cell differentiation

Figure 2. Flow cytometric analysis of CD4+ and CD8+ T-cell subset distribution in PBMC from CRSwNP (n = 38) and CRSsNP (n = 14) patients compared to healthy donors (n = 21). Percentages of naïve, effector, effector memory and central memory T cells within CD4+ or CD8+ T cells as well as of CD4+ T helper cell subsets Th1, Th2 or Th17/22 are shown.
from naïve to effector, effector memory and central memory cells was analysed for CD4+ and CD8+ T cells by specific makers. Moreover, CD4 T helper subsets TH1, TH2, and TH17/22 could be distinguished. The percentages of each subset as well as the PD-1 and PD-L1 expression levels were analysed.

Our data revealed no significant alterations of CD4+ and CD8+ T-cell subset percentages, neither in CRSwNP nor in CRSsNP patients. All analysed patients and healthy donors showed a very comparable and stable distribution of circulating T-cell subsets, unaffected by CRS (Figure 2). In addition, CD4+ and CD8+ T-cell subsets in CRS patients were analysed regarding their PD-1 and PD-L1 expression intensities (MFI). PD-L1 expression was slightly increased on CD4+ and CD8+ T cells from CRS patients whereby big individual differences could be observed (Figure 3). We further questioned whether the distribution of T-cell subsets and their PD-1/PD-L1 expression were influenced in response to 1,8-Cineol treatment (3 x 200 mg/day) over 14 days for therapeutic use. The composition of T-cell subsets showed no differences between the groups in response to 1,8-Cineol treatment (data not shown).

Redistribution of monocyte subsets in response to 1,8-Cineol
We further questioned whether the grade of monocyte subset alteration was affected by therapeutic treatment with anti-inflammatory 1,8-Cineol. Viewing all events, our investigations identified non-significant slight shifts of the abundances of all three monocyte subsets in CRSwNP and CRSsNP patients with strong individual deviations in response to 1,8-Cineol treatment (Figure 4A).

Of note, CRS patients with initially less than 75% classical monocytes showed a significant increase of this subset (p = 0.0087) accompanied by a decrease of the non-classical monocytes (p = 0.038) after 1,8-Cineol treatment (Figure 4B).

In addition, nasal polyp tissue probes before and after 1,8-Cineol treatment were analysed for the distribution of tissue infiltrating M1 and M2 macrophages. Therefore, tissue samples were cryosectioned and analysed by immunofluorescence microscopy using specific primary antibodies for human CD68 and CD163 to recognize M1 and M2 macrophages as described before. Data revealed strong individual deviations, but no significant changes in the abundance of M1/M2 macrophages in response to two-week treatment with 1,8-Cineol (Figure 4C).

Discussion
The present study was undertaken to investigate the impact of chronic rhinosinusitis with (CRSwNP) and without (CRSsNP) nasal polyps on the distribution of circulating monocyte subsets.
Monocyte alteration in CRS

and T-cell immune alterations, distinguishing between CD4+ and CD8+ T cells. Additionally, the expression levels of PD-1 and PD-L1 on the peripheral T cells of CRSwNP and CRSsNP patients and healthy donors were analysed for CD3+CD4+ and the CD3+CD8+ subsets and monocytes using flow cytometry. Furthermore, the M1/M2 macrophage distribution in nasal tissue and polyps was investigated by immunofluorescence staining.

The normal monocyte distribution in peripheral blood under healthy conditions is composed of 85% “classical” CD14++CD16- monocytes, and each 2-10% “intermediate” CD14++CD16+ and “non-classical” CD14dim+CD16+ monocytes (19). Whereas classical monocytes are characterized by phagocytosis, endothelial transmigration and production of pro-inflammatory cytokines, are CD16+ subsets linked to more specialised antigen presentation functions, defence against viruses and patrolling behaviour (33). Under various inflammatory conditions, an increase of CD16+ intermediate and non-classical subsets have been reported (34).

Zagolski and colleagues found that in CRSwNP, peripheral blood leukocytes, neutrophils, monocytes and eosinophils were higher than in CRS without nasal polyps, thus further subtyping of different immune cells was not performed (35). Our study revealed a significant decrease of the percentage of classical monocytes accompanied by increased CD16+ monocyte subsets in the peripheral blood of both CRSwNP as well as CRSsNP patients compared to healthy donors. The decrease of classical monocytes was even more pronounced in CRSsNP patients by tendency. An increase of CD16+ subsets was repeatedly linked to various chronic inflammatory conditions, including asthma (25). About 28% of our patients (21 out of 76) suffer from asthma and 25% (19 out of 76) have also allergies. Unexpectedly, there was no correlation or differences of the monocyte subset distribution between patients with these comorbidities and those without. Our findings indicate that the monocyte subset composition in peripheral blood is primarily affected by the chronic rhinosinusitis itself.

The life cycle and cellular interplay of circulating monocyte subsets has been the subject of several recent studies. Basically, these studies suggest a linear differentiation relationship between the three monocyte subsets. In two studies a sequential enrichment of classical monocytes, then intermediate monocytes and finally non-classical monocytes in the peripheral blood has been shown using in vivo cell labelling with 6,6-2H2-glucose in healthy volunteers (21, 36). Furthermore, classical monocytes reappeared in the blood 7 days after an autologous stem cell transplantation, followed by intermediate monocytes and then non-classical monocytes after 10 days (37). These observations

Figure 4. Flow cytometric analysis of monocyte subsets before (pre) and after (post) 1,8-Cineol treatment. A: Whole blood analysis revealed only slight shifts of the percentages of all three monocyte subsets in CRSwNP (n = 17)/CRSsNP (n = 8) patients with strong individual deviations in response to 1,8-Cineol treatment. B: CRS patients with severe monocyte alterations in peripheral blood (n = 9; less than 75% CM) showed significantly increased percentages of classical monocytes as well as decreased percentages of intermediate monocytes in response to 1,8-Cineol treatment. C: Immunofluorescence stainings of nasal polyps before (pre) and after (post) two-week 1,8-Cineol treatment. Specific primary antibodies for human CD68 and CD163 to recognize M1 and M2 macrophages revealed no significant changes in the abundance of M1/M2 macrophages in response to two-week treatment with 1,8-Cineol.
indicate a differentiation process from classical to CD16\(^+\) monocyte subsets. Our study revealed a reconstitution of classical monocytes after 1,8-Cineol intake in patients with less than 75% classical monocytes pre 1,8-Cineol treatment. Questions remain whether 1,8-Cineol is able to block the differentiation to CD16\(^+\) monocyte subsets or if only those increasingly disappear from circulation and how long the effect remains. It has been shown that circulating classical monocytes have a short lifespan of about 1–2 days, before differentiating into CD14\(^+\)CD16\(^-\) monocytes or migrating into tissue and differentiate into macrophages (21, 36, 38). These data suggest a short-term restorative influence of anti-inflammatory therapeutic approaches regarding the healthy balance of circulating monocyte subsets.

It has repeatedly been reported that the inflamed sinunasal and polyp tissue is predominantly infiltrated by M2 macrophages that induce and maintain a Th2 type inflammation which mediates the humoral immune cell response. But there was also no significant difference within the M1/M2 macrophage distribution in our examined polyp tissues after oral 1,8-Cineol therapy. Possible explanations might be the lacking effect of 1,8-Cineol on the macrophage differentiation within the nose or a rather slow-working long-term influence. We investigated the macrophage distribution after two-week 1,8-Cineol treatment. Tissue resident macrophages are known to stay present for several months, so the impact might not be visible after two weeks. This has to be investigated in further studies.

With respect to monocyte immune functions and their immunological interplay our study revealed some new insights. We found a positive correlation between the percentage of CD14\(^+\)CD16\(^-\) classical monocytes and serum IgE levels in CRSwNP patients \((p = 0.048)\). One study likewise reported elevated serum IgE levels correlating with monocyctic CD14 expression in patients with allergic asthma which was also validated in-vitro (39). They showed an increased monocyctic secretion of the pro-inflammatory cytokines TNF-\(\alpha\) and IL-6 after IgE cross-linking and impaired phagocytotic functions of these monocytes.

Furthermore, our data revealed a significant increase of PD-L1 expression on monocytes in CRS patients, suggesting a crucial mechanism for the regulation of T-cell responses preventing overreaction. Recently, significantly higher levels of CD80, CD274, and CD273 have been identified in nasal polyp tissues compared to peripheral blood from patients with CRSwNP, indicating an important role of the PD-1/PD-L1 pathway as a potential therapeutic target in CRS (40). In addition, we found an increase of PD-L1 by tendency and low significance in naive and effector CD4\(^+\) and CD8\(^+\) T cells, respectively which implies an activated but regulatory state of these T cells (41, 42). An imbalanced PD-L1 signalling has been repeatedly associated with chronic viral or bacterial infections that also exist during chronic rhinosinusitis (43, 46).

Astonishingly, our data revealed no significant alterations of peripheral CD4\(^+\) and CD8\(^+\) T-cell subset percentages, neither in CRSwNP nor in CRSsNP patients, since recent studies corroborated alterations of T-cell differentiation within the microenvironment of nasal polyps (32). All analysed patients and healthy donors showed a very comparable and stable distribution of circulating T-cell subsets, unaffected by CRS and observed monocyte subset alterations. These data suggest a spatial separation of monocyte and T-cell subset redistribution in the peripheral blood of CRS patients compared to the inflamed sinunasal tissue and nasal polyps, respectively.

**Conclusion**

The present study revealed new insights into the peripheral monocyte as well as tissue macrophage composition in CRS patients with respect to oral 1,8-Cineol treatment. Our data hint to a beneficial, anti-inflammatory effect of 1,8-Cineol since monocyte subsets were restored to healthy levels in some patients. Thus, further studies need to be performed in order to understand the immunological interplay and to select profiting patient cohorts.

**Authorship contribution**

CP and KL performed the molecular studies, data analysis and statistical evaluation. CI, DW and MH conducted the medical examination and sample collection. KLB and RP participated in the design, coordination and evaluation of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

**Acknowledgments**

We thank all doctors and technical staff of the ENT department, University Hospital Schleswig Holstein, Lübeck for supporting sample collection. This work was supported by the Cassella-med GmbH & Co. KG, Cologne, Germany.

**Funding**

There was no funding for this report.

**Ethics approval and consent to participate**

The patient gave consent for publication to KMB

**Consent for publication**

Not applicable

**Availability of data and materials**

Not applicable

**Conflict of interest**

The authors declare no conflict of interest.
References


29. Sifei Y, Shen C, Qianying L et al. Local IL-17 positive T cells are functionally associated with neutrophil infiltration and their development is regulated by mucosal microenvironment in nasal polyps. Inflamm Res. 2020.


