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Exaggerated inflammatory responses mediated by *Burkholderia cenocepacia* in human macrophages derived from Cystic fibrosis patients

Benjamin T. Kopp [a,*], Basant A. Abdulrahman [b,c], Arwa A. Khweek [b], Surender B. Kumar [b], Anwari Akhter [b], Richard Montione [d], Mia F. Tazi [b], Kyle Caution [b], Karen McCoy [a], Amal O. Amer [b]

[a] *Section of Pediatric Pulmonology, Nationwide Children's Hospital, Columbus, OH, United States*
[b] *Department of Microbial Infection and Immunity and the Department of Internal Medicine, The Ohio State University, Columbus, OH, United States*
[c] *Biochemistry and Molecular Biology Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt*
[d] *Campus Microscopy and Imaging Facility, The Ohio State University, Columbus, OH, United States*

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ABSTRACT

Cystic fibrosis (CF) is accompanied with heightened inflammation worsened by drug resistant *Burkholderia cenocepacia*. Human CF macrophage responses to *B. cenocepacia* are poorly characterized and variable in the literature. Therefore, we examined human macrophage responses to the epidemic *B. cenocepacia* J2315 strain in order to identify novel anti-inflammatory targets. Peripheral blood monocyte derived macrophages were obtained from 23 CF and 27 non-CF donors. Macrophages were infected with *B. cenocepacia* J2315 and analyzed for cytokines, cytotoxicity, and microscopy. CF macrophages demonstrated significant increases in IL-1β, IL-10, MCP-1, and IFN-γ production in comparison to non-CF controls. CF patients on prednisone exhibited globally diminished cytokines compared to controls and other CF patients. CF macrophages also displayed increased bacterial burden and cell death. In conclusion, CF macrophages demonstrate exaggerated IL-1β, IL-10, MCP-1, and IFN-γ production and cell death during *B. cenocepacia* infection. Treatment with corticosteroids acutely suppressed cytokine responses.

1. Introduction

Cystic fibrosis (CF) is the most common life-limiting autosomal recessive disease among people of European descent, affecting over 70,000 people worldwide [1]. CF causes a wide range of systemic symptoms due to viscous mucus plugging in epithelial lined organs, but is most recognized for chronic, progressive respiratory infections which contribute to over 85% of patient deaths [2]. One of the major contributors to the chronic respiratory symptoms of CF patients is an exaggerated systemic and lung inflammatory state with sustained pro-inflammatory mediators present in CF patients from an early age in response to infection [3], as well as neutrophilic lower airway inflammation independent of infection [4]. An area of investigation into the sustained pro-inflammation in CF has been the role of polymorphisms in IL-1β genes that are thought to help modulate CF lung disease, as patients with similar genotypes often manifest different disease phenotypes based on their particular polymorphism [5]. CF patients identified with two particular IL-1β gene single nucleotide polymorphisms demonstrated increased long term disease severity, underscoring the importance of IL-1β in CF and implicating a contribution of genetic variation in IL-1β in the overall pathogenesis of lung function decline in CF [5].

CF patients are affected by numerous bacterial pathogens, but a particularly pathogenic bacterium among CF patients is *Burkholderia cenocepacia*. This microorganism is a member of the Burkholderia Cepacia Complex, which is known to cause exaggerated inflammation in CF patients and is rapidly transmissible from patient to patient [6–8]. Clinical disease can range from a severe and often fatal sepsis state termed 'cepacia syndrome', to a chronic progressive deterioration of CF lung disease associated with worsened outcomes for CF patients [9]. Due to poor lung transplant post-operative outcomes and high antibiotic resistance, infection with *B. cenocepacia* is usually an exclusion criterion from lung transplant eligibility in most centers, thereby offering little hope to infected CF patients for prolonged survival [10,11]. *B. cenocepacia* can also infect patients with chronic granulomatous disease (CGD), a disorder characterized by defective microbial phagocytosis and increased caspase-1 dependent IL-1β production in the absence of reactive oxygen species [12].

Abbreviations: CF, Cystic fibrosis; CGD, Chronic granulomatous disease; IL-1β, Interleukin 1β; IL-10, Interleukin 10; IFN-γ, Interferon gamma; LDH, Lactate dehydrogenase; MCP-1, Monocyte chemotactic protein 1; MDM, Monocyte derived macrophage.

* Corresponding author. Fax: +1 614 722 4755.
E-mail address: Benjamin.Kopp@NationwideChildrens.org (B.T. Kopp).

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Because of the refractory nature and sustained inflammation of *B. cenocepacia* infections in CF, we are investigating inflammatory pathways to better understand responses to CF pathogens and uncover new therapeutic targets. Recently, we demonstrated that murine CF macrophages had increased IL-1β release in response to *B. cenocepacia* infection in comparison to normal controls [13]. Murine models also demonstrated a caspase-1 dependent IL-1β production similar to that seen in CGD [13]. However, human CF studies are varying in the reported impact of IL-1β production, and incomplete in characterization of responses to *B. cenocepacia* infection. Longitudinal studies have shown very little change in IL-1β production over the first years of life [14], whereas others have shown increased IL-1β within subsets of CF patients infected with viruses or various bacterial pathogens [3]. In addition, non CF human macrophages have demonstrated increased IL-1β in response to *B. cenocepacia* [15], but it is unclear how this response compares to CF macrophages and the overall impact on inflammation in CF. In this study, using the virulent *B. cenocepacia* J2315 strain, we found increased levels of IL-1β, IL-10, MCP-1, and IFN-γ in infected human CF macrophages, along with increases in cell death and bacterial survival compared to non CF macrophages.

2. Materials and methods

2.1. Bacterial strains and culture

B. cenocepacia strain J2315 was originally isolated from a CF patient of the prototypic epidemic ET12 lineage [16]. Strain J2315 was the index strain from which patient-to-patient spread of this lineage was first reported in Edinburgh, Scotland [17]. The bacterial strain was grown in Luria–Bertani (LB) broth at 37 °C overnight with high amplitude shaking.

2.2. Monocyte-derived Macrophages (MDMs) with Ethics Statement

Monocytes were isolated from heparinized blood of 23 CF and 27 non CF human donors aged 2–46 years (mean CF 23.4 years, non CF 23 years). Patient demographics are listed in Table 1. No patients were colonized with *B. cenocepacia* and subjects were excluded if on any chronic immunosuppressants. Non CF patients represented a mixed population of healthy volunteers and asthma exacerbations to represent a model of airway inflammation for comparison to CF. 5 CF and 3 non CF patients received 60 mg of prednisone one time 16–20 h prior to blood collection as part of their clinical care. Written informed consent was obtained from all subjects as approved by the Institutional Review Board of Nationwide Children's Hospital. Written consent from legal guardians of minors was obtained as well as written assent from minors aged 9–17 years. Monocytes were separated from the whole blood using Ficol-Hypaque (Sigma). Isolate monocytes were re-suspended in RPMI (Gibco) plus 10% human AB serum (Lonza) to a concentration of 2×10^6 cells/mL and incubated for 5 days at 37 °C. On day 5 macrophages were isolated and infected with J2315 at a multiplicity of infection (MOI) of 10.

Table 1
Patient Demographics.

	Cystic fibrosis	Non cystic fibrosis
Age (mean years)	23.4	23.0
Caucasion	100%	100%
Male	50%	48%
P. aeruginosa colonization	62%	N/A
MRSA colonization	33%	N/A
Average FEV1% predicted	50% +/− 23%	N/A

2.3. Bioplex

MDMs were infected with J2315 for 24 h and the culture supernatant was collected, centrifuged, and stored at −20 °C until assayed for cytokine content. The concentration of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ, MCP-1, GM-CSF, G-CSF, and MIP-1β in the supernatant was determined by Bioplex assay following the manufacturer's protocol (BioRad, Hercules, CA).

2.4. Enzyme-Linked Immuno Sorbent Assay (ELISA)

MDMs were infected with J2315 for 24 h and culture supernatants were centrifuged and stored at −20 °C until assayed for cytokine content. The quantification of IL-1β, IL-8, IL-10, and TNF-α in the supernatant was determined by sandwich ELISA following the manufacturer's protocol (R&D system Inc, Minneapolis, MN, USA) as previously described [18].

2.5. Cytotoxicity

MDMs were infected with J2315 for 24 h and the culture supernatants were collected and centrifuged. Histone-associated DNA fragments were detected using the cytotoxicity detection photometric assay kit according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Experiments were performed in triplicate.

2.6. Confocal microscopy

2.0×10^5 MDMs cultured on 12 mm glass cover slips in 24 well tissue culture plates were infected synchronously with J2315 at an MOI of 10. Nuclei were stained with the nucleic acid dye 4′,6′-diamino-2-phenylindole (DAPI) and converted to green for pictures. Lysosomes were stained red with Lysotracker Red (L7528, Invitrogen, Carlsbad, CA). One hundred bacteria were scored for each condition, and scoring was verified by blinded reviewers. Experiments were performed in triplicate. Samples were analyzed with an Olympus FV10i Spectral Confocal microscope.

2.7. Transmission electron microscopy

MMDs were isolated and infected with J2315 at an MOI of 10 for 2 h. Cells cultured on Permanox (Lab-Tek) chamber slides were fixed with 2.5% gluteraldehyde in 0.1 M phosphate buffer with 0.1 M sucrose. These were post fixed with 1% osmium tetroxide in phosphate buffer then en bloc stained with 2% uranyl acetate in 10% ethanol, dehydrated in a graded series of ethanols and embedded in Eponate 12 epoxy resin (Ted Pella Inc. USA). Ultrathin sections were cut on a Leica EM UC6 ultra microtome (Leica microsystems, Germany), collected on copper grids, and then stained with lead citrate and uranyl acetate. Images were acquired with an FEI Technai G2 Spirit transmission electron microscope (FEI, USA), Macrofire (Optronics) digital camera and AMT image capture Software.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.0). Unpaired *t*-tests and ANOVA were used when appropriate. Statistical significance was determined with a two-tailed $p < 0.05$.

3. Results

3.1. IL-1β, IL-10, MCP-1, and IFN-γ are increased in blood MDMs derived from CF patients infected with J2315

Previous murine models have demonstrated increased pro-inflammatory IL-1β production in CF macrophages after infection with *B. cenocepacia* K56-2, but studies in humans are controversial [19,20]. Therefore, we assessed multiple inflammatory cytokines to determine differential responses to the J2315 strain in CF patients. Human CF and non CF peripheral MDMs were infected for 24 h with the J2315 strain of *B. cenocepacia* and supernatants of infected macrophages were collected and analyzed for IL-1β, IL-10, MCP-1, and IFN-γ release. IL-1β, IL-10, MCP-1, and IFN-γ production was significantly increased in macrophages derived from CF patients compared to non CF controls (Fig. 1A-D). P values were 0.0105 (IL-1β), 0.0478 (IL-10), <0.001 (MCP-1) and 0.025 (IFN-γ). There was no significant difference in cytokine production between male and female patients (data not shown). There was also no difference when comparing CF patients to matched non CF asthmatic and healthy controls. These results demonstrate that both pro inflammatory IL-1β, MCP-1, and IFN-γ and anti-inflammatory IL-10 are increased in production in response to *B. cenocepacia* infection in macrophages from CF patients. There was no significant difference in IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12, IL-13, IL-17, GM-CSF, G-CSF, MIP-1β, and TNF-α production between CF and non CF patients in response to J2315 infection (data not shown). These results indicate that although these cytokines may be important in other CF pathogen states, their production is not increased in response to *B. cenocepacia* infections in CF patients.

3.2. Corticosteroids decrease inflammatory and anti-inflammatory cytokine production from CF monocyte-derived macrophages

Although control of heightened inflammation during *B. cenocepacia* infection is extremely difficult, corticosteroids have been shown to be acutely beneficial in case reports of *Burkholderia cepacia* sepsis [21]. We therefore analyzed cytokine production in five CF patients (mean age 25.8 years) and three non CF patients (mean age 17 years) on corticosteroid treatment. CF and non CF patients were given one dose of 60 mg prednisone as part of their standard treatment 16–20 h prior to blood isolation. Isolated MDMs were infected with J2315 for 24 h and supernatants were analyzed. There was a significant decrease in IL-1β, IL-10, MCP-1, and IFN-γ production in the CF patients on prednisone in comparison to CF patients not on steroids (Fig. 1A–D). Therefore, exaggerated pro and anti-inflammatory responses in CF patients to *B. cenocepacia* infection may be substantially reduced with corticosteroid treatment.

3.3. B. cenocepacia avoids lysosomal degradation in CF macrophages

Defective macrophage clearance of *B. cenocepacia* has been reported previously as a potential contributor to *B. cenocepacia*

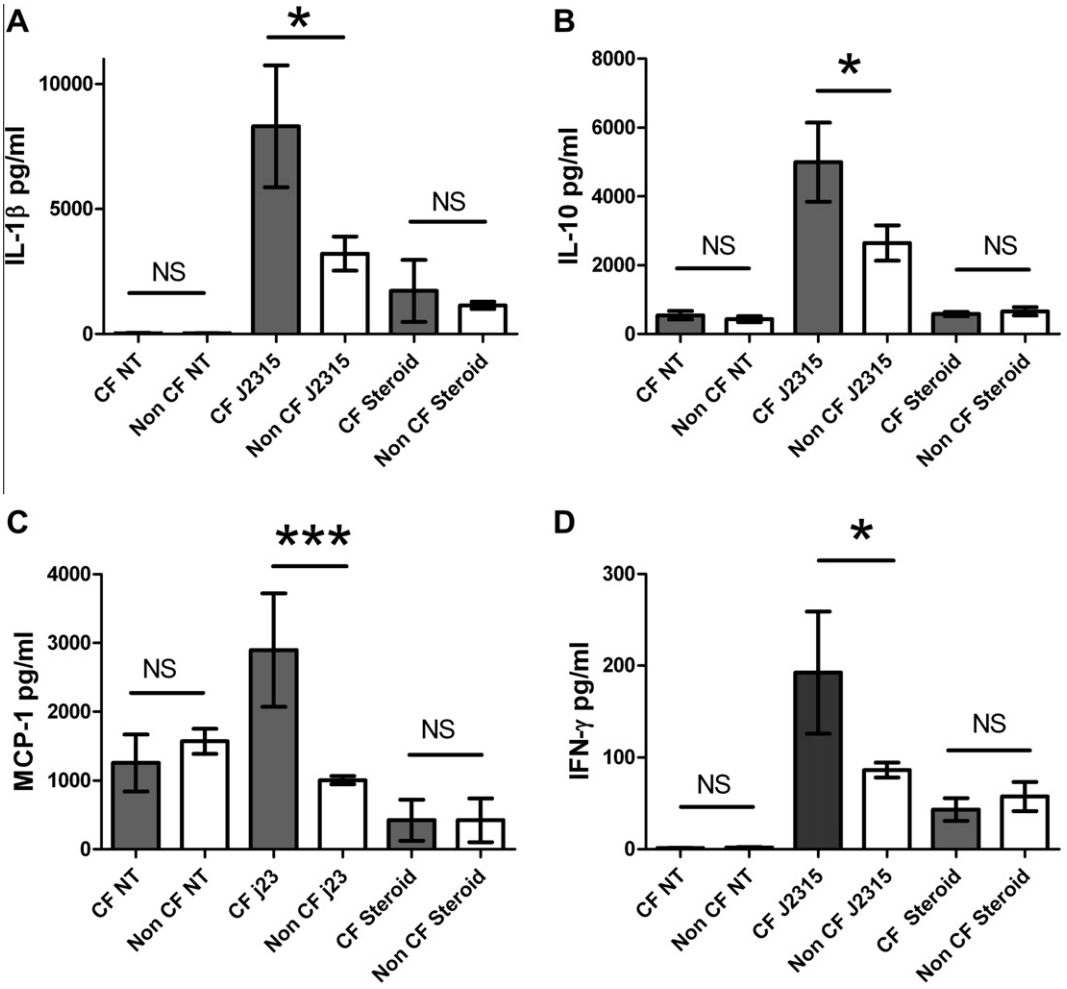

Fig. 1. Cytokine concentrations. (A) IL-1β, (B) IL-10, (C) MCP-1, and (D) IFN-γ levels for CF and non CF human MDMs infected with *B. cenocepacia* J2315 for 24 h. CF patients on steroids were on 60 mg of prednisone daily at collection. Supernatant concentrations are in pg/mL.

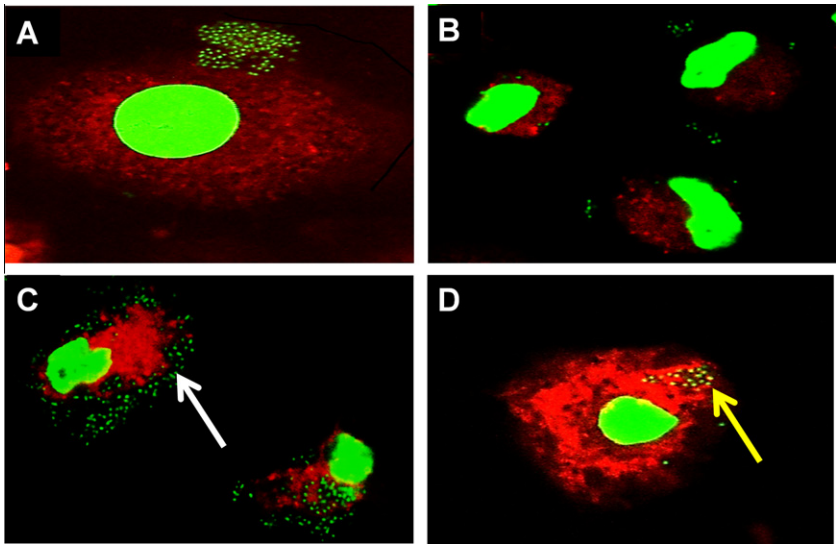

Fig. 2. Macrophage derived blood monocytes from CF (A, C) and non CF patients (B, D) were infected with J2315 for 30 min (A, B) and 1 h (C, D) and examined by confocal microscopy. The white arrow highlights increased bacteria. The nucleus is bright green, lysosome stained red, and bacteria displayed in dark green. Co-localization of bacteria in the lysosome is indicated in yellow and increased co-localization in non CF macrophages is marked by a yellow arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

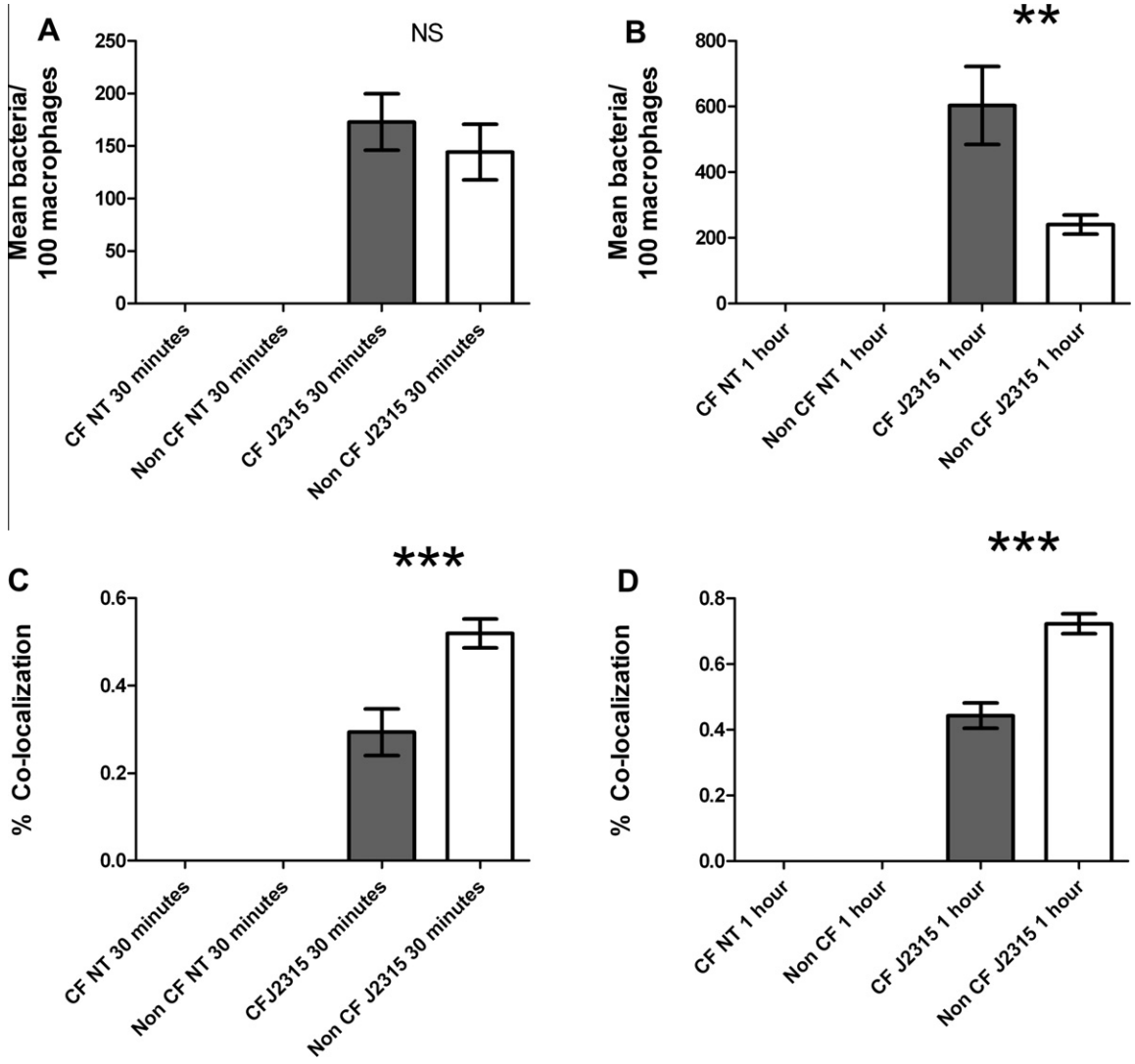

Fig. 3. Confocal microscopy scoring. CF and non CF macrophages infected with *B. cenocepacia* J2315. Mean bacteria presence per 100 macrophages after 30 min (A) and 1 h (B). Percent co-localization of bacteria to the lysosome after 30 min infection (C) and 1 h (D).

virulence in CF [22–24]. We used confocal microscopy to determine co-localization of J2315 to the lysosome where bacterial degradation would take place to determine any differences in bacterial trafficking between CF and non CF MDMs that could contribute to the increased inflammation seen in CF MDMs. CF and non CF MDMs were infected with J2315 for 30 min and 1 h and then a fluorescent lysotracker label was added. Lysotracker label is weakly basic and concentrates in the acidic lysosome. First, we scored the presence of bacteria associated per 100 macrophages. After 30 min of infection there was no significant difference in mean bacteria present between CF and non CF macrophages (p value 0.4548), but at one hour post infection there was a significant increase in bacteria present in CF macrophages compared to non CF macrophages (Figs. 2A–D, 3A and B, p value < 0.0060). Second, we evaluated the percent of bacteria localized with lysotracker red. After 30 min of infection there was a significant increase in lysosomal co-localization of bacteria observed in non CF macrophages (p value 0.010) with a continued significant increase in lysosomal co-localization of bacteria after one hour in non CF macrophages (Fig. 3C and D, p value < 0.0001). Taken together, these data indicate that *B. cenocepacia* avoids trafficking to the lysosome in CF macrophages leading to increased bacterial survival.

3.4. B. cenocepacia bacterial burden is increased in CF macrophages

Given that *B. cenocepacia* avoids trafficking to the lysosome in CF MDMs, transmission electron microscopy was used to further characterize the macrophage interaction with J2315 in CF and non CF patients. Macrophages were infected with J2315 for 2 h and transmission electron microscopy images were obtained as described in the methods. CF macrophages infected with J2315 demonstrated an increased presence of active bacteria as indicated by lipid droplets, with more than twice the amount of active bacteria present in the CF macrophage compared to the non CF macrophage (Fig. 4A and B). There was also an increased number of bacteria with signs of degradation in the non CF macrophages (Fig. 4B). Bacteria were noted to be in well defined vacuoles in the non CF macrophages (Fig. 4B). These observations suggest that CF macrophages exhibit an increased bacterial burden during *B. cenocepacia* infection.

3.5. Cell death is increased in macrophages derived from CF patients

To determine the correlation between increased inflammatory cytokine production and cell injury, an LDH assay was performed to assess cell death. MDMs were infected for 24 h with J2315 and supernatants were assessed for LDH release. There was a significant increase in cell death in CF macrophages compared to non CF macrophages after 24 h of infection with J2315 as measured by percent LDH release (not shown, p value 0.043). No difference in basal cell death between the two groups in untreated macrophages was observed. Therefore, increased cell death in CF patients is attributable to *B. cenocepacia* infection and is not a baseline derangement in CF macrophages.

4. Discussion

Cystic fibrosis patients endure exaggerated inflammation that is further increased during *B. cenocepacia* infections leading to poor clinical outcomes and may result in rapid death [8,9]. While ongoing research into potential curative therapies for CF is promising, patients currently infected with *B. cenocepacia* continue to suffer from poor outcomes and pose serious risks for transmitting infection to other patients due to a lack of effective therapeutics. It is therefore vital to develop new medications to help combat the heightened inflammatory cascade observed in these infections.

While an increased IL-1β inflammatory response to *B. cenocepacia* has been previously demonstrated in murine models of CF [19,20], human CF macrophage responses to *B. cenocepacia* are still poorly characterized with controversial findings. Previous studies have shown normal IL-1β levels in young CF patients not infected with *B. cenocepacia* [14,25], elevated levels in adult CF alveolar macrophages not infected with *B. cenocepacia* [26], and increased IL-1β in non CF macrophages in response to *B. cenocepacia* [15]. Therefore, it is not clear if IL-1B is specifically increased in CF macrophages upon *B. cenocepacia* infection. Here we demonstrate exaggerated human macrophage IL-1β responses to *B. cenocepacia* J2315. Given the findings in this study, in addition to the availability of anti-IL-1β monoclonal antibodies which have proven efficacy in clinical syndromes characterized by excessive IL-1β production [27], IL-1β could prove to be an interesting target for acute and chronic *B. cenocepacia* infections in CF patients.

Additionally, we also demonstrate evidence of increased MCP-1, IFN-γ, and IL-10 production in CF patients. These findings may be related to the need for more effective macrophage bacterial clearance and antigen presentation during infection as evidenced by the increased bacterial burden in CF patients seen using microscopic analysis. Interestingly, IFN-γ has been shown to be an effective therapy in CGD, the other clinical entity affected by *B. cenocepacia*. In CGD, IFN-γ works by improving the phagocytic clearance of apoptotic cells [28] and therefore could be another target of similar clinical application in CF patients. Previous studies have also shown a protective role for MCP-1 in *Burkholderia mallei* infections

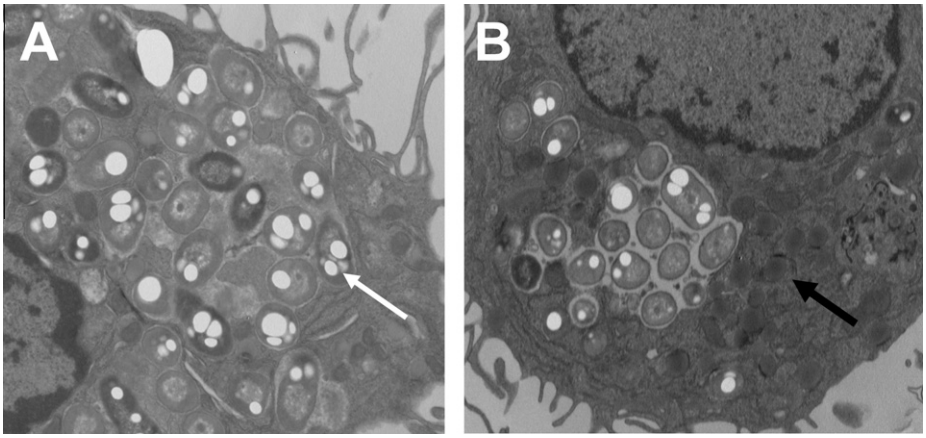

Fig. 4. Electron microscopy data. Macrophages derived from blood monocytes of CF (A) and non CF (B) patients were infected with *B. cenocepacia* for 2 h, and then processed for electron microscopy. The black arrow indicates degraded bacteria and the white arrow indicates active bacteria.

and a close relationship with IFN-γ in order to clear pulmonary infection [29]. IL-10's role in CF is less clear, but decreases in IL-10 production are thought to contribute to airway inflammation present during acute and chronic infections [30]. However, the acute increase in IL-10 seen in this study must be taken with caution, as elevated IL-10 has been linked to immunoparalysis and poor outcomes during sepsis states [31], which would need to be considered in the treatment of 'cepacia syndrome'. All together these findings lend further support to our theory that the aberrations in the cytokines observed in this study may be appropriate targets for future therapeutic interventions.

In addition to potential specific cytokine directed therapies, there have been reports of cases of *B. cepacia* sepsis alleviated by corticosteroids [21], but the exact influence of broad immunomodulators such as corticosteroids acutely and chronically remains to be elucidated in CF patients with *B. cenocepacia* infection. The markedly diminished inflammatory responses observed in the subset of CF patients who had been placed on corticosteroids prior to infection, suggests the possibility of acutely blocking the intense inflammatory response elicited by *B. cenocepacia* through broad immunosuppression. However, the exact timing and duration of steroid therapy needs to be clarified in future studies examining human macrophage responses as the patients in this study received corticosteroids prior to infecting their macrophages.

In summary, CF macrophages have exaggerated inflammatory responses to *B. cenocepacia* infection along with host trafficking defects that are potential targets of future studies and therapeutic interventions.

Authorship

B.K. designed, performed, analyzed results and wrote the manuscript. B.A., A.A., S.K., A.K., M.T. and K.C contributed to the performance of the experiments and editing of the manuscript. R.M. generated the transmission electron microscopy images. K.M. edited and analyzed the manuscript. A.O.A. helped design experiments, analyze results, and write the manuscript.

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