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The use of novel lung alveolar macrophage model, Max Planck Institute cells, to demonstrate the differential effects of smooth and rough *mycobacterium abscessus* forms on IL-6 and IL-1B induction

Thanthri Peiris

Project Advisor: Gyorgy Fejer, School of Biomedical Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA

Abstract

Mycobacterium abscessus frequently initiates respiratory infections in humans and is of clinical concern in patients with cystic fibrosis who develop secondary pulmonary infections. Alveolar macrophages and innate responses play a crucial role in the pathogenesis of M. abscessus. The virulence potential of M. abscessus was suggestive of cell surface transition of glycopeptidolipid loss from the smooth biofilm producing strain to transform into the rough invasive variant. This study focuses on innate responses of three strains of M. abscessus; NCTC rough morphotype, CL-B (DC088-B) smooth morphotype and CL-C (DC088-C) mixed morphotype; each isolated and used as heat and formalin killed stimulants. A novel Max Planck Institute (MPI) macrophage model was used to replicate the alveolar lung macrophage responses, additionally benefiting from self-renewing properties and ease of availability. MPI- macrophages were stimulated and cytokines IL-6 and IL-1β were studied over time periods of 5, 24, 48, 72 and 120 hours. As our primary aim, host inflammatory responses were analysed for variances in the levels of cytokines produced by different strains of M. abscessus and if heat or formalin inactivation had any impact. Bacteria were inactivated to prevent replication but still induce a cytokine response from the macrophages. Previous research concluded rough colony type was more aggressive in potentiating the pathogenic process of M. abscessus. Therefore, we studied variances between the smooth and rough morphotypes. Results indicate rough colony NCTC strain presented overall highest IL-6 levels when the heat-killed, rather than the formalin killed form, was used. Smooth colony type, CL-B strain had consistently low IL-6 levels across both inactivation types. Formalin killed mixed colony type CL-C strain induced higher secretion of IL-6 cytokine compared to formalin killed N-strain and B-strain. IL-1β was poorly induced across all three strains, however the formalin killed bacteria seemed to induce a relatively high IL-1β compared to the heat-killed method with all three strains. CL-C strain relatively displayed the highest IL-1β titres across both inactivation types. In the Max Planck Institute macrophage model, the M. abscessus mixed colony variant induced a larger cytokine response than the rough or the smooth variant. Results obtained from stimulation with MPI-cells potentially benefit personalized therapy towards patients via isolation of the bacteria and modification of treatment depending on the M. abscessus strain.

Keywords: Lung alveolar macrophage model, Max Planck Institute cells, MPI cells, cell culturing, *mycobacterium abscessus*, *M. abscessus,* ELISA IL-6, ELISA IL-1B, cytokines, IL-1β, proteins

Introduction

Mycobacterium abscessus is a soil and water derived complex of the mycobacterium species, responsible for skin and respiratory infections in humans (Chhotaray et al., 2020). M. abscessus has high clinical relevance in cystic fibrosis patients (CF) and lung transplantation cases (Howard et al., 2006). Evidence exists that transmission of this bacteria could occur from human to human especially in a hospital setting (Bryant et al., 2016). The abscessus complex is found present in 2.6%-13% of mycobacterial pulmonary infections in the USA (Lee et al., 2015). However, Taiwan has record high rates of 1.7 cases per 100, 000 (Lee et al., 2015). Infection develops with persisting symptoms leading to lung dysfunction, soft tissue infections and dissemination of the bacteria via the lymph nodes (Griffith et al., 2007). Emerging resistance in M. abscessus poses a number of treatment challenges, though some bacteriostatic effect is shown with a select few antibiotics such as cefotoxin, amikacin and clarithromycin (Chhotaray et al., 2020).

Mycobacterium subspecies *abscessus*, *massiliense* and *bolletii* form the *M. abscessus* complex. The abscessus complex can exist as both rough morphotype (RM) and smooth morphotype (SM), normally with five major glycopeptidolipids, which vary by location on the cell wall and the number of sugar/acetyl moieties attached to it (Howard *et al*., 2006). The difference exists within the RM where there is no presentation of glycopeptidolipids. *M. abscessus* has shown the ability to transform from the smooth biofilm producing, non-invasive morphotype to the rough, invasive strain (Howard *et al*., 2006; Chhotaray *et al*., 2020). Distinct cell wall components of the RM were believed to cause this aggressive pulmonary immune reaction. Contrastingly, the SM has the ability to form a biofilm, which the rough variety lacks, thus allowing for more persistent, long-term infections (Chhotaray *et al*., 2020).

M. abscessus supposedly acquires the SM through the environment, leading to colonization and aggressive infection by the RM (Chatherinot *et al*., 2009). T-helper 1 skewed immune response is triggered by infection and phagocytosis of the bacteria is initiated by macrophages which leads to slowed bacterial growth within the immune cells. (Orme and Ordway, 2014). In cytokine profiling of *M. abscessus* in human peripheral blood mononuclear cells and murine bone marrow-derived macrophages, tumour necrosis factor and interferon gamma were primarily induced, followed closely by the upregulation of IL-1β (Sampaio *et al*., 2008). The cytokines under our investigation have been chosen based on the fact that macrophages have the tendency to secrete IL-6 when Toll-like receptor 2 is stimulated by the rough variant of *M. abscessus* (Shin *et al*., 2008). Additionally, IL-6 and IL-1β are notably activated in the pro- inflammatory pathway. IL-6 is a basic macrophage pro-inflammatory cytokine, shown to have a part in NOS2 promoter activation and IL-1β is produced from cytosolic signals that lead to inflammasome activation (Shin *et al*., 2008). Inflammasome activation determines the production of mature IL-1β. Involved in this process are Nod-like receptors, in particular NLRP3, which facilitates inflammasome activation via ligand binding. This induction occurs due to the extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinases (MAPKs) as well as the nuclear factor-kB translocation (NFkB) pathway (Lee and Schorey, 2005). It remains unclear the true immunopathological mechanisms involved in infection with *M. abscessus*. Nevertheless, based on previous research, it was speculated that after macrophage engulfment, the glycopeptidolipids present on the cell wall of the

smooth variant were responsible for averting TLR-2 signalling. However, after the transformation into RM, unmasked components on the bacterial cell wall would proceed to antagonize the TLR- 2, thus stimulating the signalling mechanisms (Sampaio *et al*., 2008).

Macrophages are versatile immune cells originating from various systemic locations, with many subsets that release pro- and anti-inflammatory cytokines in response to infection (Fejer *et al*., 2013; Gordon and Pluddemann, 2017). The majority of these macrophages have limited life spans and develop as a result of macrophage colony stimulating factor (MCSF) and/or granulocyte-macrophage stimulating factor (GM-CSF) signalling that propagate monocytes down the bone marrow haematopoietic stem cell lineage and into eventual specialisation (Geissmann *et al*., 2010). Macrophages play a vital role in intracellular mycobacteria pathogenesis, as shown when interaction with *Mycobacterium tuberculosis*, leads to the uptake of the pathogen by alveolar macrophages (AM) and subsequent survival and proliferation within them (Queval *et al*., 2017). Interaction between the macrophage and the mycobacteria result in the vital cellular processes of apoptosis, antigen presentation, autophagy and inflammasome activation.

The most prominently studied structure is the Macrophage Receptor with Collagenous structure (MARCO) which together with Toll-like receptor 2 induces the release of pro-inflammatory cytokines down the NF-κB transcription factor pathway (Queval *et al*., 2017). Macrophages used in this study were cultured with GM- CSF which was also shown to stimulate growth in AM. GM-CSF is critical in AM growth and homeostasis (Fejer *et al*., 2013; Fejer *et al*., 2015). AM are unique subset of antigen- presenting cells responding to infections of the lung caused by pulmonary pathogens. In comparison to other subsets within the body, AMs present with an unusual phenomenon of phenotypically mirroring dendritic cells (DC) by expressing CD11c levels comparable to that expressed by DC (Guth *et al*., 2009). Studies show that AM exhibited better antigen- presentation and suggested that the lung environment consisting of high GM-CSF concentrations, high oxygen tension and surfactants SP-D and SP-(A) were all key factors in the development of AM into DC-like macrophages (Guth *et al*., 2009). From this study, AM was believed to inhibit the initiation of destructive inflammatory pathways by silencing antigen presentation (Guth *et al*., 2009). This mechanism is most likely stimulated by pathogens such as *Mycobacterium tuberculosis*. As demonstrated in a study by Lafuse *et al*., (2019) the $CD11c_+$ $CD11b_+$ mouse AM subset with monocytic markers, more readily induced inflammation when stimulated with *M. tuberculosis* compared to resident $CD11c_+$ $CD11b_-$ AMs, proven to be immunoregulatory in nature.

Max-Planck Institute (MPI) cells were used in the described stimulation procedure. MPI cells were developed to provide a model of alveolar macrophages for the study of pulmonary diseases. MPI-cells are self-renewing, non-transformed, GM-CSF dependent, differentiated mouse macrophages (Fejer et al., 2013). They were shown to be distinctly advantageous over the current macrophage models, especially AM which are difficult to obtain and use for study. Further limitations include complications in developing and obtaining appropriate purity levels as well as having suitable quantities of the model available and tackling ethical issues. (Fejer et al., 2013). However, using MPI macrophage cells would ensure that the innate immune responses of AM are faithfully reproduced thus making any potential results

applicable to a clinical setting. Moreover, MPI cells are extremely sensitive to many pathogen associated molecular patterns including Mycobacteria components, Lipopolysaccharide (LPS) and cord factor (Fejer et al., 2013). Comparative to bone marrow macrophages (BMM) which have limited life spans, MPI cells can be cultured for extremely long-term use in unlimited quantities which alleviates the need for alveolar macrophages (Fejer et al., 2013).

M. abscessus RM have been linked to highly dangerous forms of lung infections (Chatherinot et al., 2009). Thus, using alveolar macrophage models, it is important to investigate the cytokine responses of morphologically different M. abscessus strains. This area of research is especially valid in cases of CF where susceptibility to lung infections with pulmonary non- tuberculous mycobacteria are constantly an issue (Chatherinot et al., 2009). In this study, it was considered vital to understand how bacteria elicit immune responses in AM. Using the novel MPI-macrophage model conquers the primary hurdle of AM insufficiency with the added capability of mimicking the cytokine responses. Stimulation experiments were carried out to assess interleukin-6 (IL-6) and interleukin-1β (IL-1β) quantities released from interaction between MPI-macrophages and three isolated M. abscessus strains with various morphological types. The MPI-model was used to test the hypothesis that the RM would induce higher IL-6 and IL-1b levels than the SM or mixed variant (MM).

Methods

Bacterial Culture

Mycobacterium abscessus strains NCTC 13031, CL-B (DC008-B) and CL-C (C008-C) exhibiting rough, smooth and mixed colony phenotypes respectively were cultured in 7H9 middle-brook agar (ThermoFisher - R454012). Bacteria were grown to mid-log phase in suspension and CFU were determined by plating on solid 7H9 middle-brook agar (ThermoFisher - R454012). Inactivated bacteria were washed and resuspended in PBS. Suspensions were stored at -20°C. Initial bacteria were grown for 20 hours at 37 °C, 5% CO^2 when the Optical density (OD) at 600nm was determined using fresh medium and when 0.442 was reached. Then 1ml aliquots of the culture were added to an Eppendorf tube and centrifuged at x10,000g for 10 minutes until pellet was seen. Supernatant was discarded and cells were resuspended in PBS pre-infection. The NCTC strain conc. 1.2 $x10^8$/ml, B strain conc. 6.1 $x10^8$/ml and C strain conc. 1.25 $x10^8$/ml.

Heat inactivation of M. abscessus strains

Heat inactivated versions of Mycobacterium abscessus strains were killed at 95°C. Strains were resuspended in PBS and stored at -20°C.

Formalin inactivation of M. abscessus strains

Each strain grown in 7H9 agar after OD at 600nm calculated. Strains were then centrifuged at x10,000g for 10 minutes until pellet seen. 300 ul of 4% formaldehyde diluted in PBS (SLS – 17-512F) was added and the bacterial pellet was resuspended. After rapid vortex, the 4% formaldehyde containing bacterial strain solution was incubated for 15 minutes at room temperature. Solution was centrifuged at x10,000g for another 10 minutes to form a pellet. The supernatant containing formaldehyde was removed and pellet washed in 300 ul of PBS to remove any

residual formaldehyde. Solution was spun again at x10,000g for 10 minutes and the supernatant formed was removed again. The pellet was resuspended in a final solution of 1ml of PBS and stored at 4°C.

Cell Culturing with MPI Macrophages

Culturing MPI cells required precise measures of 4 reagents: DPBS, 0.0095M (PO4) without Calcium and Magnesium (500ml) (Lonza, SLS – 17-512F), PBS-EDTA (500ml) (Lonza, SLS- LZBE-02-017), Complete RPMI – 10% vol/vol FBS, 2mM L-Glutamine, 100Ul/ml Penicillin/Streptomycin (500ml) and X-63-GM-CSF. Complete RPMI contains 50ml FBS (LabPro, SLS-SLS1108), 5ml Penicillin/Streptomycin (10, 000 Ul/ml) (Lonza, SLS-LZBE17 – 602E) and 5ml L-Glutamine (200mM) (Lonza, SLS-LZBE12-702F). Each passage started with checking cells under a light microscope to assess confluency. Confluency between 70-90% was considered appropriate and ready to split. If appropriate confluency was not reached, then cells were left in incubator for another 24 hours.

After confluency was determined, the rest of the procedure was carried out in a Category 2 fume hood. Initially, pre-warmed DPBS, was added to detach cells from walls of the flask. From this point onwards, all washings were removed into a 50ml tube. This is followed by PBS-EDTA to de-clump any remaining cells. After an incubation in the CO2 incubator (5% CO2 levels; 37°C), cells were examined again under a light microscope to check for full detachment. After another wash with DPBS, cells were centrifuged at 1000rpm to form a pellet. The pellet was resuspended in complete RPMI (10% vol/vol FBS, 2mM L-Glutamine, 100UI/ml). Cell count was done at this point to calculate amounts of RPMI, cells and GM-CSF needed for next passage. GM-CSF is added at 1% vol/vol. Cells are then reseeded with the reagents according to the cell count.

Cell Counting and Plating for Stimulation

Cells were counted using a haemocytometer with Trypan Blue, 0.4% (Sigma Aldrich – T8154). 10ul sample of resuspended cells transferred onto surface of haemocytometer and 1:1 volume of Trypan Blue was added. Under the light microscope, blue cells that indicated death; were left out of the count. Counting all other cells, multiplied by 10/ml and multiplied by 2, taking into consideration the dilution. Cells were reseeded in 24-well plates with each well containing 1.5x105 cells/ml.

MPI Bacterial Stimulation

Pre-infection, optical density was calculated, and bacterial concentrations were determined for each strain. The bacteria were then added to 24-well stimulation plates. According to the multiplicity of infection (MOI), it was calculated that the ratio of MPI cells to bacteria would be 1:10. In total, for 24 wells, 3.45 x106 MPI cells were resuspended with 13.8ml fresh RPMI and 138ul of GM-CSF. Wells were separated into heat and formalin killed categories for each strain. The NCTC strain conc. 1.2 x108/ml, B strain conc. 6.1 x108/ml and C strain conc. 1.25 x108/ml. Total counts of bacteria for each well, were calculated depending on the number of timepoints stimulation would be carried out over. Incubation was carried out over 5, 24, 48, 72- and 120- hours at 37°C, 5% CO2, to allow the cells to adhere to the bottom of the well. Also included in the 24 hour well plate was negatives that contained just RPMI and MPI cells. The cell supernatants were collected at each time-point and stored in

appropriately labelled 96-well storage plates. Plates were then frozen at -20°C until use in ELISA.

Cytokines were quantified using sandwich ELISA with antibody pairs following the manufacturer's protocols for IL-6 (ThermoFisher Scientific - KMC0061) and IL-1b (ThermoFisher Scientific - BMS6002). All data points were measured in duplicates, and sample pairs of concentrations, plus blank, generated by recombinant cytokines were measured to make standard curve and calculate sample values.

ELISA IL-6

IL-6 was measured according to the manufactures protocol for the ELISA kit, the procedure is recommended to be done over three days. On day 1, diluted purified anti-murine IL-6 antibody 2ug/ml (ThermoFisher Scientific – 14-7061-85) in PBS was used to coat plate. The second day consisted of washing plate with 1x PBS, 0.05% Tween 20 (Sigma-Aldrich – P1379) before blocking it with 100ul/well 2% Bovine Serum Albumin (100ml): 2g BSA – (Sigma-Aldrich – A2153), 100ml DPBS (SLS – LZBE12-512). Recombinant murine IL-6 (ThermoFisher Scientific – 14-8061-62) used to prepare standards in Eppendorf's using serial dilutions (2000pg/ml, 1000pg/ml, 500pg/ml 250pg/ml, 125pg/ml, 62.5pg/ml, 31.25pg/ml). Plate washed again and standards from Eppendorf's added in duplicates to columns 1-2 and row A-G with H1 and H2 acting as blanks. Standards and samples are incubated at 4°C overnight. Day three consists of washing plates and adding diluted Biotin conjugated anti-murine IL-6 antibody (ThermoFisher Scientific – 13-7112-85) in PBS, 2ug/ml. Plates are coated with 50ul/well of biotin IL-6-PBS 3ug/ml solution and left to incubate at room temperature for 1 hour. Avidin-HRP (ThermoFisher Scientific – 18-4100-51) diluted with PBS, 1:500 to detect biotin antibodies. After incubation and washing, SuperAqua Blue ELISA substrate (ThermoFisher Scientific – 00-4203-58) added per well. After 10-30 minutes incubation, ELISA plate absorbance is read at a wavelength of 450nm.

ELISA IL-1B

IL-1b was measured according to the manufactures protocol for the ELISA kit, the procedure is recommended to be done over three days. On day 1, diluted purified anti-murine IL-1β antibody 2ug/ml (ThermoFisher Scientific – 14-7012-85) in PBS was used to coat plate. The second day consisted of washing plate with 1x PBS, 0.05% Tween 20 (Sigma-Aldrich – P1379) before blocking it with100ul/well of 2% Bovine Serum Albumin (100ml): 2g BSA – (Sigma- Aldrich – A2153), 100ml DPBS (SLS – LZBE12-512). Recombinant murine IL-1β (ThermoFisher Scientific – 14-8012-62) used to prepare standards in Eppendorf's using serial dilutions (2000pg/ml, 1000pg/ml, 500pg/ml 250pg/ml, 125pg/ml, 62.5pg/ml, 31.25pg/ml). Plate washed again and standards from Eppendorf's added in duplicates to columns 1-2 and row A-G with H1 and H2 acting as blanks. Standards and samples are incubated at 4°C overnight. Day three consists of washing plates and adding diluted Biotin conjugated anti-murine IL-1β antibody (ThermoFisher Scientific – 13-7112-85) in PBS, 3ug/ml. Plates are coated with 50ul/well of biotin IL-1β-PBS 3ug/ml solution and left to incubate at room temperature for 1 hour. Avidin-HRP (ThermoFisher Scientific – 18-4100-51) diluted with PBS, 1:500 to detect biotin antibodies. After incubation and washing, SuperAqua Blue ELISA substrate (ThermoFisher Scientific – 00-4203-58) added per well. After 10-30 minutes incubation, ELISA plate absorbance is read at a wavelength of 450nm.

Statistical Analysis

Two experimental replicates were performed for each cytokine. Samples were tested as two technical duplicates and all graphs plus statistics were produced and analysed by statistical software (Minitab19, c2019). A two-sample t-test was used to analyse comparisons between heat and formalin inactivated strains (Minitab19, c2019). General Linear Model Fit ANOVA was used to assess comparisons between the three M. abscessus strains (Minitab19, c2019). A P-value of less than 0.05 was considered significant.

Results

Mycobacterium abscessus induced cytokine secretion profiles in MPI cells

The cytokine secretion profiles of MPI cells against infection with heat killed (HK) and formalin killed (FK) strains of Mycobacterium abscessus were quantified. Three different strains containing rough – NCTC (N-strain), smooth – CL-B (B-strain) and mixed – CL-C (C-strain) colony morphology were assessed across time points ranging from 5 hours to 120 hours. As seen in Figure 1a of IL-6 Experiment 1, infection of MPI cells with HKN bacteria showed the production of IL-6 24-hours and 48-hours post infection, whereas secretion was barely detected at the initial timepoint of 5-hours, the same was true later at 72-hours. In the same Figure 1a, FKN bacteria showed drastically lower titres of IL-6 secretion - yet again, at 24-hours and 48-hours post infection only. However, Figure 1b from IL-6 ELISA

Experiment 2 shows that secretion of IL-6 from the N-strain is possible at 5 hours as well as 72 hours. Cytokine induction can persist 120-hours post infection. Levels of IL-6 from FKN in Figure 1b, have also increased drastically. In both Experiment 1 and 2, induction is relatively highest past the 5-hour point with both HKN and FKN bacteria. In Figure 1c, B-strain stimulated MPI cells, indicate moderate levels of IL-6 secreted by both heat and formalin killed strains. However, non-detectable values below the range were prominent especially at 5 hours and 72 hours for all the strains in IL-6 Experiment 1. IL-6 Experiment 2 (Fig 1d and 1e) overall, had higher induction levels, however only N-strain and C-strain showed a consistent trend over the time period, with B-strain fluctuating between the hours. It can be observed in Figure 1d and 1c that HKB induced more secretion than formalin killed bacteria. Figure 1d ($P=.002$), p-value$<.01$; indicates a statistically significant difference between levels of IL-6 pg/ml produced by HKB and FKB. The levels of IL-6 secretion by C-strain, as seen in Figure 1e, indicate that both heat and formalin killed bacteria release comparably high levels of IL-6.

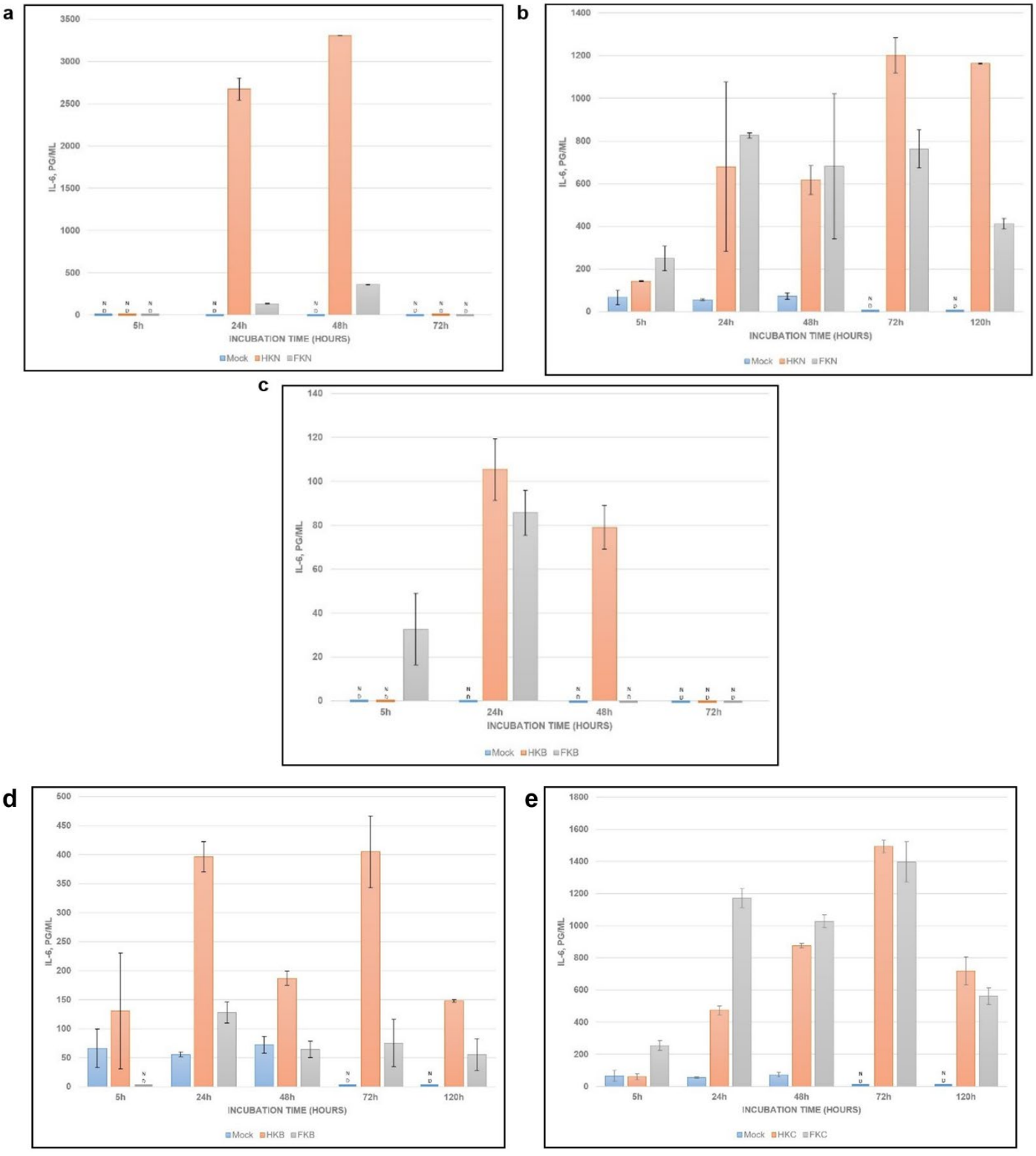

Figure 1: a-e IL-6 cytokine induction from stimulation of MPI-cells with heat-killed (HK) or formalin-killed (FK) Mycobacterium abscessus NCTC strain (N), CL-B strain (B) and CL-C strain (C). Bar graphs indicate variances in cytokine levels between heat and formalin methods of killing each Mycobacterium abscessus strain. Cytokines were quantified at 5, 24, 48, 72 hours for both Experiment 1 and 2 and cytokine levels at 120 hours were additionally quantified for Experiment 2. Data are representative of one ELISA output per experiment and values shown are expressed as mean ± SD. Statistical significance between heat killed

and formalin killed strains are described in the text. ND indicates values that are not detectable. No indication given for non-significant results.

Figure 2a and 2b supports the overall trend that FKC induced a higher secretion of IL-6 cytokine compared to FKN and FKB. However, this is not the case when considering the heat- killed bacteria, in which N-strain seems to stimulate higher levels of IL-6. With regards to B- strain, IL-6 levels remained consistently lowered compared to the other two strains. Figure 2a (P=.002), p-value<.01; (P=.001), p-value<.001; indicating a significant difference between IL- 6 pg/ml produced FKN and FKC, respectively, compared to FKB. In the same Figure 2a, significant differences were also established between FKN and FKC (P=.001), p-value <.001. Figure 2c (P=.015), p-value<.05; (P=.002), p-value<.01; indicating significant difference between levels of IL-6 pg/ml produced by HKN and HKC, respectively, compared to HKB. Figure 2d helps confirms significant relationship between HKN and HKC bacteria; (P=.043), p-value<.05.

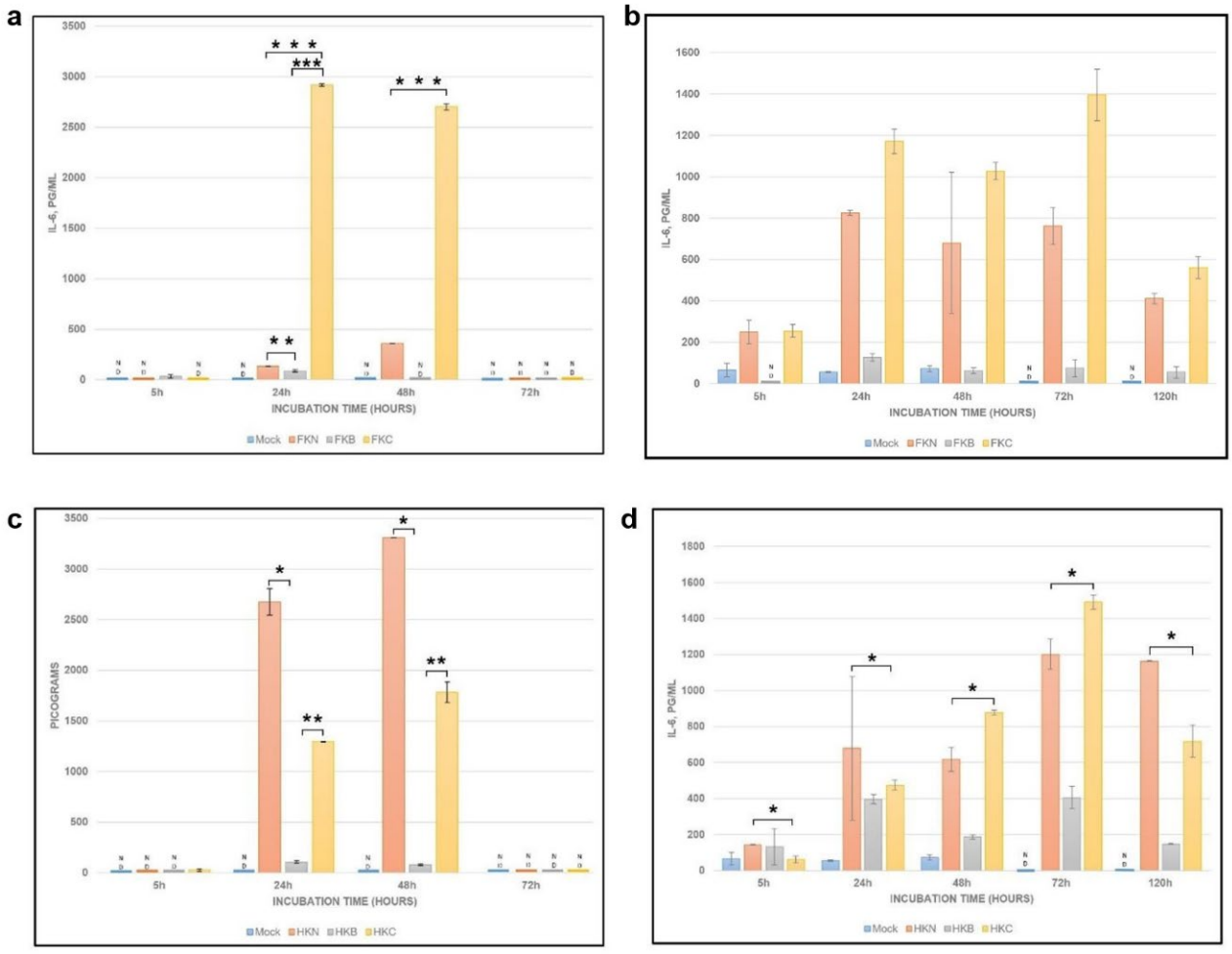

Figure 2 a-d: Collective IL-6 cytokine induction of all heat-killed (HK) and all formalin-killed (FK) *Mycobacterium abscessus* NCTC strain (N), CL-B strain (B) and CL-C strain (C). Bar graphs indicate variances in cytokine levels between *Mycobacterium abscessus* strains that were either heat or formalin killed. Cytokines were quantified at 5, 24, 48, 72 for both Experiment 1 and 2 and cytokine levels at 120 hours were additionally quantified for Experiment 2. Results from IL-6 ELISA experiments and expressed as mean values ± SD for two technical replicates. Statistical significance between M. abscessus strains are described in the text. *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*- value < 0.001. ND indicates values that are not detectable. No indication given for non-significant results.

Shifting the focus to the IL-1b secretory profile, it can be established from Figure 3a, IL-1β Experiment 1, that infection of MPI cells with HKN and FKN showed a high secretory profile for the formalin killed method compared to heat-killed forms. This is the general trend that can be observed from IL-1β Experiment 1 across B-strain and C-strain. Generally, there was no secretion detected 5-hours post-infection with all the *Mycobacterium abscessus* strains. However, in comparison to IL-1β Experiment 1, Figures 3b-3d representing Experiment 2, indicate moderately high titres 5-hours post infection. An inconsistent trend in all IL-1β secretion values over the time points for Experiment 2 can be seen. However, a point of note is that C-strain seems to generally induce comparatively higher IL-1β levels across both Experiment 1 and 2.

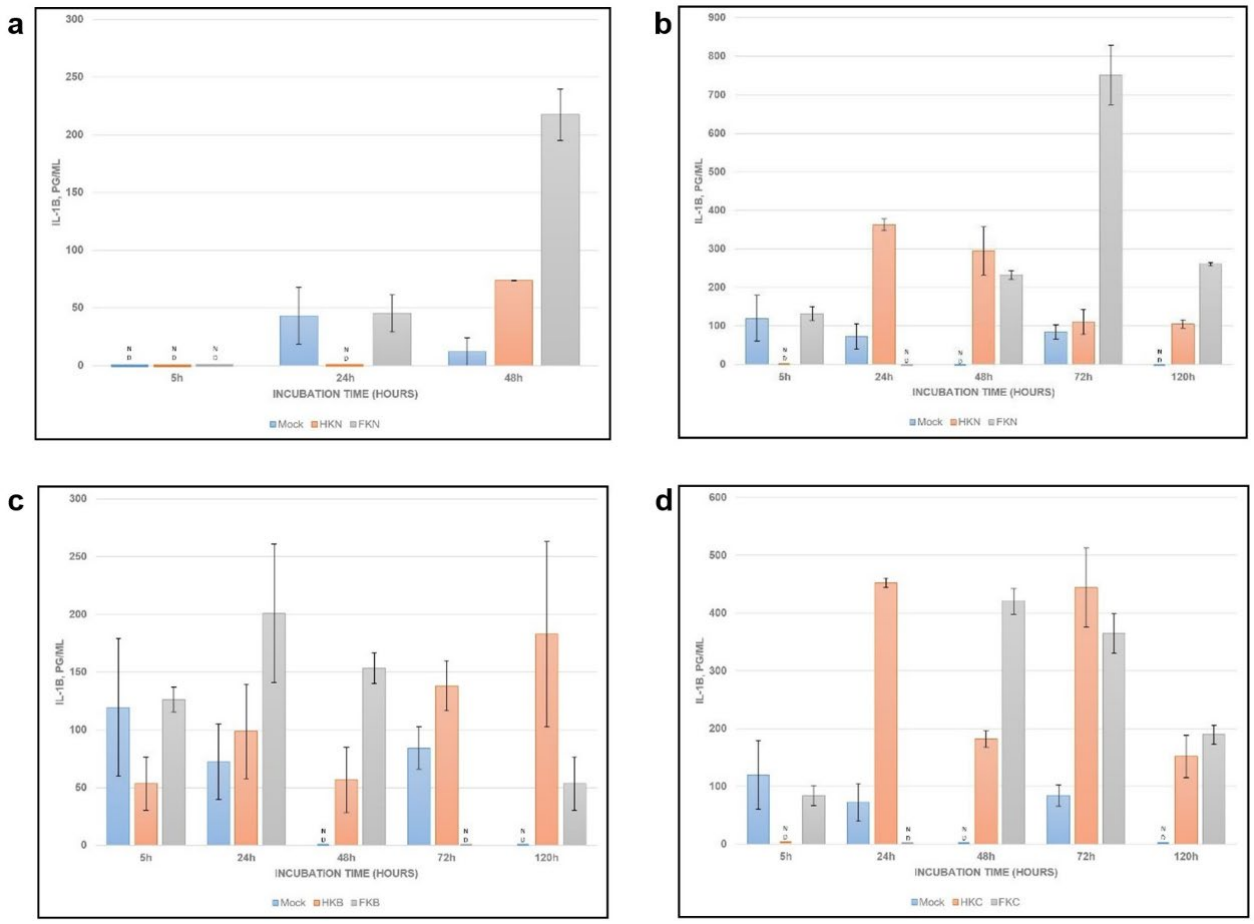

Figure 3 a-d: IL-1b cytokine induction from stimulation of MPI-cells with heat-killed (HK) or formalin-killed (FK) *Mycobacterium abscessus* NCTC strain (N), CL-B strain (B) and CL-C strain (C). Bar graphs indicate variances in cytokine levels between heat and formalin methods of killing each *Mycobacterium abscessus* strain. Cytokines were quantified at 5, 24, 48 hours for both Experiment 1 and 2 and cytokine levels at 72 and 120 hours were additionally quantified for Experiment 2. Data are expressed as mean ± SD for two technical replicates. Statistical significance between heat killed and formalin killed strains are described in the text. ND indicates values that are not detectable. No indication given for non-significant results.

Figure 4a, 4b, and 4d display higher observable C-strain titres across both heat-killed Experiment 1 and 2 (Figure 4a, 4c) and formalin killed Experiment 1 (Figure 4b) bacteria. In IL-1β Experiment 1, Figure 4a, ANOVA established a significant difference between heat- killed C-strain compared to heat-killed B-strain (P=.007), p<.01; and N-strain (P=.029), p<.05. The significance between C-strain and N-strain extended into formalin-killed forms (Figure 4b) with (P=.004), p<.01. Likewise, In Experiment 2 (Figure 4d), ANOVA deemed heat-killed N- strain and C-strain to have a significant difference (P=.041), p<.05. However, in the case of Experiment 2 with formalin-killed bacteria (Figure 4c), the only significant differences were between B-strain compared to N-strain (P=.014), p<.05; and C-strain (P=.485), p<.05.

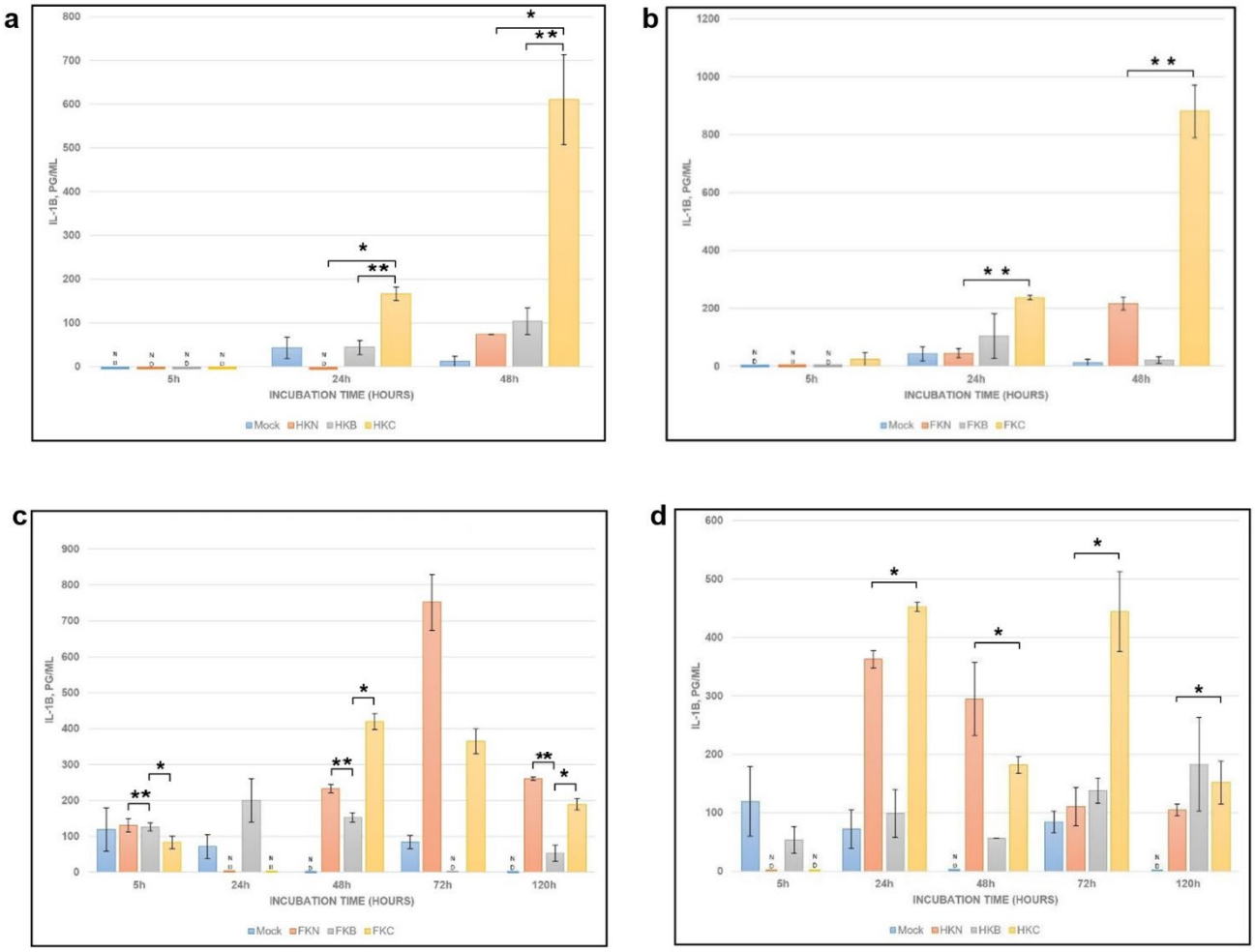

Figure 4 a-d Collective IL-1B cytokine induction of all heat-killed (HK) and all formalin-killed (FK). Mycobacterium abscessus NCTC strain (N), CL-B strain (B) and CL-C strain (C). Bar graphs indicate variances in cytokine levels between Mycobacterium abscessus strains that were either heat or formalin killed. Cytokines were quantified at 5, 24, 48 hours for both Experiment 1 and 2 and cytokine levels at 72 and 120 hours were additionally quantified for Experiment 2. Collective data expressed as mean values ± SD for two replicates. Statistical significance between cytokine secretion of M. abscessus strains are described in the text. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001. ND indicates values that are not detectable. No indication given for non-significant results.

Discussion

The described data indicates that the stimulation of MPI cells with mixed colony type C-strain overall induced a higher cytokine response across both Experiment 1 and 2. This result is mirrored in cystic fibrosis patients, where studies have observed the mix of colonization by rough and smooth variants of M. abscessus (MABS) as a preliminary source of morbidity (Bernut et al., 2016). Previous studies have demonstrated replication of MABS variants in various macrophage models and identification of the glycopeptidolipid in the S variant as cause for the biofilm formation that the R variant lacks (Bernut et al., 2016). Immune response to MABS involves recognition of the uncovered phosphatidyl-myo-inositol mannosides on the R variant that interact with Toll-like receptor 2 which responds by producing a host of inflammatory cytokines including IL-6 and IL-1β from alveolar macrophages (Roux et al., 2016).

IL-1β was generally poorly induced by all three M. abscessus strains with the highest in formalin-killed C-strain (~800 pg/ml) compared to IL-6 which had (~3000 pg/ml) from heat- killed N-strain. IL-6 and IL-1β both have induction pathways that involve NFκB transduction and MAP-kinase (Duque and Descoteaux, 2014). However, the key difference lies in the fact that IL-1β is activated via the production of an inflammasome whereas IL-6 is a basic pro- inflammatory cytokine that is more readily induced as a part of the acquired immunity against many mycobacteria (Jeon et al., 2009). In the case of mycobacteria survival within macrophages, studies suggest a large part of this evasive mechanism can be attributed to resistance of phagosome maturation and acidification. This in turn, contributes towards protection from reactive oxygen and nitrogen intermediates (Jeon et al., 2009). Studies investigating the inflammasome show that IL-1β requires induction from the inflammasome complex which acts on Pro-IL-1β resulting in the production of mature IL-1β. Mature IL-1β can then proceed to mediate immune responses (Briken et al., 2013). The components needed for the assembly of the inflammasome are located intracellularly and activation depends on the sterility of the host cytoplasm being compromised by bacterial products released from bacterium (Mayer-Barber et al., 2011). A study investigating Salmonella spp. in an in-vivo model highlighted that higher cytokine levels were produced by IL-6 than IL-1β which showed no significant change in secretory levels as the study continued (Crowley et al., 2016). From this evidence in combination with results from the described study, it can speculated that the reason for poor IL-1β response is due to the fact that, IL-6 is at the forefront of the protective response by the host system. Comparatively, it can be argued that production of IL-1β needs to fulfil a larger criteria than IL-6 for successful induction thus taking a longer period of time for the observable titres to present on the ELISA. To truly assess the IL-1β response from macrophages, further studies could be carried out over a longer period of time to ensure that IL-1β has enough time to be fully induced via the inflammasome.

Heat and formalin inactivation were used in this study as a way to investigate the different signalling mechanisms within the macrophages. The use of live bacteria in experiments can be helpful in cases where observing mechanisms of replication in bacteria are of benefit to the investigator. However, in the case of this study, inactivating M. abscessus strains with heat and formalin paved the way to separately study the different processes of cytokine secretion and the direct effects of MPI-cell stimulation. Across both IL-6 and IL-1b induction, the formalin inactivated strains had

better induction levels than the heat-inactivated strains. There is a clear lack of investigation into the effects of different Mycobacteria inactivation and its consequences on cytokine levels. However, in two different vaccine studies involving formalin- inactivated viruses, Coxsackievirus and West Nile Virus, we can suggest a minor similarity concerning the effects of formalin, based on the intracellular habitats that the viruses and M. abscessus have in common. Results from these studies found formalin-fixed samples had far greater responses. The experimenters attributed this to the ability of formalin to stabilize particles on the surface of the pathogen even at high temperatures, thus preserving morphology. It can be presumed that the formalin inactivation preserved the integrity of the cell wall structure due to the cross-linkage formed between the proteins on the cell surface of the morphotypes. Thus, the higher cytokine responses observed in the formalin killed group could be due to preservation of proteins that can successfully bind with their receptors in the inflammatory pathway (Chowdhury et al., 2015; Hankaniemi et al., 2019). There could be an as yet unidentified structural reason why the MABS strains might behave differently following heat killing. The same is true for why formalin inactivated C-strain induced the overall greatest cytokine response. It can be speculated that heat can denature the components of the cell wall, thus altering proteins would lead to changes in receptor interaction, possibly poor receptor binding.

Herein, we established that the smooth variant had the poorest cytokine induction. This could be due to a survival mechanism by which, residing inside macrophages would reduce the likelihood of an immune response. A study by Roux et al., (2016) corroborates this theory, by suggesting that the electron translucent zone that surrounds the smooth colony type prevents the formation of phagosomes within the macrophage which would lead to increased survival within. However, the opposite is true for the rough variant – which most likely extends to the mixed colony type as well, that due to the induction of a high cytokine profile as seen in the present study, there is likely larger apoptotic action against the macrophages which could possibly lead to extravascular dissemination of the invasive variant resulting in a more aggressive disease (Roux et al., 2016).

In this present study, MPI-cells were used to observe the levels of IL-6 and IL-1β cytokines released via stimulation with the three M. abscessus strains. We believe this was the most appropriate and innovative macrophage model in comparison to models used in other studies. This particular macrophage model is novel and dependant on GM-CSF with availability in unlimited quantities (Fejer et al., 2013). Woo et al. (2018) demonstrates the efficacy of MPI cells, stimulated by live and heat killed Mycobacterium tuberculosis, to induce cytokine production. A range of cytokines were analysed amongst which included IL-6 and IL-1b. Results showed successful cytokine induction by both live bacteria and heat killed, however responses were more distinctly robust in live compared to heat-killed bacteria (Woo et al., 2018). Another study by Caverly et al. (2015) used a murine model in which the intratracheal inoculation, suspended in thrombin and fibrinogen solution, aims to trap the bacteria in the fibrin plugs within the distal airways. Caverly et al. (2015) investigated the BALF neutrophil count produced by cystic fibrosis patients with pulmonary infection involving both smooth and rough colony types of M. abscessus. It was determined that the rough morphotype greatly increased the number of BALF neutrophils compared to the smooth variant. (Caverly et al., 2015). This is reflected

in the higher levels of IL-6 and IL-1β produced by MPI cells when stimulated with rough colony NCTC strain compared to smooth colony CL-B. These findings theoretically support the notion that the invasive potential of the rough morphotype is greater than the smooth. In relation to other literature, prolonged experiments using Mycobacteria have used mice with compromised immune systems or injected the organism of interest through the tail. However, this was reported to increase the systemic burden over the threshold of what is clinically seen in the lung. Thus, it produces variation in the host immune response (Alexander, 2011). The MPI-macrophage model was developed for this very reason – to ensure that pulmonary infection models presented more biological relevance. Similar to alveolar macrophages, studies have shown that MPI cells are able to reproduce into multinucleated giant cells in the presence of GM-CSF (Fejer et al., 2013). The functional relationship between AM and MPI-cells is further corroborated by the shared cell morphology, high sensitivity to airborne pathogens, specialized pro-inflammatory cytokine response and selective surface markers (Fejer et al., 2013). There are minor disadvantages of cell lines such as MPI, having the disposition to present with complications at the genetic level as well as faults in signalling mechanisms (Mendoza and Castanon, 2016). In cases where this could occur, it could be useful to consider the alternative of a murine model with fibrin plug bacterial suspension. Fibrin, being actively present in cases of inflammatory airway complications, has showed efficacy in pulmonary infection experiments with Pseudomonas aeruginosa (Caverly et al., 2015).

Another stimulation experiment by Andrea et al., (2017) using M. abscessus in Macrophage J-774A.1 in vitro model, established that rough strains had a higher rate of phagocytosis compared to smooth strains (P =.0003), p-value<.01. This significance is mirrored in the described study where the rough morphotype NCTC induced larger cytokine quantities. This could be in correlation with a higher rate of phagocytosis. Although the mouse model has a wide popularity, The MPI-model has been recognized by the present and previous studies as a popular alternative option to study M. abscessus and other pulmonary pathogens due to high success rates with cytokine responsiveness (Fejer et al., 2013). This was attributed to the nature by which MPI have the unique ability to self-renew and functionally imitate lung alveolar macrophage (Fejer et al., 2013). Regarding the IL-1β cytokine response, a stimulation experiment using a human peripheral blood mononuclear model discovered altered cytokine profiles between M. abscessus rough strain and smooth strain. Results indicated that the rough isolates induced significantly more IL-1β compared to the smooth variant (Jonsson et al., 2012). This is not reflected in the present study conducted and could be due to an experimental error such as limited time exposure and small sample size as overall IL-1β numbers were low compared to IL-6. Additionally, the study revealed IL-6 levels were produced in relatively equal amounts by both smooth and rough M. abscessus colonies (Jonsson et al., 2012). By contrast, findings from the present study showed a significant difference in the IL-6 levels between the rough and smooth variants (P=.015), p-value<.05. However, this previous study used UV, instead of heat or formalin, to inactivate the M. abscessus which could explain the differences in findings. Despite the lack of literature investigating mixed-colony morphology, the present study explored CL-C strain to understand the effects of a heterogenic cell wall on the pathogenic process. Previous studies also suggest that the rough variant exists as clumps and is engulfed by the macrophages such that there is an uneven ratio of bacteria per

macrophage present. However, the smooth variant is engulfed as a lone component (Brambilla et al., 2016). Evidently, it is useful to consider the possibility that larger cytokine induction by the rough colony could be credited its higher bacterial count within the macrophage.

In both cytokine experiments there were undetectable values below the range which could be due to artefacts from preparation of killed strains. The NCTC strain was prepared from a denser culture, thus calculation errors might have underestimated the bacterial numbers so a higher count than needed was used for stimulation experiments. An adjusted Multiplicity of Infection (MOI) could rectify this issue. Additionally, dead cell components present in the culture could have stimulated MPI-cells leading to higher cytokine responses than expected. In cases where there was no significance determined by statistical tests, it should be taken into consideration that arbitrary units were used and there was a limited sample size. Thus, large variations in data which resulted in no statistical significance were detected but biological relevance could still exist. In acknowledgment of this error, future experiments should perform more assays to increase the sample size and reduce variations in data. Additionally, MPI cell culture might have been sabotaged by the use of cold reagents. Reagents were left in the water bath for an indeterminate amount of time before cell passage. However, it was impossible to measure if the reagents had reached an appropriate temperature without risking the contamination. Inadequate reagent temperature could have affected the integrity of the MPI cells resulting in cell distress and death. Either scenario would drastically affect the number of cells left in the flask and this would compromise subsequent stimulation experiments. Passage numbers 13 and 14 were faced with this issue, as not enough cells were viable for stimulation which caused a delay in experiments and the number of repeats performed.

Conclusions

The use of MPI-cells in this study both highlights the ongoing challenge of developing a clinically representative model of M. abscessus as well as presenting a better alternative to pre-existing models. The ability of the MPI model to produce observable variances in cytokine levels in reflection to the M. abscessus rough, smooth and mixed morphotypes enhances the validation of pre-published research on virulence factors and responses of the immune system. Findings in the described study could have clinical impact if MPI-stimulation experiments are to be used therapeutically as reference points for cytokine profiling in patients suffering with pulmonary complications caused by M. abscessus (Fejer et al., 2013). Initiating a treatment course for M. abscessus is complicated by lengthy procedures to identify the infection strain, antibiotic cost and drug toxicity (Caverly et al., 2015). This area is lacking in research to develop better diagnostic techniques, which subsequently hinders the clinical outcomes in CF patients. Our results to date do not suggest a mechanistic solution as to how either morphotype plays a part in M. abscessus pathogenicity. Nevertheless, it emphasizes the importance of conducting more clinical experiments to achieve a clearer understanding of how morphotype differences effect pulmonary disease, not only in M. abscessus, but in other pulmonary pathogens as well.

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