CD14⁺ monocytes contribute to inflammation in chronic nonbacterial osteomyelitis (CNO) through increased NLRP3 inflammasome expression

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Abstract

The pathophysiology of chronic nonbacterial osteomyelitis (CNO) remains incompletely understood. Increased NLRP3 inflammasome activation and IL-1β release in monocytes from CNO patients was suggested to contribute to bone inflammation. Here, we dissect immune cell infiltrates and demonstrate the involvement of monocytes across disease stages. Differences in cell density and immune cell composition may help to discriminate between BOM and CNO. However, differences are subtle and infiltrates vary in CNO. In contrast to other cells involved, monocytes are a stable element during all stages of CNO, which makes them a promising candidate in the search for “drivers” of inflammation. Furthermore, we link increased expression of inflammasome components NLRP3 and ASC in monocytes with site-specific DNA hypomethylation around the corresponding genes *NLRP3* and *PYCARD*. Our observations deliver further evidence for the involvement of pro-inflammatory monocytes in the pathophysiology of CNO. Cellular and molecular alterations may serve as disease biomarkers and/or therapeutic targets.

~~1.~~1 Introduction

Chronic nonbacterial osteomyelitis (CNO) is an autoinflammatory bone disorder. While all age groups can be affected, peak onset ranges between 7 and 12 years. A subset of CNO patients develops inflammatory lesions in single bones that can sometimes be timely limited. Most patients, however, experience chronic recurrent courses with more or less symmetric lesions at multiple sites. Such courses are usually referred to as chronic recurrent multifocal osteomyelitis (CRMO) [1–3]. Particularly cases with high disease activity, and/or delayed diagnosis and treatment initiation are prone to develop additional inflammatory symptoms, including palmoplantar pustulosis, arthritis or bowel inflammation. Some patients experience severe sequelae, such as vertebral body fractures. Timely diagnosis and initiation of effective treatment can be challenging in individual cases caused by the absence of prospectively tested disease biomarkers or diagnostic criteria [3,4]. Thus, CNO/CRMO remains a diagnosis of exclusion that requires the consideration of differential diagnoses, including infectious osteomyelitis, malignancies and other rare conditions (such as the metabolic bone disease hypophosphatasia and other autoinflammatory conditions). In unclear cases, differential diagnoses, namely bone infection or malignancies, require to be excluded through bone biopsies [2,3,5].

To date, the exact molecular pathophysiology of CNO/CRMO remains unknown. Descriptive analyses of bone biopsies suggest the presence of more or less distinct disease stages [2,6,7]. “Early lesions” suggest a central contribution of innate immune cells, namely neutrophils and monocytes, in disease pathophysiology, while “later stage” bone biopsies are characterized by infiltrates of lymphocytes and plasma cells. However, based on the rarity of CNO, the fact that only a relatively small subset of patients requires diagnostic bone biopsies, and lastly and most importantly the fact that the interval between disease-onset and bone biopsy remains unclear in all cases, a clear definition of “early” *vs.* “late” was impossible, and lastly based on the visual impression in histopathological samples. The presence of innate immune cells particularly in presumably “early lesions”, contributed to the conclusion, that CNO/CRMO is an autoinflammatory bone disorder. However, the contribution of adaptive immune cells in “late phase” bone lesions has not been investigated and remains elusive.

While the exact molecular pathophysiology of CNO is incompletely understood, recent data suggest reduced IL-10 expression and uninhibited NLRP3 inflammasome activation in monocytes from CNO patients as central contributors to bone inflammation and resulting clinical symptoms [8–10]. Scianaro et al. (2014) detected increased mRNA expression of IL-1β and NLRP3 inflammasome components NLRP3 (*NLRP3* gene), ASC (*PYCARD* gene) and caspase-1 (*CASP1* gene) in PBMCs from active CRMO patients [11]. We recently demonstrated increased IL-1β expression and release in monocytes from CRMO patients [8]. Furthermore, we and others identified NLRP3 and IL-1β protein in inflamed bone regions from CRMO patients [8]. Whether monocytes or other immune cells (*e.g.* neutrophils) are a source of these proteins and whether individual cells produce increased amounts of NLRP3 inflammasome-associated mediators remained unclear. Furthermore, the exact molecular mechanisms that contribute to increased inflammasome expression and resulting IL-1β activation and release remained unknown.

In the present study, we aimed to investigate immune cell infiltrates in bone biopsies from CRMO patients, particularly the presence of monocytes at different disease stages, to assess their potential contribution to both early phase and chronic bone inflammation. Furthermore, we examined the expression of pro-inflammatory IL-1β, and NLRP3 inflammasome-associated genes (*NLRP3*, *CASP1*, *PYCARD*) in monocytes and DNA methylation patterns that may contribute to gene dysregulation in CNO/CRMO [8].

~~2.~~2 Materials and ~~M~~methods

~~2.1.~~2.1 Patients and controls

All patients with CNO/CRMO or bacterial osteomyelitis (BOM) included in this study were diagnosed at the Department of Pediatrics, Universitätsklinikum Carl Gustav Carus, TU Dresden, Dresden, Germany or University Children's Hospital Würzburg, University of Würzburg, Würzburg, Germany. Bone tissue was collected during routine diagnostic workup (CNO/CRMO and BOM) or during elective bone osteotomy procedures. Lithium heparin blood was collected from treatment naïve CRMO patients and healthy controls (patients undergoing elective surgery) for the isolation of monocytes. The use of tissue from routine bone biopsies, immune staining, collection of blood samples and immune cell isolation and use was permitted by local ethics committees.

~~2.2.~~2.2 Immunohistochemistry

Paraffin embedded bone samples from 4 CNO patients, 3 patients with bacterial osteomyelitis (BOM) as disease controls, and 2 healthy individuals were decalcified, and mounted on slides in preparation for antibody staining. Demographics of CNO/CRMO patients, bacterial osteomyelitis patients and controls were comparable. None of the patients received systemic treatment other than NSAID at the time of surgery.

Slides were processed using the Ventana Benchmark ultra-automated system with proprietary reagents (Roche/Ventana Medical Systems). Heat induced antigen retrieval method was used in Cell Conditioning solution (CC1; Roche/Ventana). The following antibodies were used for immunostaining: anti-CD14 (Ventana/Roche; EPR 3653) for monocytes, anti-CD15 (Cell Marque; MMA) for neutrophils, anti-CD138 (Cell Marque; B-A38) for plasma cells, anti-

CD20 (DAKO; L26) for B lymphocytes, and anti-CD3 (Ventana; 2GV6) for T lymphocytes. Antibody detection was performed using UltraView universal DAB and UltraView universal alkaline phosphatase red kit (Roche/Ventana). Hematoxylin II and bluing reagent (Ventana/Roche) were used for chromogenic counterstaining following standard protocols. Cell numbers were determined in 4–10 visual fields (32,188.77 μm^2) per individual adding up to 36 in CNO, 25 in BOM, and 12 in the control group. The number of investigated visual fields per individual depended on the presence and size of bone lesions in the mounted tissue and sample quality.

~~2.3.~~2.3 Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were collected by density gradient centrifugation (Lymphoprep, Nycomed). From PBMCs, $CD14^+$ monocytes were isolated by negative selection (Stemcell Technologies). Monocytes were cultured at a concentration of 1×10^6 cells/mL in DMEM with 10% FCS. Some cells were treated with lipopolysaccharide (10 ng/mL) for 16 h. Cells were harvested for subsequent qRT-PCR and DNA methylation analyses as indicated.

~~2.4.~~2.4 Real-time PCR

Semiquantitative real-time PCR (qRT-PCR) was performed in samples from 6 CNO/CRMO patients and 6 controls, using GoTaq qPCR Master Mix Real time PCR system (ProMega) according to manufacturer's instructions. Results were normalized to GAPDH and a calibrator of pooled human cDNA derived from *ex vivo* isolated "untouched" $CD14^+$ monocytes (Stemcell Technologies) included on all PCR plates. Real-time PCR data were analyzed using the comparative CT method [12].

~~2.5.~~2.5 Western blotting

Resting or LPS stimulated (see above) $CD14^+$ monocytes (Stemcell Technologies) from 4 CNO/CRMO patients and 4 controls were lysed and processed as reported previously [13]. After centrifugation (16,400 *g*; 30 ~~minutes~~min; 4 °C), supernatants were collected and an identical amount of protein from each lysate (5 µg/well) was separated on NuPAGE 4%–12% Bis-Tris Gel (Life Technologies). Proteins were transferred to a nitrocellulose membrane, which was subsequently blocked for 1 ~~hour~~h using 2% BSA in PBS and incubated at room temperature with anti-NLRP3 (AG-20B-0014, AdipoGen), anti-IL-1β (sc-7884, Santa Cruz), anti-ASC (AL177, AdipoGen), anti-caspase-1 (sc-622, Santa Cruz), and anti-GAPDH (H86504M-BU, Meridian Life Science). The membrane was washed with TBS-T and incubated with a 1:3,000 dilution of secondary antibody (goat anti-mouse IgG or donkey anti-rabbit IgG) coupled with HRP (Jackson Immunoresearch). The Imaging System c300 (Azure Biosystems) was used for detection.

~~2.6.~~2.6 DNA methylation qPCR

Mouse and human *IL1B, NLRP3,* and *PYCARD* genes were aligned, and conserved non-coding sequences (CNS) regions were determined (VISTA Genome Browser). CNS regions were defined as regions with sequence homology of over 75% between the human and mouse genes over at least 200 base pairs. Based on the degree of conservation, distribution of CpG sequences and the presence of methylation-sensitive restriction sites (MSRE sites), five regions of interest were identified across the genes (Fig~~ure~~. 4A-C). OneStep qMethyl kit (Zymo Research) was used as a real-time procedure for the determination of DNA methylation of specific sequences by MSRE digestion and quantitative polymerase chain reaction (PCR), following manufacturer's instructions. Human genomic DNA derived from *ex vivo* isolated "untouched" $CD14^+$ monocytes from 6 CNO/CRMO patients and 6 controls was used as template.

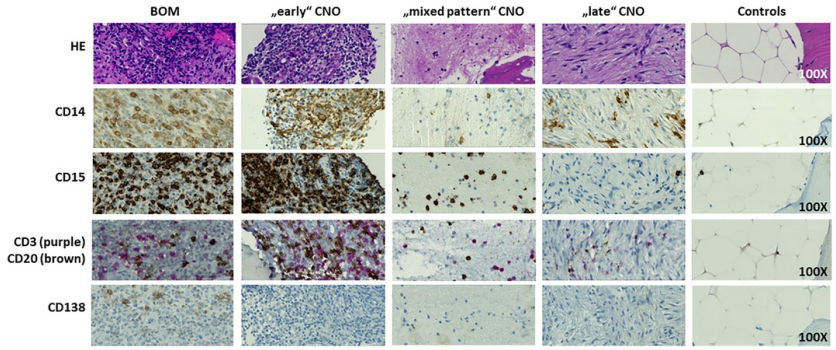
~~2.7.~~2.7 Statistical analysis

Statistical analysis was performed using STATA 12.0 software (StataCorp, College Station, TX, USA). Non-parametric tests were used due to small sample size and skewness of our data. Differences between two groups were analyzed using two-tailed Wilcoxon rank-sum (Mann–Whitney) tests for continuous variables. Kruskal-Wallis test was used to evaluate significance between independent variables of three groups. For all analyses, two-sided p values of <0.05 were considered statistically significant.

~~3.~~3 Results

~~3.1.~~3.1 Immune infiltrates in CNO/CRMO

The composition of immune cell infiltrations was determined in bone biopsies from CNO/CRMO patients, BOM patients, and healthy controls (children undergoing osteotomy) (Fig~~ure~~. 1). In line with the existing literature, immune cell infiltrates in CNO/CRMO patients were variable, potentially reflecting different disease stages. Based on immune cell infiltrates, and definitions from previous reports, we divided investigated power fields (32,188.77 μm^2 each) in three groups based on their visual appearance in hematoxylin and eosin (HE) stains: i) "early phase noninfectious osteomyelitis" with mainly segmented nuclear cells (neutrophils) and large mononuclear (monocyte) infiltrates, ii) "mixed pattern" infiltrates with small mononuclear blood cells (lymphocytes) and interspersed conglomerates of neutrophils and monocytes, and iii) "late phase noninfectious osteomyelitis" with mostly lymphocyte infiltrates (Fig~~ure~~. 1A).



~~Figure 1~~Fig. 1 Morphological appearance of bone lesions in BOM and CNO/CRMO. The composition of immune cell infiltrations was determined in bone biopsies from 4 CNO/CRMO patients, 3 BOM patients, and 2 healthy controls. CNO/CRMO patients exhibited variable appearances of immune cell infiltrates based on HE, and immune cell surface molecule staining. Based on the visual appearance of immune infiltrates, we divided included visual fields in three groups: “early” phase noninfectious osteomyelitis with mainly segmented nuclear cells (neutrophils) and large mononuclear (monocyte) infiltrates (left panel), “mixed pattern” infiltrates with small mononuclear blood cells (lymphocytes) and interspersed conglomerates of neutrophils and monocytes (middle panel), and “late” phase noninfectious osteomyelitis with mostly lymphocyte infiltrates (right panel).

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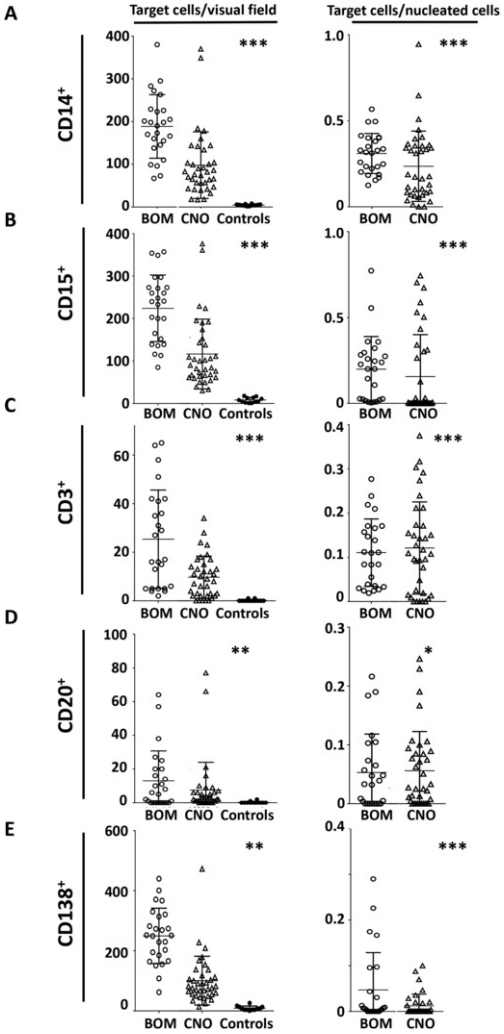
Next, we performed a cell count in all included power fields, and determined the number of cells and the proportion of monocytes ($CD14^+$), neutrophils ($CD15^+$), T lymphocytes ($CD3^+$), B lymphocytes ($CD20^+$), and plasma cells ($CD138^+$) as a fraction of all nucleated cells in the area (Fig~~ure~~s. 1, 2). Significant differences in absolute numbers of monocytes ($p = 0.0001$), neutrophilic granulocytes ($p = 0.0001$), T lymphocytes ($p = 0.0001$), B lymphocytes ($p < 0.002$) and plasma cell ($p = 0.0001$) have been detected between BOM, CNO and healthy controls with increased counts in both included forms of osteomyelitis (~~table~~Table 1).



~~Figure 2~~Fig. 2 Quantification on immune cell distribution in BOM, CNO/CRMO and controls. Paraffin embedded bone samples from 4 CRMO patients (36 power fields), 3 patients with bacterial osteomyelitis (25 power fields) as disease control, and 2 healthy individuals (12 power fields) were used for immune cell quantification through immune histochemistry. Absolute numbers (left panel) and proportions of immune cells relative to all nucleated cells in the power field (right panel) were determined. In bacterial (BOM) and chronic nonbacterial osteomyelitis (CNO) monocyte (A), neutrophil (B), T cell (C), B cell (D), and plasma cell (E) counts were increased as compared to controls. Numbers of B lymphocytes were only significantly increased in BOM (D) as compared to healthy bone, while numbers were not significantly increased in CN (*p < 0.05; **p < 0.01, ***p < 0.001).

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Table 1 Quantification of tissue immune cells.

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Cell type	Absolute numbers: CNO vs. BOM vs. controls (Fig~~figure~~. 2, left panel)	Immune cells relative to nucleated cells: BOM vs. CNO (Fig~~figure~~. 2, right panel)
monocytes (CD14⁺)	p < 0.001	p < 0.05

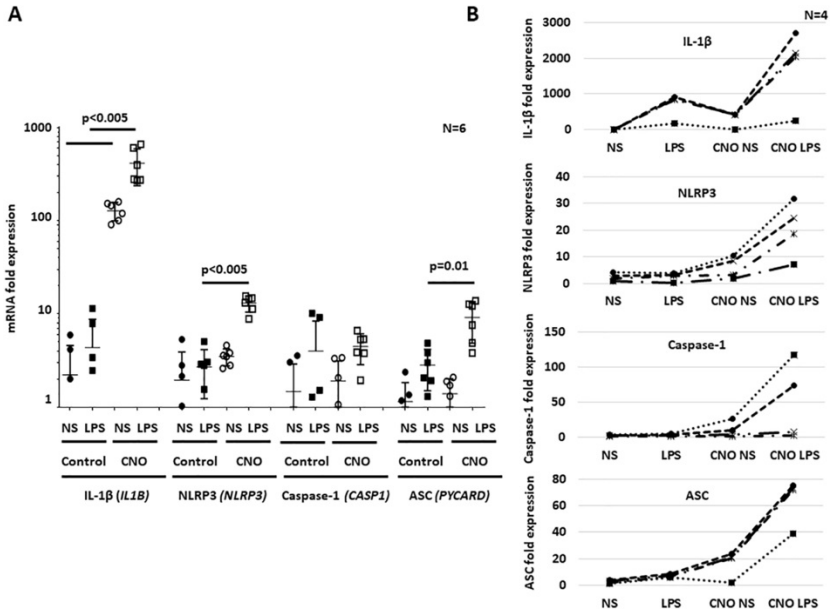
neutrophils ($CD15^+$)	$p < 0.001$	$p < 0.02$
T lymphocytes ($CD3^+$)	$p < 0.001$	ns
B lymphocytes ($CD20^+$)	$p = 0.002$	ns
plasma cells ($CD138^+$)	$p < 0.001$	$p < 0.02$

Kruskal-Wallis tests provide one p value representing potential differences between all groups included in statistical analyses (here: 3 groups comparing absolute numbers between CNO, BOM and controls). Wilcoxon rank-sum (Mann-Whitney) tests were used comparing relative numbers of BOM *vs.* CNO samples. Two-sided p values of <0.05 were considered statistically significant.

Morphological descriptions in the literature suggest that early stage biopsies of CNO patients and biopsies from BOM patients may be characterized by predominant infiltrates of neutrophils and monocytes. Thus, we used morphological appearance in HE stains to divide CNO samples in three groups ("early", "mixed pattern", and "late" stage CNO) (Figures 1, 2, Supplement 1). Absolute numbers of immune cells in samples from CNO, BOM patients and controls were compared and analyzed, applying Kruskal-Wallis tests. Significant differences between the three groups were present in the numbers of all immune cell types investigated: $CD14^+$ monocytes, $CD15^+$ neutrophilic granulocytes, $CD3^+$ T lymphocytes, $CD20^+$ B lymphocytes, and $CD138^+$ plasma cells (Figure 2, left panel). Next, immune cells ratios as a fraction of nucleated cells within visual fields were compared between BOM and CNO using Wilcoxon rank-sum (Mann–Whitney) test (Figure 2, righ panel). Normal controls were excluded from data sub-analysis, since low total cell numbers in visual fields from healthy controls may have resulted in datasets of limited biological relevance. Relative numbers of monocytes ($p < 0.05$), neutrophilic granulozytes ($p < 0.02$) and plasma cells ($p < 0.02$) based on the number of total nucleated cells were elevated in BOM as compared to CNO. Lastly, cell numbers and proportions in bone tissue from CNO patients with "early", "mixed pattern" or "late stage" bone changes were compared (Supplement 1). Differences in absolute cell numbers were detected in monocytes ($p < 0.05$) and B lymphocytes ($p < 0.05$), which were elevated in "early" CNO. Neutrophilic granulozyte ($p < 0.001$), T cell ($p < 0.05$), and B lymphocyte ($p < 0.002$) proportions relative to the number of nucleated cells were elevated in "early" and "mixed pattern" CNO as compared to "late" stages. Plasma cells trended to be more common in "late" CNO as a proportion of nucleated cells. This, however, failed to reach statistical significance. As mentioned above, absolute and relative immune cell numbers were comparable between BOM and CNO tissue.

Increased expression of NLRP3 inflammasome-associated genes in CNO/CRMO – Based on findings from previous reports and the observations in Figure 1, monocytes are likely to be central players in the immune pathogenesis of CNO/CRMO. To assess their potential contribution to bone inflammation, we monitored mRNA (Figure 3A) and protein (Figure 3B, Supplement 2A) expression of IL-1, NLRP3, caspase-1, and ASC (*PYCARD* gene). Expression of IL-1β was increased in monocytes from CNO/CRMO patients under resting conditions and in response to stimulation with TLR4 ligand LPS on the mRNA level and increased on the protein level after stimulation. Furthermore, secretion of mature IL-1β was increased in both resting and stimulated (LPS) monocytes from CNO/CRMO patients when compared to controls (Supplement 2B). Expression of NRLP3 mRNA and protein was increased in monocytes from CNO/CRMO patients in response to stimulation when compared to monocytes from controls. Other than in a previous study that targeted mRNA expression in PBMCs from CNO/CRMO patients, caspase-1 mRNA expression was comparable in monocytes from CRMO patients and controls. However, there was a trend towards increased protein expression in cells from CNO/CRMO patients. The (mRNA) expression of the adapter molecule ASC (*PYCARD*) was increased in monocytes from CRMO patients after stimulation.

DNA Methylation patterns in monocytes from CNO/CRMO patients – Previous studies suggested altered epigenetic marks to contribute to altered cytokine expression in CNO [8,10]. To investigate site-specific DNA methylation patterns across the (between CNO patients and controls) differentially expressed human *IL1B, NLRP3*, and *PYCARD* genes, we applied bioinformatics approaches to define regions of interest (ROI, Figure 4A-C). Using the Vista genome browser inline tool (http://pipeline.lbl.gov/cgi-bin/gateway2), we aligned mouse and human *IL1B, NLRP3,* and *PYCARD* genes to identify conserved non-coding sequences (CNS; pink), exons (blue) and untranslated regions (UTR; turquoise). Here, CNS sites were defined as regions with sequence homology of >75% between human and mouse over a length of at least 200 bp. CNS, UTR or exons were defined ROIs when CpG sequences (black filled circles below alignments in Figure 4A-C) were present and DNA sequences included methylation-sensitive restriction sites (MSRE sites) that allowed the chosen PCR based analytic approach (OneStep qMethyl kit, Zymo Research).



~~Figure 3~~Fig. 3 Expression of NLRP3 inflammasome-associated genes in monocytes from CNO/CRMO patients. A) We determined mRNA expression of IL-1β, NLRP3, caspase-1, and ASC (encoded by *PYCARD*) in unstimulated and stimulated (LPS) monocytes from CRMO patients and matched controls. Monocytes from CRMO patients express increased levels of IL-1β as compared to controls under resting conditions and in response to stimulation. Furthermore, expression of inflammasome components NLRP3 and ASC is increased in monocytes from CRMO patients after stimulation with LPS. No significant differences were seen in caspase-1 expression. B) Protein expression was monitored in monocytes from 4 CRMO patients and 4 controls (densitometry, normalized to GAPDH expression). Samples from one experiment (including one CNO/CRMO patient and one matched control), are connected by dotted lines. NLRP3 and IL-1β protein expression were increased in monocytes from CRMO patients in response to stimulation. Furthermore, we determined a trend towards increased expression of ASC in CRMO monocytes under all conditions, and a trend towards increased caspase-1 protein expression in monocytes from CRMO patients in cells stimulated with LPS.

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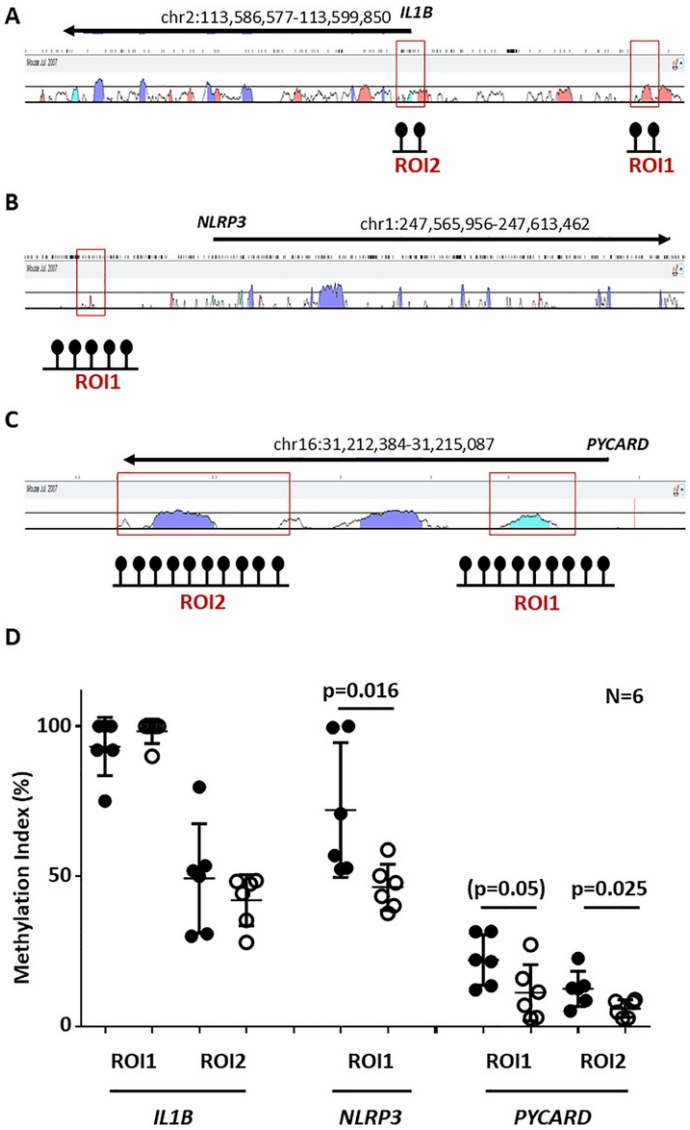

~~Figure 4~~**Fig. 4** DNA methylation of NLRP3 inflammasome-associated genes in monocytes from CRMO patients. Using the Vista genome browser (http://pipeline.lbl.gov/cgi-bin/gateway2), we aligned mouse and human *IL1B* (A), *NLRP3* (B), and *PYCARD* (C) genes to identify conserved non-coding sequences (CNS; pink), exons (blue) and untranslated regions (UTR; turquoise). "High" degrees of conservation were defined by sequence homology of >75% between human and mouse over a length of at least 200 bp. Regions of interest (ROI) were defined in CNS, UTR or exons when CpG sequences (black filled circles below alignments) were present and DNA sequences included methylation-sensitive restriction sites (MSRE sites). D) DNA methylation patterns varied between monocytes from CRMO patients (open circles) and controls (filled circles). ROI1 of *NLRP3* and ROI1 and 2 of *PYCARD* exhibited significantly reduced DNA methylation levels in monocytes from CRMO patients as compared to cells from matched healthy controls. No differences were determined between ROI1 and 2 of the *IL1B* gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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While no significant differences were determined between ROI1 and 2 of the *IL1B* gene, ROI1 of *NLRP3* and ROI1 and 2 of *PYCARD* exhibited significantly reduced DNA methylation levels in monocytes from CRMO patients as

compared to cells from matched healthy controls (Figure 4D).

4.4 Discussion

Chronic nonbacterial osteomyelitis has been classified as autoinflammatory bone disorder because of the involvement of spontaneous activation of innate immune mechanisms in its pathophysiology [1,3,14,15]. A central step on the way was the descriptive analysis of immune cell infiltrates in bone biopsies from CNO/CRMO patients. To date, it is appreciated that immune infiltrates involve innate immune cells, particularly during “early” disease stages that are replaced or complemented by lymphocytes and plasma cells in “later” disease stages [2,7]. However, none of the available reports provide exact quantification of immune cells and mostly rely on the description of morphologic features in HE stains. In a first step, we quantified immune cells in bone lesions of BOM *vs.* CNO/CRMO patients as compared to healthy bone tissue. Immune cell infiltrates were denser in BOM as compared to CNO/CRMO with higher absolute and relative numbers of monocytes, neutrophils, T and B lymphocytes. They furthermore, exhibited significant differences in immune cell numbers as compared to healthy controls (Table 1). Next, we for the first time quantified immune cell infiltrates in bone biopsies from CNO/CRMO patients at various disease stages (“early” *vs.* “late” stage) and compared findings to bone from healthy individuals (osteotomy) or bacterial osteomyelitis (BOM). Biopsies taken at any stage contained monocytes, neutrophils, T and B lymphocytes, and plasma cells. However, “early” stage inflammation was characterized by increased absolute numbers of monocytes, and B lymphocytes as compared to later stages. Furthermore, neutrophilic granulozyte, B and T lymphocyte proportions were elevated in “earlier” stages of CNO as compared to chronic stages. Only plasma cells appeared to increase in numbers over time and were more prevalent in “late stage”-appearing bone lesions. However, since plasma cells are also prevalent in bone samples from BOM patients, the sometimes-used term “plasma cellular” osteomyelitis for CNO/CRMO appears somewhat misleading, since plasma cellular infiltrates are also present in BOM and therefore not specific to CNO/CRMO. Indeed, dense plasma cell infiltrates should trigger the consideration of differential diagnoses, such as lymphoma. Here reported observations provide evidence for a coexistence of various “disease stages” or rather mutual involvement of innate and adaptive immune components in bone inflammation during both CNO/CRMO and BOM, thus underscoring that innate and adaptive immune responses cannot be seen as individual events. Furthermore, the coexistence of innate (monocytes and neutrophils) and adaptive (lymphocyte) immune cell infiltrates in CNO/CRMO may also suggest a central contribution of innate immune cells to maintaining inflammation, disease flares or phases with enhanced disease activity [16]. This may be in analogy to *Munro* micro-abscesses in psoriasis, a disorder associated with CNO/CRMO that may also share molecular pathomechanisms. *Munro* micro-abscesses are local infiltrates of neutrophils in the epidermis of psoriasis patients that are considered to promote skin inflammation and/or trigger flares in plaque psoriasis [17,18].

The constitutive and almost unchanged presence of monocytes in morphologically diverse infiltrates in CNO/CRMO may not only suggest ongoing interplay between components of the innate and adaptive immune response, but also suggests a central contribution of monocytes to disease pathology. This is further supported by the previous observation that monocytes from CRMO patients express and release increased amounts of pro-inflammatory IL-1β [8]. The exact molecular mechanisms that contribute to increased gene expression and activation, however, remained unknown. In the present study, we provide evidence for increased mRNA and protein expression of inflammasome associated NLRP3, ASC, and IL-1β. Increased abundance of inflammasome components NLRP3 and ASC, as well as the caspase-1 substrate IL-1β contribute to enhanced inflammasome assembly and subsequent IL-1β activation and release [19,20]. Of note, intracellular protein levels of IL-1β may even underestimate the total extend of IL-1β expression in monocytes, since activated mature IL-1β is secreted to the extracellular compartment, which is also significantly increased in monocytes from CNO/CRMO patients (as seen in Supplement 1B).

Methylation of CpG dinucleotides within DNA sequences resembles a potent mechanism to prevent recruitment of transcription factors and RNA polymerases to regulatory regions [21,22], thereby regulating gene expression. In previous studies, we demonstrated that altered epigenetic marks across the *IL10* cytokine gene cluster contribute to impaired gene expression (8, 10). Thus, we tested whether disturbed epigenetic patterns may also contribute to increased NLRP3 inflammasome associated gene expression. Indeed, we provide evidence suggesting that reduced DNA methylation at highly conserved elements around the *NLRP3* and *ASC* genes in monocytes from CNO/CRMO patients promote gene expression. This is in agreement with recent data from Vento-Tormo et al. (2017) who demonstrated that chronic inflammasome activation in a group of genetic inflammatory disorders referred to as cryopyrin-associated periodic syndromes (CAPS) is promoted by reduced DNA methylation, enhancing the transcription of inflammasome components [23]. Indeed, we detected increased mRNA expression of NLRP3 and ASC/PYCARD in monocytes from CNO/CRMO patients reflecting reduced DNA methylation. The *IL1B* gene, which is transcriptionally more active in samples from CNO/CRMO patients, exhibited comparable DNA methylation patterns between both groups at the sites investigated. More extensive DNA methylation mapping, including more distal elements may provide additional information. On the other hand, *IL1B* may be more resistant to changes in DNA methylation and be subject to dysregulation through additional events in monocytes from CRMO patients.

Currently, it remains unknown which molecular mechanisms mediate DNA demethylation at inflammasome-associated genes in monocytes from CNO/CRMO patients. It is tempting to speculate that reduced expression of immune regulatory cytokines IL-10 and IL-19 and the resulting dysbalance ~~toward~~towards pro-inflammatory cytokine expression in monocytes from CNO/CRMO patients may mediate altered DNA methylation [8]. However, this remains to be determined in future studies and is beyond the scope of the study presented. Another possible contributor to DNA demethylation is the reduced activation of mitogen-activated protein kinases (MAPKs) ERK1 and 2 in monocytes from CRMO patients [9,10]. Indeed, T cells from patients with the autoimmune disorder systemic lupus erythematosus undergo DNA demethylation as a result of reduced protein kinase activation (involving MAPK), which centrally contributes to the pro-inflammatory effector phenotype of these cells [24,25].

The study presented is limited by the low numbers of patients and controls, which was caused by the rarity of the included diagnoses BOM and CNO, the fact that most patients with these diagnoses do not undergo routine bone biopsies, and difficulties identifying otherwise healthy individuals requiring osteotomy surgery (of note, osteopenic individuals were excluded). Future studies are warranted to confirm observations in larger cohorts and to address open questions of whether reduced expression of immune regulatory cytokines, increased expression of pro-inflammatory cytokines, other mechanisms such as altered MAPK activation, or a combination of the aforementioned contribute to DNA demethylation in monocytes from CRMO patients. For these investigations, international collaborations are required to allow the inclusion of larger cohorts with sufficient numbers of patients at different disease stages and with differential disease presentation. The recently published CARRA consensus treatment plan (CTP) initiative aiming at treatment harmonization and the comparison of available treatment options includes the collection of clinical datasets and biosamples [26]. Thus, it is a unique opportunity for international collaborations targeting the molecular pathophysiology of CNO/CRMO.

5.5 Conclusions

Cellular infiltrates in bacterial (BOM) and noninfectious CNO/CRMO include innate immune cells (neutrophils and monocytes) and lymphocytes. Generally, immune cell infiltrates are denser in BOM as compared to CNO/CRMO potentially reflecting higher inflammatory activity that may explain more acute onset of symptoms. In BOM and in “later stage” CNO lesions, plasma cells are present. Thus, discrimination between BOM and CNO solely based on histopathology remains difficult using currently available tools. Monocytes are prominent cellular components of inflammatory infiltrates in CNO/CRMO at all stages, which makes them a likely contributor to bone inflammation. Monocytes from CRMO patients express increased levels of NLRP3 inflammasome components NLRP3 and ASC, as well as IL-1β. Altered gene expression is promoted by site-specific DNA demethylation at the corresponding genes *NLRP3* and *PYCARD* (ASC). Taken together, reported observations provide evidence for the involvement of monocytes in the immune pathogenesis of CNO and provide additional arguments for cytokine blocking strategies and/or inflammasome inhibition in CNO/CRMO refractory to standard treatment with NSAIDs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2018.04.011.

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References

[1] PJP.J. Ferguson and MM. Sandu, Current understanding of the pathogenesis and management of chronic recurrent multifocal osteomyelitis, *Curr Rheumatol Rep.Curr. Rheumatol. Rep.* **14** (2), 2012, 130–141.

[2] CMC.M. Hedrich, SRS.R. Hofmann, JJ. Pablik, HH. Morbach and HJH.J. Girschick, Autoinflammatory bone disorders with special focus on chronic recurrent multifocal osteomyelitis (CRMO), *Pediatr. Rheumatol. Online J.* **11** (1), 2013, 47.

[3] SRS.R. Hofmann, AA. Schnabel, AA. Rosen-Wolff, HH. Morbach, HJH.J. Girschick and CMC.M. Hedrich, Chronic Nnonbacterial Oosteomyelitis: Ppathophysiological Cconcepts and Ccurrent Ttreatment Sstrategies, *J Rheumatol. Rheumatol.* **43** (11), 2016, 1956–1964.

[4] AA. Schnabel, UU. Range, GG. Hahn, RR. Berner and CMC.M. Hedrich, Treatment Rresponse and longterm Ooutcomes in Cchildren with Cchronic Nnonbacterial Oosteomyelitis, *J Rheumatol.J. Rheumatol.* **44** (7), 2017, 1058–1065.

[5] AA. Schnabel, UU. Range, GG. Hahn, TT. Siepmann, RR. Berner and CMC.M. Hedrich, Unexpectedly high incidences of chronic non-bacterial as compared to bacterial osteomyelitis in children, *Rheumatol Int.Rheumatol. Int.* **36** (12), 2016, 1737–1745.

[6] PDP.D. Gikas, LL. Islam, WW. Aston, RR. Tirabosco, AA. Saifuddin, TWT.W. Briggs, et al., Nonbacterial osteitis: a clinical, histopathological, and imaging study with a proposal for protocol-based management of patients with this diagnosis, *J Orthop Sci.J. Orthop. Sci.* **14** (5), 2009, 505–516.

[7] HJH.J. Girschick, HIH.I. Huppertz, DD. Harmsen, RR. Krauspe, HKH.K. Muller-Hermelink and TT. Papadopoulos, Chronic recurrent multifocal osteomyelitis in children: diagnostic value of histopathology and microbial testing, *Hum Pathol.Hum. Pathol.* **30** (1), 1999, 59–65.

[8] SRS.R. Hofmann, ASA.S. Kubasch, CC. Ioannidis, AA. Rosen-Wolff, HJH.J. Girschick, HH. Morbach, et al., Altered expression of IL-10 family cytokines in monocytes from CRMO patients result in enhanced IL-1beta expressior

and release, ~~*Clin Immunol.*~~*Clin. Immunol.* **161** (2), 2015, 300–307.

[9] ~~SR~~S.R. Hofmann, ~~H~~H. Morbach, ~~T~~T. Schwarz, ~~A~~A. Rosen-Wolff, ~~HJ~~H.J. Girschick and ~~CM~~C.M. Hedrich, Attenuated TLR4/MAPK signaling in monocytes from patients with CRMO results in impaired IL-10 expression, ~~*Clin Immunol.*~~*Clin. Immunol.* **145** (1), 2012, 69–76.

[10] ~~SR~~S.R. Hofmann, ~~T~~T. Schwarz, ~~JC~~J.C. Moller, ~~H~~H. Morbach, ~~A~~A. Schnabel, ~~A~~A. Rosen-Wolff, et al., Chronic non-bacterial osteomyelitis is associated with impaired Sp1 signaling, reduced IL10 promoter phosphorylation, an reduced myeloid IL-10 expression, ~~*Clin Immunol.*~~*Clin. Immunol.* **141** (3), 2011, 317–327.

[11] ~~R~~R. Scianaro, ~~A~~A. Insalaco, ~~L~~L. Bracci Laudiero, ~~R~~R. De Vito, ~~M~~M. Pezzullo, ~~A~~A. Teti, et al., Deregulation of the IL-1beta axis in chronic recurrent multifocal osteomyelitis, *Pediatr. Rheumatol. Online J.* **12**, 2014, 30.

[12] ~~KJ~~K.J. Livak and ~~TD~~T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) ~~M~~method, ~~*Methods.*~~*Methods* **25** (4), 2001, 402–408.

[13] ~~MC~~M.C. Heymann, ~~S~~S. Winkler, ~~H~~H. Luksch, ~~S~~S. Flecks, ~~M~~M. Franke, ~~S~~S. Russ, et al., Human procaspase-1 variants with decreased enzymatic activity are associated with febrile episodes and may contribute to inflammation via RIP2 and NF-kappaB signaling, ~~*J Immunol.*~~*J. Immunol.* **192** (9), 2014, 4379–4385.

[14] ~~PJ~~P.J. Ferguson and ~~HI~~H.I. El-Shanti, Autoinflammatory bone disorders, ~~*Curr Opin Rheumatol.*~~*Curr. Opin. Rheumatol.* **19** (5), 2007, 492–498.

[15] ~~SR~~S.R. Hofmann, ~~A~~A. Roesen-Wolff, ~~G~~G. Hahn and ~~CM~~C.M. Hedrich, Update: ~~C~~cytokine ~~D~~dysregulation in ~~C~~chronic ~~N~~nonbacterial ~~O~~osteomyelitis (CNO), *Int. J. Rheumatol.* **2012**, 2012, 310206.

[16] ~~CM~~C.M. Hedrich, Shaping the spectrum - ~~F~~from autoinflammation to autoimmunity, ~~*Clin Immunol.*~~*Clin. Immunol.* **165**, 2016, 21–28.

[17] ~~E~~E. Christophers, ~~G~~G. Metzler and ~~M~~M. Rocken, Bimodal immune activation in psoriasis, ~~*Br J Dermatol.*~~*Br. J. Dermatol.* **170** (1), 2014, 59–65.

[18] ~~JL~~J.L. Harden, ~~JG~~J.G. Krueger and ~~AM~~A.M. Bowcock, The immunogenetics of Psoriasis: ~~A~~a comprehensive review, ~~*J Autoimmun.*~~*J. Autoimmun.* **64**, 2015, 66–73.

[19] ~~S~~S. Winkler, ~~CM~~C.M. Hedrich and ~~A~~A. Rosen-Wolff, Caspase-1 regulates autoinflammation in rheumatic diseases, ~~*Z Rheumatol.*~~*Z. Rheumatol.* **75** (3), 2016, 265–275.

[20] ~~S~~S. Winkler and ~~A~~A. Rosen-Wolff, Caspase-1: an integral regulator of innate immunity, ~~*Semin Immunopathol.*~~*Semin. Immunopathol.* **37** (4), 2015, 419–427.

[21] ~~CM~~C.M. Hedrich, ~~K~~K. Mabert, ~~T~~T. Rauen and ~~CG~~G.C. Tsokos, DNA methylation in systemic lupus erythematosus, *Epigenomics* **9** (4), 2017, 505–525.

[22] ~~D~~D. Alvarez-Errico, ~~R~~R. Vento-Tormo and ~~E~~E. Ballestar, Genetic and ~~E~~epigenetic ~~D~~determinants in ~~A~~autoinflammatory ~~D~~diseases, ~~*Front Immunol.*~~*Front. Immunol.* **8**, 2017, 318.

[23] ~~R~~R. Vento-Tormo, ~~D~~D. Alvarez-Errico, ~~A~~A. Garcia-Gomez, ~~J~~J. Hernandez-Rodriguez, ~~S~~S. Bujan, ~~M~~M. Basagana, et al., DNA demethylation of inflammasome-associated genes is enhanced in patients with cryopyrin-associated periodic syndromes, ~~*J Allergy Clin Immunol.*~~*J. Allergy Clin. Immunol.* **139** (1), 2017, 202–211, (e6).

[24] ~~G~~G. Gorelik, ~~AH~~A.H. Sawalha, ~~D~~D. Patel, ~~K~~K. Johnson and ~~B~~B. Richardson, T cell PKCdelta kinase inactivation induces lupus-like autoimmunity in mice, ~~*Clin Immunol.*~~*Clin. Immunol.* **158** (2), 2015, 193–203.

[25] ~~GJ~~G.J. Gorelik, ~~S~~S. Yarlagadda, ~~DR~~D.R. Patel and ~~BC~~B.C. Richardson, Protein kinase Cdelta oxidation contributes to ERK inactivation in lupus T cells, *Arthritis Rheum.* **64** (9), 2012, 2964–2974.

[26] ~~Y~~Y. Zhao, ~~EY~~E.Y. Wu, ~~MS~~M.S. Oliver, ~~AM~~A.M. Cooper, ~~ML~~M.L. Basiaga, ~~SS~~S.S. Vora, et al., Consensus ~~T~~treatment ~~P~~plans for ~~C~~chronic ~~N~~nonbacterial ~~O~~osteomyelitis ~~R~~refractory to ~~N~~nonsteroidal ~~A~~anti-~~i~~inflammatory ~~D~~drugs and/or with ~~A~~active ~~S~~spinal ~~L~~lesions, *Arthritis Care Res.* 2017.

E-Extra

Morphological descriptions in the literature suggest that early stage biopsies of CNO patients and biopsies from BOM patients may be characterized by predominant infiltrates of neutrophils and monocytes. Thus, we used morphological appearance in HE stains to divide CNO samples in three groups (“early”, “mixed pattern”, and “late” stage CNO) (Figures 1, 2, Supplement 1). Absolute numbers of immune cells in samples from CNO, BOM patients and controls were compared and analyzed, applying Kruskal-Wallis tests. Significant differences between the three groups were present in the numbers of all immune cell types investigated: $CD14^+$ monocytes, $CD15^+$ neutrophilic granulocytes, $CD3^+$ T lymphocytes, $CD20^+$ B lymphocytes, and $CD138^+$ plasma cells (Figure 2, left panel). Next, immune cells ratios as a fraction of nucleated cells within visual fields were compared between BOM and CNO using Wilcoxon rank-sum (Mann–Whitney) test (Figure 2, righ panel). Normal controls were excluded from data sub-analysis, since low total cell numbers in visual fields

from healthy controls may have resulted in datasets of limited biological relevance. Relative numbers of monocytes ($p < 0.05$), neutrophilic granulozytes ($p < 0.02$) and plasma cells ($p < 0.02$) based on the number of total nucleated cells were elevated in BOM as compared to CNO. Lastly, cell numbers and proportions in bone tissue from CNO patients with “early”, “mixed pattern” or “late stage” bone changes were compared (Supplement 1). Differences in absolute cell numbers were detected in monocytes ($p < 0.05$) and B lymphocytes ($p < 0.05$), which were elevated in “early” CNO. Neutrophilic granulozyte ($p < 0.001$), T cell ($p < 0.05$), and B lymphocyte ($p < 0.002$) proportions relative to the number of nucleated cells were elevated in “early” and “mixed pattern” CNO as compared to “late” stages. Plasma cells trended to be more common in “late” CNO as a proportion of nucleated cells. This, however, failed to reach statistical significance. As mentioned above, absolute and relative immune cell numbers were comparable between BOM and CNO tissue.

Increased expression of NLRP3 inflammasome-associated genes in CNO/CRMO -- Based on findings from previous reports and the observations in Figure 1, monocytes are likely to be central players in the immune pathogenesis of CNO/CRMO. To assess their potential contribution to bone inflammation, we monitored mRNA (Figure 3A) and protein (Figure 3B, Supplement 2A) expression of IL-1, NLRP3, caspase-1, and ASC (*PYCARD* gene). Expression of IL-1β was increased in monocytes from CNO/CRMO patients under resting conditions and in response to stimulation with TLR4 ligand LPS on the mRNA level and increased on the protein level after stimulation. Furthermore, secretion of mature IL-1β was increased in both resting and stimulated (LPS) monocytes from CNO/CRMO patients when compared to controls (Supplement 2B). Expression of NRLP3 mRNA and protein was increased in monocytes from CNO/CRMO patients in response to stimulation when compared to monocytes from controls. Other than in a previous study that targeted mRNA expression in PBMCs from CNO/CRMO patients, caspase-1 mRNA expression was comparable in monocytes from CRMO patients and controls. However, there was a trend towards increased protein expression in cells from CNO/CRMO patients. The (mRNA) expression of the adapter molecule ASC (*PYCARD*) was increased in monocytes from CRMO patients after stimulation.

The constitutive and almost unchanged presence of monocytes in morphologically diverse infiltrates in CNO/CRMO may not only suggest ongoing interplay between components of the innate and adaptive immune response, but also suggests a central contribution of monocytes to disease pathology. This is further supported by the previous observation that monocytes from CRMO patients express and release increased amounts of pro-inflammatory IL-1β [8]. The exact molecular mechanisms that contribute to increased gene expression and activation, however, remained unknown. In the present study, we provide evidence for increased mRNA and protein expression of inflammasome associated NLRP3, ASC, and IL-1β. Increased abundance of inflammasome components NLRP3 and ASC, as well as the caspase-1 substrate IL-1β contribute to enhanced inflammasome assembly and subsequent IL-1β activation and release [19,20]. Of note, intracellular protein levels of IL-1β may even underestimate the total extend of IL-1β expression in monocytes, since activated mature IL-1β is secreted to the extracellular compartment, which is also significantly increased in monocytes from CNO/CRMO patients (as seen in Supplement 1B).

E-component

The following are the supplementary data related to this article.

Multimedia Component 1

Supplement 1 Quantification on immune cell distribution in “early”, “mixed pattern” and “late” CNO/CRMO. Paraffin embedded bone samples from 4 CRMO patients (36 power fields), 3 patients with bacterial osteomyelitis (25 power fields) as disease control, and 2 healthy individuals (12 power fields) were used for immune cell quantification through immune histochemistry. Absolute numbers (left panel) and proportions of immune cells relative to all nucleated cells in the power field (right panel) were determined. Differences in absolute numbers of immune cells between the three groups of CNO samples (“early” CNO (eCNO); “mixed pattern” CNO (mCNO); “late stage” CNO (lCNO)) were detected in monocytes and B cells. Neutrophilic granulocyte and B cell proportions relative to the number of nucleated cells were elevated in “earlier” stages of CNO as compared to “late” stages. Plasma cells trended to be more common in “later” CNO stages. This, however, failed to reach statistical significance (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$).

alt-text: Supplement 1

Multimedia Component 2

Supplement 2 Expression of NLRP3 inflammasome-associated genes in monocytes from CNO/CRMO patients. A) Protein expression was monitored in monocytes from 4 CRMO patients and 4 controls. One representative Western blot is provided. B) Secretion of mature IL-1β protein from monocytes from CNO/CRMO patients and controls under resting conditions and in response to stimulation with LPS. In the upper panel, absolute values of IL-1β protein in the supernatant are displayed; the lower panel indicates fold increase of IL-1β protein secretion from monocytes based on unstimulated cells from healthy controls in each experiment. Median values, standard deviations, and p values from Mann-Whitney tests are provided.

alt-text: Supplement 2

Highlights

- Immune infiltrates in CNO involve cells of the innate and adaptive immune system.
- Innate immune cells may drive flares in analogy to *Munro* abscesses in psoriasis.
- Monocytes are a stable and almost unchanged element of all CNO/CRMO stages.
- Monocytes from CNO patients over-express NLRP3, ASC, and IL-1β
- Gene expression is promoted by DNA hypomethylation at *NLRP3* and *PYCARD*.

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