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Periodontitis in Chédiak-Higashi Syndrome: An Altered Immunoinflammatory Response

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Abstract: *Chédiak-Higashi syndrome (CHS), a rare autosomal recessive disorder caused by mutations in the lysosomal trafficking regulator gene (LYST), is associated with aggressive periodontitis. It is suggested that LYST mutations affect the toll-like receptor (TLR)-mediated immunoinflammatory response, leading to frequent infections. This study sought to determine the periodontal status of patients with classic (severe) and atypical (milder) forms of CHS and the immunoregulatory functions of gingival fibroblasts in CHS patients. In contrast to aged-matched healthy controls, atypical (n = 4) and classic (n = 3) CHS patients presented with mild chronic periodontitis with no evidence of gingival ulceration, severe tooth mobility, or premature exfoliation of teeth. As a standard of care, all classic CHS patients had undergone bone marrow transplantation*

*(BMT). Primary gingival fibroblasts obtained from atypical and BMT classic CHS patients displayed higher protein expression of TLR-2 (1.81-fold and 1.56-fold, respectively) and decreased expression of TLR-4 (–2.5-fold and –3.85-fold, respectively) at baseline when compared with healthy control gingival fibroblasts. When challenged with whole bacterial extract of *Fusobacterium nucleatum*, both atypical and classic CHS gingival fibroblasts failed to up-regulate TLR-2 and TLR-4 expression when compared with their respective untreated groups and control cells. Cytokine multiplex analysis following *F. nucleatum* challenge showed that atypical CHS gingival fibroblasts featured significantly increased cytokine expression (interleukin [IL]–2, IL–4, IL–5, IL–6, IL–10, IL–12, interferon- γ , tumor necrosis factor- α), whereas classic CHS cells featured similar/*

decreased cytokine expression when compared with treated control cells. Collectively, these results suggest that LYST mutations in CHS patients affect TLR-2 and TLR-4 expression/function, leading to dysregulated immunoinflammatory response, which in turn may influence the periodontal phenotype noted in CHS patients. Furthermore, our results suggest that atypical CHS patients and classic CHS patients who undergo BMT early in life are less susceptible to aggressive periodontitis and that hematopoietic cells play a critical role in mitigating the risk of aggressive periodontitis in CHS.

Knowledge Transfer Statement:

Results from this study can be used to create awareness among clinicians and researchers that not all CHS patients exhibit historically reported aggressive periodontitis, especially if

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*they have atypical CHS disease or have received bone marrow transplantation. *LYST* mutations in CHS patients may affect TLR-2 and TLR-4 expression/function leading to dysregulated immunoinflammatory response, which in turn may influence the periodontal phenotype noted in CHS patients.*

Keywords: lysosomal trafficking regulator (*LYST*) protein, phagocyte bactericidal dysfunction, immunodeficiency, toll-like receptors, periodontal disease, lysosomes

Background

Chédiak-Higashi syndrome (CHS) is a rare autosomal recessive disorder (~500 cases reported worldwide) caused by mutations in the lysosomal trafficking regulator gene (*LYST*; Barbosa et al. 1996; Nagle et al. 1996). Because of defective intracellular trafficking, giant lysosomal granules develop within neutrophils and other cells (e.g., melanocytes, fibroblasts, neural Schwann cells), leading to abnormal lysosome fusion with phagosome, which subsequently results in impaired function of multiple body cells (Faigle et al. 1998; Introne et al. 1999). CHS is characterized by oculocutaneous albinism, bleeding diathesis, recurrent bacterial infections, immunodeficiency, and neurological dysfunctions (Faigle et al. 1998; Introne et al. 1999; Ward et al. 2002). About 85% to 90% of CHS patients develop the severe form of these phenotypes and are diagnosed as having “classic” CHS. The remaining 10% to 15% develop milder phenotypes and are diagnosed as having “atypical CHS” (Karim et al. 2002; Weisfeld-Adams et al. 2013). Classic CHS is often noted in children who are at high risk of progressing to the accelerated phase, and their average life span is estimated to be around 6 y without bone marrow transplantation (BMT; Haddad et al. 1995; Karim et al. 2002). In contrast, atypical CHS is noted in adults who never develop the accelerated phase, but eventually become debilitated due to neurological dysfunction (Weisfeld-Adams et al. 2013).

Previous studies have noted that humans and animals (e.g., mink, mice, cattle, and cats) with CHS disease are more prone to developing aggressive periodontitis (Bailleul-Forestier et al. 2008; Charon et al. 1985; Delcourt-Debruyne et al. 2000; Gustafson 1969; Hamilton and Giansanti 1974; Khocht et al. 2010; Kramer et al. 1977; Lavine et al. 1976; Page and Schroeder 1982; Tempel et al. 1972; Weary and Bender 1967). However, the molecular mechanisms regulating periodontal tissue destruction in CHS remain unclear. It is believed that neutropenia and congenital neutrophil dysfunction in CHS lead to bacterial invasion and rapid destruction of periodontal tissues at a very early age (Delcourt-Debruyne et al. 2000; Shibutani et al. 2000). Similar to CHS, other syndromes associated with neutrophil dysfunction such as leukocyte adhesion deficiency and Papillon-Lefèvre syndrome also present with aggressive periodontitis early in life (Hart et al. 2000; Moutsopoulos et al. 2014). The aggressive periodontitis noted in CHS is generally unresponsive to mechanical removal of oral bacterial biofilm and/or antibiotics (Deas et al. 2003; Shibutani et al. 2000). Furthermore, in contrast to CHS, individuals with chronic granulomatous disease are less susceptible to aggressive periodontitis despite featuring defective neutrophil bactericidal activity (Nussbaum and Shapira 2011). Collectively, these findings suggest that the periodontal disease noted in CHS may involve mechanisms in addition to defective immune control of the periodontal microbiota.

Fibroblasts, one of the most abundant resident cells in the periodontal tissues, form the first line of defense against microorganisms apart from neutrophils (McCulloch and Bordin 1991). Beyond their structural role in synthesizing and remodeling the extracellular matrix, fibroblasts secrete and respond to cytokines, chemokines, and growth factors (McCulloch and Bordin 1991). While several studies (Faigle et al. 1998; Ward et al. 2002), including our recent study on natural killer (NK) cells

(Gil-Krzewska et al. 2016), have focused on the effects of *LYST* mutations on immune cells, the effects of *LYST* mutations on fibroblasts remain unclear. Furthermore, recent evidence suggests that toll-like receptor (TLR) signaling is functionally intertwined with the endosomal/phagosomal trafficking system, and *LYST* functions as a physiological regulator of TLR-mediated proinflammatory response (Westphal et al. 2017). To date, little is known about how the impaired endosomal/phagosomal trafficking system affects TLR signaling in immune and nonimmune cells of CHS patients. This study sought to understand if the *LYST* mutation affects TLR expression/function and proinflammatory response in gingival fibroblasts and how the underlying process may potentially contribute to the development of periodontal disease in classic and atypical CHS patients. In addition, to the best of our knowledge, this is the first study to report the periodontal findings in atypical CHS patients.

Materials and Methods

Patients

This study was approved by the National Human Genome Research Institute (protocol 00-HG-0153, ClinicalTrials.gov identifier NCT00005917) and Piracicaba Dental School (protocol 078/2014) Institutional Review Boards. Written informed consent was obtained from all patients or their legal guardians according to the guidelines of the Declaration of Helsinki. From a cohort of CHS patients followed at the National Institutes of Health (NIH), 3 patients with classic form of CHS and 4 patients with atypical form of CHS were enrolled in this study. Individuals were diagnosed as having classic CHS if they had 2 nonsense or truncating mutations, while individuals with atypical CHS had 1 missense and 1 nonsense or truncating mutation or 2 missense mutations (Introne et al. 1993). In addition, classic CHS patients had minimal *LYST* protein expression, while atypical CHS patients had substantial residual *LYST* protein expression.

Clinical Periodontal Examination

All CHS patients underwent a comprehensive periodontal examination including a digital panoramic radiograph. A trained examiner (V.T.M.) measured probing depth (PD), clinical attachment level, and bleeding on probing (BOP) at 6 sites on all teeth and measured gingival index and plaque index on 6 index teeth (Ramfjord 1967). Periodontal measurements were also obtained from age-matched healthy controls for comparison (courtesy of Patricia Lenton, University of Minnesota School of Dentistry, and Dr. Ann L. Griffen, The Ohio State University, College of Dentistry). Blood studies were completed for all CHS patients as part of their annual medical examination.

Cell Isolation, Culture, and Treatment

Primary gingival fibroblasts were obtained from atypical ($n = 3$) and bone marrow transplanted classic CHS patients ($n = 3$) at the NIH and from a different group of healthy controls at Piracicaba Dental School ($n = 3$, mean age = 20.7 ± 4.7 y, gender = 2 males and 1 female). All assays including control cells and CHS cells were performed at the same time. Twenty-four hours after plating the cells at 2×10^5 cells/well in 12-well culture plates, cells were challenged with whole extract of *Fusobacterium nucleatum* (2 μ g/mL) for 3 h. Cells from passages 2 to 6 were used for all experiments. Details about fibroblast isolation are described in the Appendix Materials and Methods section.

Preparation of *F. nucleatum* Extract

Preparation of *F. nucleatum* extract is described in detail in the Appendix Materials and Methods section. *F. nucleatum* strain ATCC25586 was used in this study.

SDS-PAGE and Western Blotting

For protein analysis, total cellular proteins were extracted from cells using a lysis and extraction kit (Thermo Fisher Scientific). EDTA-free protease inhibitor cocktail (Thermo Fisher Scientific) was

included to prevent protein degradation during the extraction process. Protein concentration was determined using the Bradford assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as previously described (Wang et al. 2014). Primary antibodies against TLR-2, TLR-4, and GAPDH (all from Abcam) were used to detect proteins of interest. Chemiluminescent and fluorescent signal from blots were documented and quantified by the automated image acquisition program Alliance 4.7 Chemiluminescence and fluorescence systems (UVITEC).

Secretome Multiplex Analysis

Bio-Plex assay (Cat No. 171A1001P, Bio-Rad) was used to determine inflammatory target cytokine levels (Appendix Table 1) in the supernatants from serum-free *F. nucleatum*-treated and -untreated gingival fibroblasts of classic CHS, atypical CHS, and control subjects. Details about the assay are described in the Appendix Materials and Methods section.

Statistical Analysis

All experiments were performed in triplicate and repeated at least twice. Values are presented as mean $\pm$ standard deviation. Intragroup protein expression data were analyzed by the Student's *t* test (untreated versus treated cells), whereas intergroup analysis was performed by 1-way analysis of variance followed by the Bonferroni's test ($\alpha = 0.05$) to compare the treated groups (GraphPad Prism software version 6 [GraphPad Software] and BioEstat 5.3 [Mamiraua Institute]). Periodontal measurements of CHS and control subjects were compared using a 2-sample *t* test.

Results

Patient Characteristics

The demographic characteristics of the study population are shown in Table 1. All CHS patients were Caucasians and exhibited some degree of oculocutaneous albinism, silvery gray

hair, bleeding diathesis, or a history of excessive infections in childhood. Most of the patients also presented with skin infections, respiratory infections, photophobia, gait abnormalities, higher body mass index, and a varying degree of neurologic manifestations. Notably, the 3 classic CHS patients had undergone BMT with conditioning regimen for correction of hematologic and immunologic defects associated with CHS. Subject CHS 1 was treated for hemophagocytic lymphohistiocytosis at the age of 7 y, prior to BMT at the age of 10 y. None of the atypical CHS patients underwent BMT. All CHS patients exhibited mutations in the *LYST/CHS1* gene, except CHS patient 1, in whom the precise mutation is currently being established. Appendix Table 2 and the Appendix Figure present the genotype data. None of the families presented a history of endogamy or consanguinity.

Clinical and Radiographic Periodontal Findings

None of the atypical or BMT classic CHS patients presented evidence of generalized aggressive periodontitis, gingival ulceration, severe tooth mobility, or premature exfoliation of deciduous and permanent teeth (Fig. 1A–D). However, both atypical and classic CHS patients exhibited increased PD, clinical attachment loss (AL), and BOP when compared with age-matched healthy controls, mainly owing to irregular and poor access to dental care. Based on the American Academy of Periodontology (2015) guidelines, they were classified as having mild chronic periodontal disease. Atypical CHS patients also exhibited increased carious teeth and mild to moderate gingival inflammation. Notably, both classic and atypical CHS patients exhibited a higher number of sites with BOP (classic: 32% and atypical: 52%). Table 2 summarizes the clinical periodontal findings in atypical and BMT classic CHS patients compared with age-matched healthy controls. Panoramic radiographs from both CHS groups showed no evidence of extensive alveolar bone loss or radiolucent lesions (Fig. 1B, D).

Table 1.

Baseline Characteristics of CHS Patients.

Subjects ^a	Age, Gender	Race	Preexisting Medical Conditions	Past Medical Therapy	Active Medications
CHS 1 (classic post-BMT)	32, F	White	Peripheral neuropathy; gait abnormality; hypothyroidism (well controlled); oculocutaneous albinism; left eye amblyopia; obesity; 2 episodes of left tibial fracture	S/P bone marrow transplant and chemotherapy at age 10; eye surgery for amblyopia; left tibial internal reduction	Levothyroxine, 125 µg/d; tumeric 400 mg/d; magnesium potassium chelates; multivitamins; food-based calcium; omega-3 fish oil; 5-hydroxytryptophan
CHS 2 ^b (atypical)	32, M	White	Parkinsonism secondary to CHS; gait abnormality; partial oculocutaneous albinism; chickenpox; recurrent skin infections; recurrent epistaxis; poor wound healing	Intravenous immunoglobulin monthly from 1 y to 16 y of age	Cephalexin 500 mg/d; carbidopa/levodopa 25 mg/100 mg tid; ibuprofen 400 mg/d
CHS 3 ^b (atypical)	28, M	White	Peripheral neuropathy; photophobia; attention deficit disorder; ataxia; recurrent skin infections; recurrent epistaxis	Intravenous immunoglobulin monthly from 2 mo to 14 y of age	None
CHS 7 ^c (atypical)	29, M	White	Oculocutaneous albinism; photophobia; strabismus; gait abnormality; anosmia; easy bruising; 2 episodes of wrist fracture	NS	None
CHS 8 ^c (atypical)	23, M	White	Oculocutaneous albinism; photophobia; ataxia; hypertension; obesity	Lisinopril	None
CHS 9 (classic post-BMT)	8, M	White	Oculocutaneous albinism; attention-deficit hyperactivity disorder; chickenpox postvaccination; hepatomegaly	S/P 2 bone marrow transplants at 10 mo and 18 mo of age; second transplant preceded by cyclophosphamide, fludarabine, antithymocyte globulin, total-body irradiation, mycophenolate mofetil, tacrolimus; left lower-lung lobectomy	None
CHS 10 (classic post-BMT)	9, F	White	Oculocutaneous albinism; recurrent otitis media	S/P 3 allogenic stem cell transplants at 20, 24, and 25 mo of age; third transplant preceded by campath, thiotepa, and cyclophosphamide	None

BMT, bone marrow transplantation; CHS, Chédiak-Higashi syndrome; NS, nonsignificant; S/P, status post.

^aPatients presented in the order of recruitment.^bSiblings.^cSiblings.

Table 2.

Comparisons of Clinical Periodontal Measurements (Mean [SD]) between CHS Patients and Controls.

Characteristic	Classic (n = 3)	Control (n = 3)	P Value	Atypical (n = 4)	Control (n = 4)	P Value
Age, mean (SD), y	16.3 (13.6)	15.7 (13.3)	0.96	29.7 (3.7)	26.5 (5.1)	0.65
Gender, male/female	1/2	1/2	—	4/0	4/0	—
Number of missing teeth ^a	4.3 (0.6)	4.0 (0)	0.86	6.5 (4.5)	4.0 (0)	0.31
Mean plaque index ^b	0.65 (0.5)	—	—	0.51 (0.2)	—	—
Mean gingival index	0.56 (0.3)	0.53 (0.2)	0.90	0.70 (0.2)	0.63 (0.3)	0.64
Bleeding on probing, %	32.1 (30.0)	4.6 (4.9)	0.001	51.9 (28.9)	4.5 (5.2)	0.001
Mean full-mouth PD, mm	2.14 (0.6)	1.96 (0.8)	<0.001	2.42 (0.6)	1.94 (0.7)	0.008
Mean full-mouth AL, mm	0.84 (1.1)	0.16 (0.4)	<0.001	2.18 (1.2)	0.21 (0.4)	<0.001
% Sites PD ≥ 4 mm	0.20 (0.3)	0.25 (0.4)	0.80	5.34 (2.9)	0.45 (0.6)	<0.001
% Sites AL ≥ 3 mm	6.55 (11.3)	0 (0)	0.001	20.94 (16.3)	0 (0)	<0.001

AL, attachment loss; CHS, Chédiak-Higashi syndrome; PD, probing depth.

Boldface indicates significant P value (<0.05).

^aUnrupted permanent teeth in classic CHS patients 9 and 10 and classic controls are not counted as missing.^bMissing mean plaque index data for control subjects.

Between atypical and BMT classic CHS patients, atypical CHS patients had increased PD (2.42 ± 0.6 mm vs. 2.14 ± 0.6 mm, $P < 0.001$), AL (2.18 ± 1.2 mm vs. 0.84 ± 1.1 mm, $P < 0.001$), and percentage of sites with PD ≥ 4 mm (5.34 ± 2.9 vs. 0.20 ± 0.3 , $P = 0.037$). Furthermore, atypical CHS patients presented with increased percentage of sites with AL ≥ 3 mm (21% vs. 6%), although the difference was statistically insignificant ($P > 0.05$). The difference for missing teeth, plaque index, gingival index, and BOP was also statistically insignificant between classic and atypical patients. Some of the insignificant differences between the groups may be attributed to the small sample size (underpowered), a common issue when dealing with rare diseases.

Laboratory Findings

Successive blood counts during a 2-y medical evaluation revealed neutropenia in 3 atypical and 1 BMT classic CHS patients and monocytosis in 1 classic and all of the 4 atypical CHS patients.

The other white blood cells and red blood cells were within normal limits. Platelet count was low in 1 atypical CHS patient. Table 3 shows the laboratory values. May-Grünwald-Giemsa staining of peripheral blood smear revealed the presence of pathognomonic, abnormal, pink, spherical granules in the cytoplasm of lymphocytes and neutrophils of CHS patients (Fig. 1H-L). The granules varied in size and were single or multiple in number. In all classic CHS patients who underwent BMT, posttransplant blood smear showed normal-appearing neutrophils and lymphocytes with absence of pathognomonic spherical granules in the cytoplasm (Fig. 1M, N). Myeloperoxidase staining showed that peroxidase activity (appearing as dark blue granules) was distributed homogenously throughout the neutrophils from control patients, whereas in atypical CHS patients 7 and 8, the granules were more punctate (Fig. 1E-G). The NK cell function in atypical CHS patients (CHS 2, 3, 7, 8) and BMT classic CHS patient (CHS 1) is illustrated in Figure 2A-H. Detailed findings are

reported elsewhere (Gil-Krzewska et al. 2016).

CHS Gingival Fibroblasts Exhibit Altered Expression of TLR-2/TLR-4 at Baseline and in Response to *F. nucleatum* Treatment

To determine whether *LYST* mutation affects TLRs in gingival fibroblasts, the expression of TLR-2 and TLR-4 was measured by Western blotting (Fig. 2I). At baseline, TLR-2 protein expression was higher in atypical and BMT classic CHS cell groups compared with control gingival fibroblasts (atypical: 1.81-fold and classic: 1.56-fold). In contrast, TLR-4 protein expression was significantly lower in atypical (–2.50-fold) and classic CHS cells (–3.85-fold) compared with control cells. Upon *F. nucleatum* treatment, both atypical and classic CHS gingival fibroblasts failed to up-regulate the expression of TLR-2 (atypical: –1.56-fold, classic: –1.19-fold vs. control: 1.70-fold) and TLR-4 (atypical: 1.22-fold, classic: –1.26-fold vs. control: 4.40-fold) when compared with their respective untreated groups and control cells.

Figure 1. Clinical and radiographic images of the oral status of classic Chédiak-Higashi syndrome (CHS) patient 9 (**A, B**) and atypical CHS patient 3 (**C, D**). Note the pink, healthy gingiva in classic CHS patient 9 without evidence of inflammation, swelling, gingival ulceration, gingival recession, alveolar bone loss, or premature exfoliation of teeth. In contrast, CHS patient 3, one of the most advanced cases in the atypical CHS group, showed mild to moderate plaque accumulation with apparent signs of marginal gingival inflammation, swelling, gingival recession, alveolar bone loss, and tooth loss. In this subject, most of the teeth were lost due to dental caries. (**E, G**) Myeloperoxidase (MPO) staining illustrates peroxidase activity as dark blue granules in the cytosol. Note that in the neutrophils from the control cells, the blue granules are distributed homogeneously throughout the cells, whereas in CHS patients 7 and 8, the granules are more punctate. (**I–L**) Peripheral blood smear from atypical CHS patients 7 and 8 and pretransplant blood smear from classic CHS patient 10 shows large polymorphonuclear neutrophils and lymphocyte with pathognomonic, abnormal, pink, spherical granules in the cytoplasm. (**M, N**) Posttransplant blood smear from classic CHS patient 10 shows normal-appearing neutrophils with absence of pathognomonic spherical granules in the cytoplasm (May-Grünwald/Giemsa stain; original magnification 400 $\times$).

Table 3.
Physical Parameters and Blood Values.

Parameter	Normal Range	CHS 1 (Classic post-BMT)	CHS 9 (Classic post-BMT)	CHS 10 (Classic post-BMT)	CHS 2 (Atypical)	CHS 3 (Atypical)	CHS 7 (Atypical)	CHS 8 (Atypical)
Weight, kg	—	80	35	34	93	116	122	137
Height, cm	—	163	134	129	174	183	176	179
Hemoglobin, g/dL	11.2-17.5	12.4	11.7	13.1	15.9	14.4	14.4	15.0
Hematocrit, %	34.1-51.0	37.2	34.6	37.7	45.4	40.7	43.2	41.0
MCV, fL	79.0-94.8	90.5	82.8	82.5	82.7	79.2	84.2	83.0
WBC, K/ μ L	3.98-10.04	4.14	↓3.75	5.30	↓2.15	4.18	7.10	6.06
MPV, fL	9.2-12.4	11.6	9.2	9.2	↑12.7	10.0	10.6	9.9
Platelets, K/ μ L	161-369	173	181	227	↓118	182	275	251
Neutrophils, %	34.0-71.1	52.2	↓31.6	44.8	↓32.2	↓26.9	44.2	↓30.5
Lymphocytes, %	19.3-53.1	39.4	49.9	42.6	50.2	51.4	39.4	52.8
Monocytes, %	4.7-12.5	7.0	↑17.1	7.7	↑15.3	↑19.9	↑14.2	↑12.7
Eosinophils, %	0.7-7.0	1.0	1.1	4.3	2.3	1.4	1.8	1.0
Basophils, %	0.1-1.2	0.2	0.3	0.4	0.0	0.2	0.3	0.0
Immature granulocytes, %	0.0-0.4	0.2	0.0	0.2	0.0	0.2	0.1	NA
Total bilirubin, mg/dL	0.0-1.2	0.4	0.6	0.2	0.8	0.3	0.5	0.6
Direct bilirubin, mg/dL	0.0-0.3	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
CRP, mg/L	0.0-4.99	1.4	0.6	0.3	0.80	1.6	↑6.60	↑5.30
PT, s	11.6-15.2	13.5	13.5	13.7	↑15.7	14	14.3	14.6
aPTT, s	25.3-37.3	27.5	33.2	34.9	↑42.1	↑37.8	33.3	32.0

Abnormal values are indicated with arrows. aPTT, activated partial thromboplastin time; CHS, Chédiak-Higashi syndrome; CRP, C-reactive protein; MCV, mean corpuscular volume; MPV, mean platelet volume; PT, prothrombin time; WBC, white blood cell count.

CHS Gingival Fibroblasts Exhibit Altered Cytokine Production in Response to Whole Bacterial Extract of *F. nucleatum*

The cytokine multiplex assay showed that *F. nucleatum*-treated control and CHS gingival fibroblasts exhibited increased expression of secreted cytokines when compared with their respective untreated groups. However, the magnitude of change was significantly higher in treated atypical CHS cells compared with treated control and BMT classic CHS cells: interleukin (IL)-2 (classic: 1.71-fold, atypical: 4.41-fold), IL-4 (classic: 1.76-fold, atypical: 4.28-fold), IL-5 (classic: 1.19-fold, atypical: 1.99-fold), IL-6 (classic:

28.85-fold, atypical: 74.76-fold), IL-10 (classic: 1.24-fold, atypical: 2.94-fold), IL-12 (classic: 2.20-fold, atypical: 3.80-fold), interferon- γ (classic: 4.08-fold, atypical: 8.30-fold), and tumor necrosis factor- α (TNF- α ; classic: 11.20-fold, atypical: 104.83-fold). The fold change in control cells ranged from 1.70 to 39.7. Figure 2J and Appendix Table 3 illustrate secretome findings for the target cytokines assayed.

Discussion

To the best of our knowledge, this is one of the first studies to report the periodontal findings in atypical CHS patients along with defining the

periodontal status of BMT classic CHS patients. In contrast to previous clinical reports, atypical and BMT classic CHS patients in this study presented with mild chronic periodontitis. Primary gingival fibroblasts from CHS patients presented decreased TLR-2 and TLR-4 protein expression upon *F. nucleatum* exposure when compared with treated healthy control gingival fibroblasts. In addition, control and CHS gingival fibroblasts treated with *F. nucleatum* exhibited increased cytokine expression when compared with their respective untreated groups. However, the magnitude of change was significantly higher in treated atypical CHS gingival fibroblasts compared with treated control

Figure 2. Cytotoxicity of Chédiak-Higashi syndrome (CHS) natural killer (NK) cells and immunoinflammatory patterns of CHS gingival fibroblasts. NK cells from CHS patients exhibit significantly reduced natural and antibody-dependent cell-mediated cytotoxicity. (A) The percentage of CD3⁺CD56⁺ NK cells in the lymphocyte population of peripheral blood mononuclear cells was decreased in CHS patients when compared with their family members (note that lymphocytes were pregated on forward vs. side scatter and then gated on CD56 vs. CD3 marker). (B–D) CHS patients exhibited NK cells with significantly reduced natural cytotoxicity (against 721.221 and K562 cells) and almost completely inhibited antibody-dependent cell-mediated cytotoxicity (against SK-OV3 cells) when compared with healthy controls. (E, F) When co-cultured with K562 target cells, a significant increase in the percentage of interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α)-positive NK cells was noted in CHS patients when compared with controls. (G, H) NK cells from CHS patients secreted similar amounts of IFN- γ and TNF- α as control NK cells in response to interleukin (IL)-12, IL-15, and IL-18 stimulation, indicating that cytokine exocytosis was not overly affected in CHS NK cells. (I) Western blot result shows that in untreated and *F. nucleatum*-treated gingival fibroblasts, toll-like receptor-4 (TLR-4) protein level is significantly lower in both atypical and bone marrow-transplanted classic CHS groups when compared with the control group. In addition, in untreated gingival fibroblasts, TLR-2 protein level was found to be higher in atypical and classic CHS groups when compared with the control group. Following *F. nucleatum* treatment, TLR-2 and TLR-4 expression was reduced in both CHS cell groups compared with the control group. (J) The secretome multiplex assay showed that when compared with their respective untreated groups, treated control, atypical CHS, and bone marrow-transplanted classic CHS gingival fibroblasts presented increased expression of secreted cytokines. However, the magnitude of change was significantly higher in treated atypical CHS gingival fibroblasts when compared with treated control gingival fibroblasts. For Western blot results, the bars are representative of the mean $\pm$ standard deviation of the protein expression values for TLR-2 and TLR-4 normalized by GAPDH protein values, obtained from gingival fibroblasts of atypical CHS patients ($n = 3$), bone marrow-transplanted classic CHS patients ($n = 3$), and healthy controls ($n = 3$). Comparable results were obtained in at least 2 additional and independent experiments. For secretome results, the bars are representative of the mean $\pm$ standard deviation of the levels of secreted cytokines (pg/mL) estimated in duplicate samples from atypical CHS patients ($n = 3$), bone marrow-transplanted classic CHS patients ($n = 3$), and healthy controls ($n = 3$). *Statistical intragroup differences by Student's t test ($P < 0.05$). #Statistical intergroup differences between treated CHS versus treated control cells by 1-way analysis of variance, followed by the Bonferroni's test ($P < 0.05$). ns, nonsignificance ($P > 0.05$). †Siblings. Note that 2 B-H was adapted from “Chédiak-Higashi Syndrome: Lysosomal Trafficking Regulator Domains Regulate Exocytosis of Lytic Granules but Not Cytokine Secretion by Natural Killer Cells” (Gil-Krzewska et al. 2016). (Adapted with permission from the *Journal of Allergy and Clinical Immunology*.)

Figure 2. (continued)

and classic CHS gingival fibroblasts (Fig. 2J). The differential response to bacterial challenge by atypical and classic CHS gingival fibroblasts may be attributed to the defective trafficking of TLR-2 and TLR-4. In agreement with this hypothesis, our previous study shows that skin fibroblasts from BMT classic CHS patients exhibit defective intracellular trafficking of TLR-2 and TLR-4, which likely contributes to hyposensitive inflammatory response upon immunogenic challenge (Wang et al. 2014).

Previous reports have noted severe gingivitis or aggressive periodontitis associated with advanced bone loss and tooth mobility in CHS patients (Bailleul-Forestier et al. 2008; Delcourt-Debruyne et al. 2000; Khocht et al. 2010; Rezende et al. 2013; Shibutani et al. 2000). Most of these findings have been reported in children who developed severe periodontitis by the age of 15 to 20 y and subsequently underwent full-mouth tooth extraction (Bailleul-Forestier et al. 2008; Hamilton and Giansanti 1974; Khocht et al. 2010; Tempel et al. 1972; Weary and Bender 1967). Animal studies of CHS-affected mink and mice have also observed

severe and widespread destruction of gingival and periodontal tissues (Gustafson 1969; Lavine et al. 1976). In contrast to these findings, atypical CHS patients and BMT classic CHS patients (with restored hematopoietic cell defect) in this study exhibited mild chronic periodontitis, without evidence of extensive alveolar bone loss and premature exfoliation of teeth. These differences may be partly explained by the fact that atypical CHS patients have residual LYST protein function and retain some degree of functional immune cells, which may prevent them from developing the aggressive form of periodontitis. Also, classic CHS patients in this study had undergone BMT, which may have corrected the neutrophil, B-, T-, and NK cell dysfunction to a certain extent and hence reduced the susceptibility to severe periodontitis. Indeed, our results show that in CHS patient 1, who underwent BMT, NK cell function was restored to normalcy and was indistinguishable from NK cells isolated from healthy donors (Fig. 2B–H). Furthermore, the improved life expectancy in BMT classic CHS patients corroborates the correction of hematologic and immunologic defects.

Posttransplant blood smear from classic CHS patients shows normal-appearing neutrophils with absence of pathognomonic spherical granules (Fig. 1K–N). The correction of immune cell dysfunction in classic CHS patients may also partly explain the relatively reduced PD and AL noted in this group when compared with atypical CHS patients, who never underwent BMT. In the previously published studies, it is unclear if patients had classic CHS or atypical CHS or if they ever received BMT. Furthermore, the limited study population makes it difficult to draw conclusions based on the clinical parameters.

The pathogen-recognition receptors such as TLRs, NOD-like receptors, and receptor kinases present on the resident cells recognize microbial structures (e.g., lipopolysaccharide [LPS], peptidoglycans, bacterial DNA, and double-stranded RNA) highly conserved among periodontal pathogens, to activate immune and local inflammatory responses (Mahanonda and Pichyangkul 2007). In periodontal tissues, TLR-2 and TLR-4 are the major receptors that recognize microbial molecules (Mahanonda and Pichyangkul 2007). In

the current study, gingival fibroblasts from BMT classic CHS patients presented decreased expression of TLR-4 at baseline and following *F. nucleatum* challenge, which was associated with chemokine and cytokine production (except TNF- α) similar to control gingival fibroblasts. These results are in contrast with previous findings, which show that TLR-2^{-/-} and TLR-4^{-/-} mice exhibit diminished production of cytokines (e.g., IL-1 β , TNF- α , and CCL5) when compared with their respective wild-type mice (Gelani et al. 2009; Lima et al. 2010). Genetic variations or deficiency of TLR-2 and TLR-4 in humans and mice have been suggested to influence susceptibility to periodontitis by causing impaired recognition of periodontal pathogens (Gelani et al. 2009; Schroder et al. 2005). The molecular mechanisms contributing to the measured production of chemokines and cytokines in classic CHS cells remain unclear.

In addition, when compared with control gingival fibroblasts, atypical CHS gingival fibroblasts exhibited increased TLR-2 and decreased TLR-4 expression at baseline, which remained suppressed upon *F. nucleatum* challenge. However, chemokines and cytokines production was significantly increased in atypical CHS gingival fibroblasts following *F. nucleatum* challenge. Currently, the molecular mechanism(s) driving increased chemokines and cytokines production in atypical CHS gingival fibroblasts, despite featuring deficient TLR-2 and TLR-4, remains unclear and needs to be further investigated. Gingival fibroblasts are known to express various innate immune receptors including TLR-1 to TLR-9, MD-2, MyD88, and NOD1 and NOD2 (Uehara and Takada 2007). It is possible that other TLRs or NOD molecules may be contributing to the increased chemokines and cytokines production in CHS gingival fibroblasts. In an experimental murine periodontitis model, TLR-9-mediated inflammation has been shown to facilitate alveolar bone loss (Kim et al. 2015). In addition, our previous study suggests that the MyD88-dependent pathway that relies on

functioning cell surface TLRs is severely defective in classic CHS skin fibroblasts (Wang et al. 2014). It is possible that the MyD88-dependent TLR signaling pathway may differently affect CHS patients and thus partly contribute to the increased proinflammatory cytokine production noted in atypical CHS. Furthermore, recent evidence suggests that *LYST* selectively controls TLR-3- and TLR-4-mediated endosomal TRIF signaling pathways that are involved in the induction of proinflammatory cytokines (Westphal et al. 2017). The role of TRIF signaling mediated inflammatory response in CHS gingival fibroblasts needs to be further explored.

Although this study did not include a microbiological assessment of CHS patients, previous studies have noted bacterial invasion and persistence within gingival tissues that could directly mediate tissue destruction in CHS patients (Delcourt-Debruyne et al. 2000; Hamilton and Giansanti 1974; Khocht et al. 2010; Tempel et al. 1972). As a preliminary step in understanding the immunoinflammatory response of CHS gingival fibroblasts to periodontal pathogens, this study focused on *F. nucleatum*, one of the first and most abundant gram-negative anaerobes to become established within the dental plaque (Signat et al. 2011). Corroborating the results noted with *F. nucleatum* treatment, atypical and classic CHS gingival fibroblasts treated with *Escherichia coli* LPS showed significantly reduced TLR-2 and TLR-4 protein expression when compared with healthy gingival fibroblasts (data not shown as a supplement). These results further indicate that the TLR-2 and TLR-4 expression/function in CHS gingival fibroblasts are distinctly affected.

In addition, atypical and BMT classic CHS patients in this study exhibited a 7- to 11-fold higher mean percentage of sites with BOP (classic: 32% and atypical: 51%) when compared with healthy controls (4.5%). Similar to our results, other studies have noted increased BOP in CHS patients (Bailleul-Forestier et al. 2008; Charon et al. 1985; Delcourt-

Debruyne et al. 2000; Rezende et al. 2013). Impaired platelet aggregation due to dense body deficiency in CHS patients is suggested to result in prolonged and excessive bleeding. Prothrombin time and activated partial thromboplastin time was normal in both atypical and BMT classic CHS patients in this study, except for a marginal increase in CHS patients 2 and 3. Furthermore, atypical CHS patients reported increased tooth loss due to dental caries. Similarly, marked dental caries has been noted in a number of CHS reports (Tempel et al. 1972; Weary and Bender 1967); however, its relationship to the disease is unknown.

In conclusion, results from this study suggest that BMT classic CHS patients and atypical CHS patients are less susceptible to aggressive periodontitis when compared with classic CHS patients who never receive BMT and that periodontitis susceptibility in CHS is mainly mediated by hematopoietic cells. Furthermore, our results demonstrate that TLR-2 and TLR-4 expression/function in CHS gingival fibroblasts are distinctly affected. The immunoregulatory function of *LYST* is well conserved in human cells (Westphal et al. 2017), and mutations in the *LYST* gene may contribute to defective TLR signaling and immunoinflammatory response, leading to frequent and severe infections noted in CHS. The dysregulated immunoinflammatory response in gingival fibroblasts may have partly contributed to the periodontitis noted in CHS patients. In addition, our preliminary data show that atypical CHS patients exhibit defective neutrophil chemotaxis (data not shown as a supplement), which may have partly contributed to the disease process along with neutropenia. Furthermore, the impaired NK cell cytotoxicity noted in atypical CHS patients may have contributed to the pathogenesis of periodontitis. Previous studies have associated impaired NK cell cytotoxicity with the pathogenesis of periodontitis in several genetic diseases and acquired conditions such as Papillon-Lefèvre

syndrome, Fanconi's anemia, long-standing insulin-dependent diabetes, and smoking (Wilensky et al. 2015). Lastly, the periodontitis noted in CHS patients may be partly attributed to their irregular and poor access to dental care.

Clinicians need to be aware of the fact that not all CHS patients exhibit historically reported severe periodontitis, and most genetic disorders display a spectrum of clinical symptoms. Some of the statistically insignificant differences noted between groups may be attributed to the underpowered nature of this study rather than true lack of biological differences between the groups. The small sample size and investigation of a single periodontal pathogen (*F. nucleatum*) may be a limitation of this rare disease study. However, in comparison to the <20 previously reported case reports of CHS-associated periodontitis (Bailleul-Forestier et al. 2008; Charon et al. 1985; Delcourt-Debruyne et al. 2000; Hamilton and Giansanti 1974; Khocht et al. 2010; Rezende et al. 2013; Shibutani et al. 2000; Tempel et al. 1972; Weary and Bender 1967), this study includes the largest CHS patient population and is one of the first studies to report the clinical periodontal findings in atypical CHS patients. Detailed investigation of leukocyte adhesion deficiency, another rare disease associated with neutrophil dysfunction, has provided new insights into the role of the IL-23–IL-17 axis in driving dysbiosis and inflammatory mediated periodontal bone loss (Moutsopoulos et al. 2014). Similarly, results from this study provide a better understanding of the molecular mechanisms governing the periodontal disease process and perhaps other situations in which patients exhibit altered TLRs and immune cell dysfunction. It also raises new hypotheses for future research and the need for personalized intervention strategies tailored to an individual's host response.

Author Contributions

V. Thumbigere Math contributed to conception, design, data acquisition, analysis, and interpretation and drafted

and critically revised the manuscript; P. Rebouças contributed to conception, design, and data acquisition and drafted and critically revised the manuscript; P.A. Giovani, R.M. Puppini-Rontani, R. Casarin, L. Martins, L. Wang, and K. Krzewski contributed to conception, design, and data interpretation and critically revised the manuscript; W.J. Introne contributed to conception, design, and data interpretation and drafted and critically revised the manuscript; M.J. Somerman, F.H. Nociti Jr., and K.R. Kantovitz contributed to conception, design, data acquisition, analysis, and interpretation and drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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