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ORIGINAL ARTICLE

Identification of N-formylated Peptides with Neutrophilic Chemotactic Activity in *Mycobacterium tuberculosis*

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Abstract

Neutrophil infiltration of the lungs is associated with granuloma formation and the severity of tuberculosis infection. Although several cytokines and chemokines are known to contribute to lung neutrophil infiltration, the neutrophilic chemotactic factors of *Mycobacterium tuberculosis* (*Mtb*) remain unexplored. Therefore, we performed Transwell based chemotactic assays using neutrophils from human peripheral blood and mouse bone marrow to probe the chemotactic activity of the culture filtrates (CF) of *Mtb* H37Rv. CF of H37Rv induced chemotaxis of both human and mouse neutrophils, and this was also confirmed with CF of 9 clinical isolates and Erdman strain of *Mtb* with neutrophil chemotactic activity. Sulfasalazine, an N-formyl-Met-Leu-Phe (fMLF) receptor inhibitor, blocked the chemotaxis of neutrophils induced by CF of *Mtb*, thus indicating the involvement of the fMLF receptor in *Mtb* CF induced chemotaxis of neutrophils. Mass spectrometry analysis of CF of H37Rv identified three candidate N-formylated heptapeptides. The chemotactic activity of the identified peptides was confirmed with their synthetic mimetics that they induced neutrophil chemotaxis in a manner dependent on N-terminal formylation. For all formylated peptides and CF of *Mtb,* the induced Ca^{2+} influx in neutrophils was suppressed by sulfasalazine. Thus, we identified novel formylated *Mtb* peptides with neutrophil chemotactic activity.

Key words: *Mycobacterium tuberculosis*, neutrophil, formyl peptides, chemotaxis, innate immune response

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INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is a chronic infectious disease characterized by the growth of nodules in the lungs. As of 2020, TB was the 13th most frequent cause of human death, and caused nearly 10 million new cases and 1.6 million deaths each year worldwide [1]. There were approximately 500,000 patients with multi-drug resistant TB, including 160,000 with extensively drug resistant TB [2]. HIV infection is a risk factor for active tuberculosis infection. Countries with high rates of HIV infection have more TB cases [3] and higher mortality rates [4–6]. The continual emergence of drug resistance together with increasing rates of HIV-TB co-infection [7–9] have made TB one of the deadliest infectious diseases. Therefore, the development of effective TB vaccines and novel anti-TB drugs is imperative, but would require greater understanding of the molecular details of interactions between immune cells and the pathogen *Mtb*.

The interaction between immune cells and *Mtb* occurs in lung granulomas (tubercles), a well-known pathological hallmark of TB [10]. After inhalation of aerosolized

Mtb bacilli, local lung alveolar macrophages respond by phagocytizing the bacteria [11], thereby initiating the production of cytokines and chemokines [12,13], which activate and recruit polymorphonuclear neutrophils from the circulation to sites of infection. The infiltration of neutrophils around the foci of the infected alveolar macrophages leads to granuloma formation and even tissue damage. Under normal conditions, neutrophils are the first responders that travel to sites of infection after phagocytosis of bacilli by alveolar macrophages. Neutrophils have multiple modes of combatting bacterial infections, such as phagocytosis of the bacteria, degranulation, and release of extracellular traps. Although these effector functions of neutrophils are efficient in controlling common bacterial infections, the efficiency of these mechanisms against *Mtb* infection is unclear, and the over-accumulation of the neutrophils is often associated with local lung tissue damage and cavity formation in patients with pulmonary TB [2]. Therefore, understanding the mechanisms controlling neutrophil recruitment in TB infection is imperative.

Neutrophil infiltration in the lungs is controlled by a variety of host derived factors, including chemokines, cytokines, lipids, *Mtb* infected alveolar macrophages, lung epithelial cells and T cells. Currently, no reports have described the potential candidate pathogen released factors involved in recruitment of neutrophils to sites of *Mtb* infection. In contrast, many studies have reported the chemotactic factors released from microbial pathogens such as *Staphylococcus aureus* and *Escherichia coli* [14,15]. Studies over the years have identified a chemoattractant that is common among many other microbial pathogens [14-16]. Schiffmann et al., in 1975, studied the chemotactic activity of *E. coli* culture filtrates and demonstrated that the chemotactic factors were N-terminally blocked peptides with a low molecular weight of 150–1,500 Da [15]. This finding was further confirmed by Marasco et al. in 1983: mass spectrometry indicated that an N-formyl-Met-Leu-Phe (fMLF) peptide showed the highest chemotactic activity. Moreover, that study indicated that the tripeptide fMLF is the major neutrophil chemotactic factor produced by *E. coli* [16]. Furthermore, formyl peptides were confirmed to be recognized by specific receptors called neutrophilic formyl peptide receptors (fPRs) on neutrophil surfaces, thus resulting in chemotaxis. Therefore, we tested whether *Mtb* might also have a formyl peptide that drives neutrophil chemotaxis. Our results demonstrated that *Mtb* secretes formyl peptides with neutrophilic chemotactic capability that may act through the formyl peptide receptor.

MATERIALS AND METHODS

Preparation of culture filtrates from *Mtb*

The culture filtrates of *Mtb* were prepared as previously described [17,18] with minor modifications. Briefly, H37Rv and clinical isolates of *Mtb* were inoculated into a T25 tissue culture flask containing 10 ml of either 7H9 broth supplemented with ADC (Difco) or Sauton's culture medium prepared in house as previously described (Microbiol). The cultures were incubated at 37°C with 5% CO_2 and slow shaking. After 2 or 4 weeks of incubation, the cultures of *Mtb* were collected into a 15 ml conical centrifuge tube and spun down at 2,600 × g at room temperature for 15 minutes. The culture supernatants were filtered through a 0.2 μm syringe filter (Millipore), divided into 1 ml aliquots and stored at −80°C as culture filtrates (CF) before use.

Isolation of neutrophils

Isolation of human neutrophils from peripheral blood. Human venous blood samples from 22 healthy donors (15 women and 7 men) were used for neutrophil isolation after signed consent was obtained, according to a protocol approved by the Institutional Review Board of the University of Texas Health Science Center at Tyler. Neutrophils were isolated with a previously described protocol [19] with minor modifications. After differential centrifugation of the heparinized venous blood over Ficoll-Paque™ Plus (GE Healthcare), the plasma and PBMC layers were removed, and the pellet containing red blood cells and neutrophils was collected and reconstituted with Hanks' buffered saline solution (HBSS) (Gibco) in a total volume of 20 ml, then centrifuged at 700 × g for 10 minutes. The pellet was resuspended in 20 ml of HBSS and mixed with 20 ml of 3% dextran (Sigma) in HBSS and allowed to sediment at room temperature for 30 minutes. The upper layer containing neutrophils was collected into a new 50 ml conical tube, and the volume was adjusted to 50 ml with HBSS. The samples were then centrifuged at 400 × g for 8 minutes. The pellets were resuspended in 5 ml of sterile water by rapid mixing for 45 seconds to lyse the residual red blood cells. The reaction was stopped by addition of 800 μl of heat inactivated-fetal bovine serum (Atlanta Biologicals) and HBSS to adjust the volume to 30 ml. The cell suspension was centrifuged at 400 × g for 8 minutes, and the neutrophil pellets were resuspended in 5 ml RPMI-1640 medium. Cell numbers and viability were determined by counting the cells resuspended in 0.4% trypan blue (Gibco) on a hemocytometer under a microscope.

Isolation of mouse neutrophils from bone marrow. Mouse neutrophils were isolated from the bone marrow of four 6–8-week-old C56BL/6 female mice (Jackson Laboratory) according to a previously described protocol [17]. The mouse studies were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Tyler. Mouse bone marrow cells were first prepared with a previously described protocol [20]. For further isolation of neutrophils from bone marrow cells, a density gradient was created by layering 3 ml of 1.119 g/ml Histopaque (Sigma) and 1.007 g/ml Ficoll-Paque (Sigma) with the bone marrow solution on top. The density gradient was centrifuged at 550 × g for 30 minutes at room temperature with no brake. The middle neutrophil rich layer was removed with a syringe, and the cell viability and number were determined as described for human neutrophils.

The identity and purity of the neutrophils were determined through Giemsa staining (American MasterTech). A 10 μl aliquot of human or mouse neutrophil suspension

was deposited on a glass slide with the cytospin method (Cytospin 3 Shandon), and the cells were fixed with 100% methanol treatment for 1 minute. After being air dried at room temperature, the slides were soaked in Giemsa dye overnight. The following day, the slides were rinsed with water and mounted with a cover slide. The slide was imaged with a LionHeart microscope (Biotek) and analyzed for neutrophil identification and purity.

CFSE labeling of neutrophils

Both human and mouse neutrophils were labeled with CFSE as described previously [21]. The cells were resuspended at 5×10^7 cells/ml in HBSS and incubated with 2 μM CFSE (Invitrogen CellTrace) at 37°C. One minute after incubation, the labeling reaction was stopped by addition of 100 μl of heat inactivated FBS; this was followed by washing with HBSS. The labeled cells were then resuspended at 2.5×10^6/ml in RPMI-1640 medium and kept on ice before use.

Chemotactic assays

The chemotactic assays for human peripheral blood neutrophils and mouse bone marrow derived neutrophils were performed as described previously [14] with minor modifications. Briefly, the chemotactic assay was performed with 24-well Transwell membrane plates (Thermo Fisher) with a pore size of 3 μm for mouse neutrophils [22] and 8 μm for human neutrophils [23]. The basal chamber received 200 μl of RPMI-1640 medium and 200 μl of CF of *Mtb* strains, fMLF peptide or synthetic peptides. The apical chamber received 2.5×10^5 neutrophils in 100 μl of RPMI-1640 medium with or without sulfasalazine. For controls, we used 7H9 medium, Sauton's medium, 250 nM fMLF peptide as a positive control, HBSS buffer or 1 mM dimethyl sulfoxide (DMSO; Sigma) as a vehicle control. The cells were incubated at 37°C overnight. The next day, the Transwell inserts were removed, and the cells in each well were collected by three washes with HBSS containing 0.5 M EDTA. The total cell numbers were determined with an Attune NXT flow cytometer (Thermo Fisher) with a fixed volume of cell suspensions, and analysis by FlowJo software was performed to determine the total migrated cells.

For inhibition experiments, sulfasalazine (Sigma), a chemical inhibitor of fPRs 1 and 2, was dissolved in DMSO to produce a 75 mM stock, then added to neutrophil suspension in the apical chambers of the Transwell plate at 0.01, 0.1, 1 or 10 mM.

Mass spectrometry of *Mtb* CF and peptide synthesis

Samples were prepared for mass spectrometry analysis as described by Millipore. Four milliliters of 2-week and 4-week *Mtb* CF in 7H9 medium were filtered through a 3 kDa cutoff centrifugal filter (Amicon Ultra-4 centrifugal filter unit) at 700 × g for 30 minutes, and 100 μl of the lower and the upper filtrates was sent to the University of Texas Southwestern Medical Center Proteomics Core facility for mass spectrometry analysis. The samples were analyzed with a Proteome Discoverer 2.2 Oribtrap Elite protein identification mass analyzer to identify a peptide beginning with MLF, with a length ranging from four to nine amino acids and an N-formylated terminus. The three N-formylated peptides with the highest confidence were selected for synthesis. The peptides f-MLFRSQD, f-MLFENSR, f-MLFHQVQ and control NH2-MLFRSQD (non-formylated control) were synthesized (Thermo Fisher) in 1 mg amounts at 97–99% purity, as determined by HPLC analysis. The peptide stocks were made by dissolving the lyophilized peptides with 2 ml DMSO, and aliquots were stored at -80°C before use.

Neutrophil viability

MTT assays were performed according to our previous study [20]. At the end of the culture, 30% MTT was added to the cells and cultured at 37°C. Four hours after incubation, the supernatant was removed, and 100 μl of MTT stop solution was added to the cells. The solution was mixed, and the optical density at 570 nm was recorded with an ELISA plate reader (Molecular Devices).

Calcium mobilization assays

Intracellular calcium mobilization of neutrophils was determined with a previously described method [24] with minor modifications. Isolated human neutrophils (8×10^6 cells/ml) were incubated with a Ca^{2+} indicator fluorescent dye, fluor-4 AM (Invitrogen), at 4 μg/ml for 30 minutes at 37°C. The cells were then washed with 10 ml of HBSS and resuspended in RPMI-1640 medium at 2.5×10^6/ml. The cells were treated with RPMI-1640 medium, CF of H37Rv (7H9) or 250 nM of f-MLFRSQD, f-MLFHQVQ or NH2-MLFRSQD, and Ca^{2+} mobilization was immediately measured through flow cytometry analysis for 5 minutes. The Ca^{2+} levels that exceeded a pre-determined threshold were quantified through ImageJ analysis, normalized to the control, and expressed as the percentage above the baseline.

Statistical analysis

The data are presented as mean ± standard error of mean (SEM). Statistical significance was measured with a standard Student's T test and ANOVA, and a p-value of less than 0.05 was considered statistically significant. Multiple replicates were analyzed as independent experiments, with n values up to six.

RESULTS

Culture filtrates of *Mtb* induce chemotaxis of neutrophils

To identify the potential neutrophilic chemotactic factors of *Mtb*, we tested the culture filtrates of *Mtb* H37Rv for their neutrophilic chemotactic activity. The incubation of neutrophils with 7H9 medium as a control in the basal

chamber resulted in migration of 3.8×10^4 cells across the Transwell. In contrast, CF of H37Rv in 7H9 medium induced migration of 1.7×10^5 cells, indicating 4.5-fold higher chemotaxis of neutrophils than that in 7H9 medium alone. To perform further tests with a culture medium with a defined composition of ingredients free of animal derived components, we performed a chemotaxis assay using CF of H37Rv cultured in Sauton's medium [25]. Sauton's medium attracted very small numbers of neutrophils (approximately 2×10^3 cells). In contrast, the CF of H37Rv with Sauton's medium significantly increased chemotaxis of neutrophils (up to 2×10^4 total cells; 5.8-fold increase, $p = 0.03$; Fig 1A). This finding was consistent with the chemotaxis of neutrophils induced by CF of H37Rv in 7H9 culture medium. We also performed tests with mouse bone marrow derived neutrophils. The CF of both the 2-week and 4-week H37Rv cultures elicited significantly greater ($p < 0.001$) neutrophil chemotaxis than the 7H9 medium control (Fig 1C). The 2-week CF produced a 9-fold increase in chemotaxed neutrophils, whereas the 4-week CF produced a 26-fold increase in chemotaxed neutrophils, thus indicating a culture time dependent pattern in the chemotactic activity of CF of H37Rv. Giemsa staining of the prepared human (Fig 1B) and mouse (Fig 1D) neutrophils confirmed a purity of the neutrophils exceeding 95%. These results together demonstrated that *Mtb* strains secrete components with chemotactic activity toward both human and mouse neutrophils. To assess whether this general mechanism might be shared by other members of the *Mtb* complex, we tested the neutrophilic chemotactic activity of CF from 9 clinical isolates and Erdman strain of *Mtb*. Flow cytometry analysis of the chemotaxed neutrophils demonstrated that all 11 *Mtb* strains induced significantly ($p = 0.03$) more chemotaxed neutrophils than the 7H9 medium control (Fig 1E). These results together demonstrated that CF of H37Rv and clinical isolates induce chemotaxis of both human and mouse neutrophils.

Sulfasalazine inhibition of neutrophil chemotaxis for CF of *Mtb* and fMLF

Isolated human neutrophils combined with increasing concentrations of sulfasalazine were incubated with CF of H37Rv (7H9) or 250 nM fMLF overnight in a 24-well Transwell plate. For fMLF stimulation, both concentrations of sulfasalazine (0.1 and 1 mM) reduced fMLF induced chemotaxis of neutrophils significantly ($p < 0.0001$) with 27-fold reduction than the DMSO control (Fig 2). Likewise, sulfasalazine at 0.1 mM reduced the CF of H37Rv induced chemotaxis of neutrophils significantly with a reduction of 2.3 fold ($p < 0.02$), and at 1 mM concentration reached to 18-fold reduction of chemotaxed neutrophils (Fig 2). The lowest concentration (0.01 mM) and highest concentration (10 mM) are not shown. Cell viability was monitored with

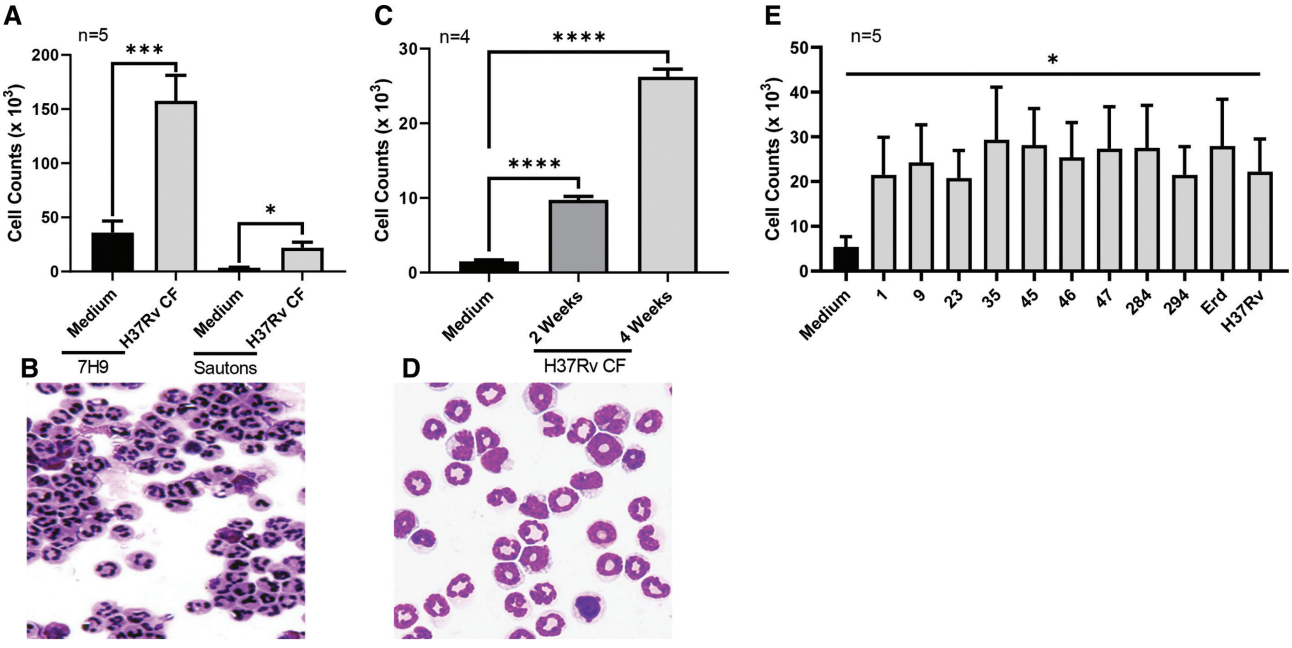

FIGURE 1 | The culture filtrates of *Mtb* induce chemotaxis of neutrophils. (A) Peripheral blood neutrophils of healthy donors were incubated in the apical chamber of a Transwell plate in 100 µl with the 4-week CF of H37Rv cultured in either Middlebrook 7H9 or Sauton's medium in the basal chamber at 37°C and 5% CO_2. At 16 hours after incubation, the cells in the basal chamber were enumerated by flow cytometry analysis. (C) Bone marrow neutrophils from naïve C57BL/6 mice were incubated with CF of H37Rv in Middlebrook 7H9 cultured for 2 weeks and 4 weeks, and the number of cells in the basal chamber was determined as described in A after 16 hours. The images of purified neutrophils from human peripheral blood (B) or mouse bone marrow (D) after Giemsa stain are shown. One representative image of three different experiments is shown. (E) Peripheral blood neutrophils from healthy donors were incubated with CF of nine clinical isolates, Erdman and H37Rv of *Mtb* cultured in Middlebrook 7H9 for 4 weeks; the number of chemotaxed cells in the basal chamber was determined as described in A. Data are expressed as mean ± SEM. *, $p < 0.05$; ***, $p < 0.005$; ****, $p < 0.0001$ compared with the respective medium control.

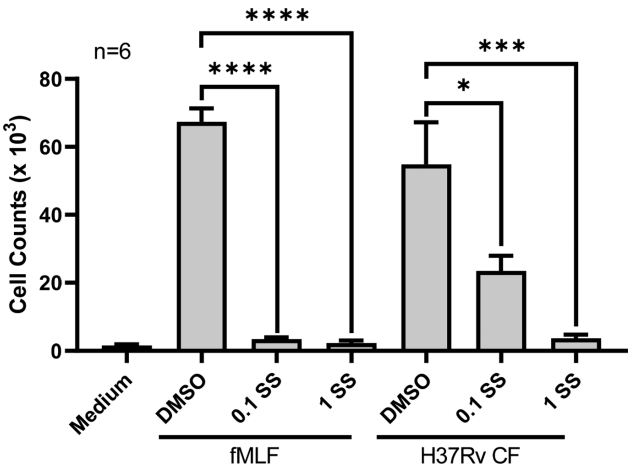

FIGURE 2 | Sulfasalazine, an inhibitor of fPRs, suppresses neutrophil chemotaxis induced by the fMLF peptide and CF of H37Rv. (A) Chemotaxis of human peripheral blood neutrophils with fMLF peptide at 250 nM or CF of H37Rv cultured in Middlebrook 7H9, assessed in the presence of sulfasalazine (SS) at the indicated concentrations (mM), or DMSO as a vehicle control and fresh Middlebrook 7H9 as a negative control (Medium), as in Fig 1. The chemotaxed cells were determined by flow cytometry analysis, and data are expressed as mean ± SEM. *, $p < 0.05$; ***, $p < 0.005$; ****, $p < 0.0001$ compared with DMSO control.

TABLE 1 | Mass spectrometry identification of three potential chemotactic candidates from the culture filtrates of H37Rv in 7H9 broth.

Confidence	Sequence	Modifications[1]	Quality Pep[2]	PSMs[3]
High	mlfeeai	1× Acetyl [N-Term]	0.205636	2
High	mlfpasp	1× Oxidation [M1]	0.285586	1
High	mlfsgpi	1× Oxidation [M1]	0.307954	2
High	mlfagiv		0.359888	2
High	mlfrsqd	1× Formyl [N-Term]	0.377808	1
High	mlfmfqv	1× Acetyl [N-Term]	0.41359	1
High	mlfensr	1× Formyl [N-Term]	0.417769	1
High	mlfhqvq	1× Formyl [N-Term]	0.417769	1
High	mlffmqv	1× Acetyl [N-Term]	0.436335	1
High	mlfgalv		0.436335	2
High	mlfhti	1× Oxidation [M1]	0.436335	2

[1]Indicative of N-terminal or internal modifications to the identified peptides.
[2]Represents measured confidence of the identified peptides, where values closer to 1 have low confidence, and values close to 0 have high confidence.
[3]PSMs (peptide spectral matches) represent the number of matching peptides in the solution.

MTT assays, which indicated that all neutrophils remained viable after incubation with sulfasalazine (Fig 5). These results indicated that the chemotactic activity of the CF of H37Rv may contain formylated peptides as a neutrophilic chemotactic factor.

Mass spectrometry identification of three candidate peptides

To identify potential chemotactic components from the CF of *Mtb*, we filtered the 4-week CF of H37Rv in 7H9 broth through a 3 kDa cut-off centrifugal filter, and analyzed the upper and lower filtrates by mass spectrometry for the identification of N-formylated peptides. No peptides matched the search criteria in the upper filtrate (components above 3 kDa); however, 11 identified peptides had an MLF starting sequence, three of which had a formylated N-terminus (Table 1). Two of the three formylated peptides had a confidence of 0.42, whereas the other had a confidence of 0.38. All three formylated peptides had peptide to spectrum matches equal to 1.

Synthetic formylated peptides induce chemotactic activity in human neutrophils

To test the neutrophilic chemotactic activity of the identified peptides, we synthesized three formyl peptides and one non-formylated peptide via a commercial source and assessed their chemotactic activity by using human peripheral blood neutrophils. Human neutrophils were incubated with 125, 250 or 500 nM of synthetic f-MLFRSQD, f-MLFENSR, f-MLFHQVQ and NH_2-MLFRSQD overnight in a 24-well Transwell plate. All concentrations of f-MLFENSR and NH_2-MLFRSQD, compared with the DMSO vehicle control, showed a non-significant increase in chemotaxed cells (Fig 3). However, both the formylated MLFRSQD and MLFHQVQ showed significant ($p = 0.003$ and $p = 0.01$) increases in chemotaxed human neutrophils for all concentrations. Formylated MLFRSQD showed an 11- to 26-fold increase in chemotaxed neutrophils, whereas f-MLFHQVQ showed a 32- to 39-fold increase in chemotaxed neutrophils and thus were selected for further analysis. To validate that these peptides stimulated chemotaxis of neutrophils through fPRs, we used sulfasalazine as an fPR inhibitor. The presence of sulfasalazine significantly ($p = 0.01$) decreased the chemotaxis of neutrophils stimulated by f-MLFRSQD, with a 56-fold difference with respect to the non-inhibited control (Fig 4). Similarly, sulfasalazine also suppressed f-MLFHQVQ elicited neutrophil chemotaxis significantly ($p = 0.01$), with a 24-fold difference with respect to the control cells without sulfasalazine (Fig 4). Cell viability was monitored with MTT assays, which indicated that all neutrophils remained viable after incubation with sulfasalazine (Fig 5).

Formyl-peptides induce substantial calcium mobilization in human neutrophils

Human neutrophils were stimulated with CF of H37Rv (7H9), f-MLFRSQD/NH_2-MLFRSQD and f-MLFHQVQ, and fMLF was used as a positive control. With RPMI-1640 medium as a baseline control, each stimulant was compared with the threshold to visualize an increase or decrease in intracellular Ca^{2+}. All stimulants except for CF of H37Rv (7H9) induced an increase in intracellular

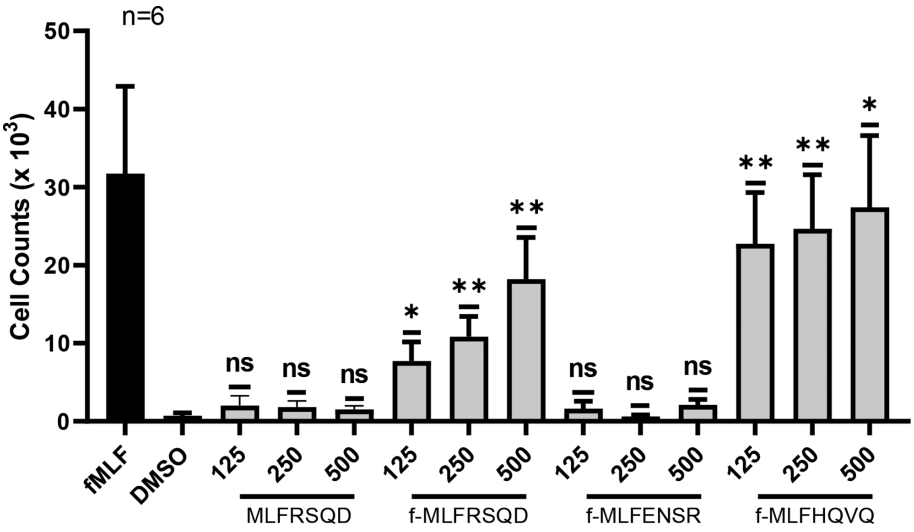

FIGURE 3 | Synthetic formylated peptides induce chemotaxis of human neutrophils. Peripheral blood neutrophils were incubated with synthetic peptides at the indicated concentrations, or DMSO as a negative control and 250 nM fMLF as a positive control, in Transwell chemotaxis assays, as described in Fig 1. The chemotaxed cells were determined with flow cytometry analysis, and data are expressed as mean ± SEM. Ns, no significance; *, $p < 0.05$; and **, $p < 0.005$ compared with DMSO vehicle control.

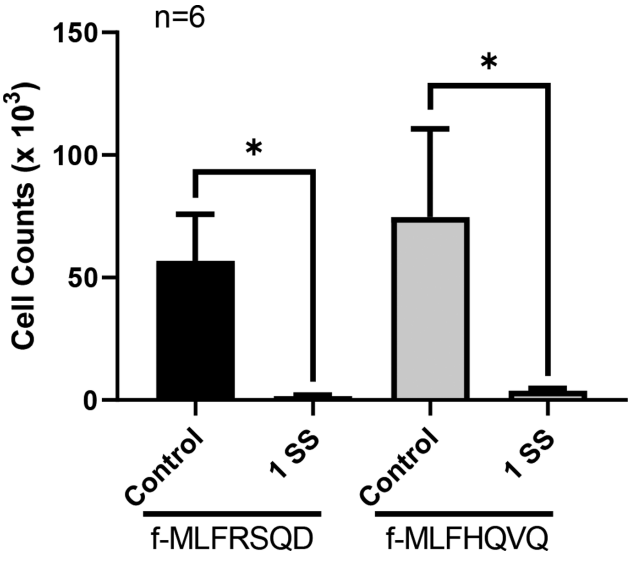

FIGURE 4 | Sulfasalazine blocks chemotaxis of human neutrophils by synthetic formylated peptides. Chemotaxis of human peripheral blood neutrophils with or without 1 mM sulfasalazine (SS) treatment, incubated with formylated peptides with the highest chemotactic activity, as previously described. The total chemotaxed cells were determined by flow cytometry analysis, and data are expressed as mean ± SEM. *, $p < 0.05$ compared with control cells.

free Ca^{2+} levels above baseline levels (Fig 6A). For quantification, the total percentage area of the level of intracellular free Ca^{2+} above baseline was normalized to that of the RPMI-1640 medium control and expressed as the relative Ca^{2+} above baseline (Fig 6B). The CF of H37Rv (7H9) and the NH_2-MLFRSQD peptide did not result in a significant increase above the baseline, whereas the three formylated peptides (fMLF, f-MLFRSQD and f-MLFHQVQ) elicited significant ($p = 0.04, 0.01, 0.001$) increases above baseline (Fig 6B).

DISCUSSION

Emerging evidence indicates that neutrophils play critical roles in TB infection, particularly in chronic infections and severe disease accompanied by granuloma formation. Although several neutrophilic chemotactic factors with host origin have been described, the chemotactic factors originating from the *Mtb* pathogen remained unexplored. Our results from this initial study demonstrated the existence of N-formylated peptides in the culture filtrates of *Mtb*. The chemotactic activity of CF of H37Rv was also confirmed with Sauton's medium with known components and without contamination from animal derived components. Even so, the chemotactic activity was lower than that of CF of *Mtb* with 7H9 broth, possibly because more bacilli were present in the 7H9 broth than Sauton's medium. The rich nutritional value of 7H9 broth supplemented with ADC and albumin might have facilitated *Mtb* growth by neutralizing the growth suppressive effects of free fatty acids produced during the growth of *Mtb*.

Interestingly, the CF of *Mtb* induced chemotaxis of both human and mouse neutrophils. Mouse neutrophils responded to the CF of H37Rv in a dose dependent manner when the cells were incubated with CF of H37Rv (7H9) harvested at 2- and 4-week culture times. The 4-week CF induced a dramatic 26-fold increase in chemotaxed cells, whereas the CF of 2-week CF induced a 9-fold increase in neutrophil chemotaxis. The chemotaxed neutrophils were 2.9-fold higher in the 4-week culture than the 2-week culture, in agreement with the greater number of bacilli in the former. The neutrophil chemotactic acitivies were also confirmed for the CF of 10 other strains of *Mtb* inlcuding *Mtb* Erdman and 9 clinical isolates of *Mtb*. These results therefore indicated that CF of *Mtb* indeed induces chemotaxis of human and mouse neutrophils.

Pathogen derived chemoattractants have been studied over 50 years. The most common chemoattractant

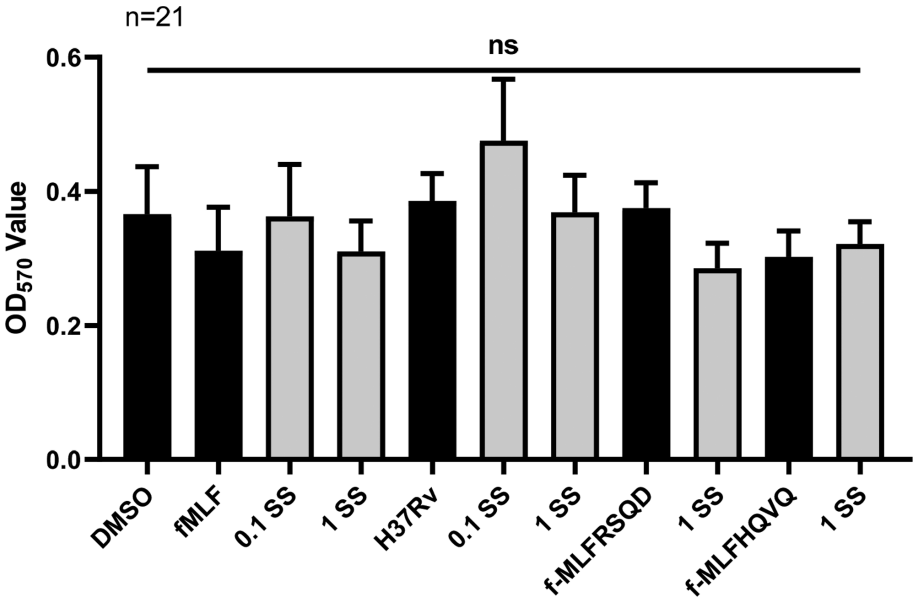

FIGURE 5 | Sulfasalazine induced suppression of neutrophil chemotaxis by formylated peptides is not due to compromised cell viability. Human neutrophils treated with sulfasalazine were incubated with MTT reagent to determine cell viability in chemotactic assays. Total cell activity was calculated according to OD_{570} absorbance, and data are expressed as mean ± SEM.

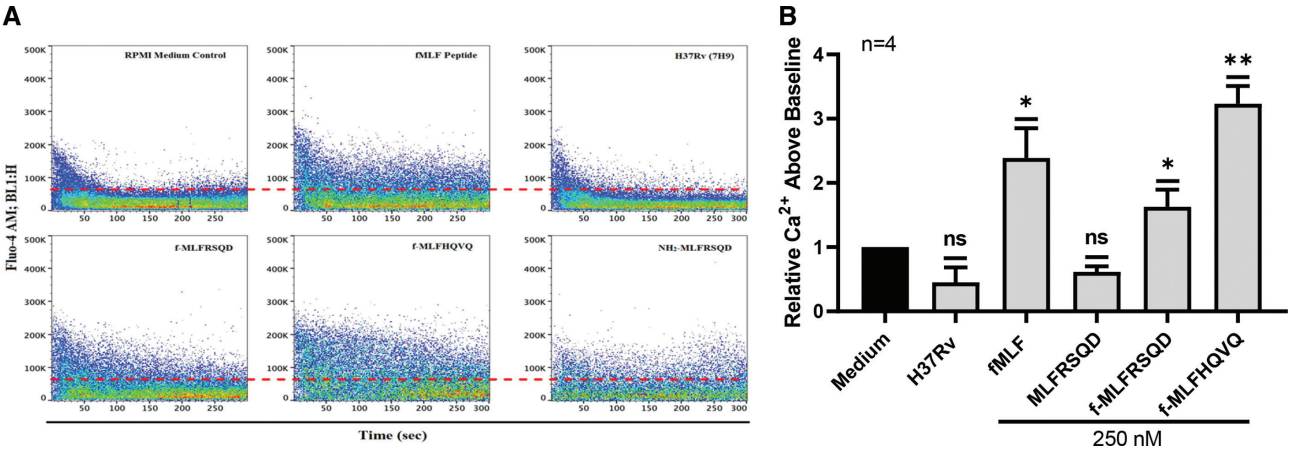

FIGURE 6 | Synthetic formylated peptides induce intracellular Ca^{2+} mobilization of neutrophils. Human peripheral blood neutrophils from four healthy donors were labeled with Fluo-4 AM and stimulated with RPMI-1640 medium control, CF of H37Rv (7H9) or 250 nM of peptides as indicated. The cells were immediately monitored for 5 minutes. (A) Flow cytometry analysis of intracellular Ca^{2+} mobilization over 5 minutes. (B) Variables were normalized to the RPMI-1640 medium control to express relative Ca^{2+} above baseline. ns, no significance; *, $p < 0.05$ and **, $p < 0.005$, fold difference in Ca^{2+} levels compared with RPMI-1640 control cells.

produced by bacterial pathogens is fMLF [15,16,26]. The N-formylation refers to the addition of a formyl group to the N-terminus of the peptide, thus effectively blocking the N-terminus from other modifications [27]. The 3-amino acid peptide is associated with the binding and activation of fPRs expressed on the membranes of mammalian neutrophils [28]. To determine fPR involvement, we applied an fPR inhibitor, sulfasalazine. This known anti-inflammatory drug has been used for more than 60 years for the clinical management of inflammatory diseases, such as rheumatoid arthritis, ulcerative colitis and inflammatory bowel disease [29-31]. This compound binds fPRs on the neutrophilic surface and blocks formylated peptide binding [32]. Isolated human neutrophils were incubated with various concentrations of sulfasalazine before incubation with CF and formylated peptides for chemotaxis. In agreement with previous published studies, sulfasalazine inhibited fMLF induced chemotaxis of neutrophils in a concentration dependent manner. Interestingly, sulfasalazine also blocked the chemotactic responses of human neutrophils to the CF of H37Rv and the synthetic formylated peptides of *Mtb* in an equivalent manner, thus indicating that the components in the CF of *Mtb* act mainly through the same fPR on neutrophils. These results suggested that the chemoattractant present in CF of H37Rv requires fPR activation to induce neutrophil chemotaxis.

Mass spectrometry analysis of CF of H37Rv after division of the filtrate into two fractions detected no peptides

that matched the search criteria of formylated peptides for the upper filtrate. However, 11 peptides starting with MLF, and between six and nine amino acids in length, were identified in the lower filtrate. Of the 11 identified peptides, three that matched the search criteria were tested for their chemotactic activity on human neutrophils by using their synthetic mimetics. Peptide 1 (f-MLFRSQD) was categorized with the highest confidence and therefore was used as the non-formylated peptide control. Only peptide 1 and peptide 3 (f-MLFHQVQ) elicited significantly greater neutrophil chemotaxis than the DMSO vehicle control and non-formylated control. However, peptide 1 did not have an efficacy comparable to that of the fMLF control. Peptide 3 induced greater numbers of chemotaxed cells than peptide 1; therefore, peptide 3 is probably the more potent chemoattractant produced by H37Rv. To confirm that these synthetic formylated peptides used the same fPR mechanism, we incubated isolated human neutrophils with the fPR inhibitor sulfasalazine and stimulated them with peptides 1 and 3 (Fig 4). The presence of sulfasalazine at 1 mM suppressed the activities of both peptides similarly. Collectively, these results suggested that synthetic peptides f-MLFRSQD and f-MLFHQVQ both induce neutrophil chemotaxis through binding fPRs on neutrophilic cell surfaces, such as fMLF.

When neutrophils are activated by cytokines, chemokines or other chemoattractants, a sequence of downstream events activate multiple phospholipases that act on membrane phospholipids [33]. Activation of the phosphoinositide-specific phospholipase Cβ then generates inositol triphosphate, which consequently initiates the mobilization of Ca^{2+} from intracellular stores [33-35]. Typically, very rapid immobilization of intracellular Ca^{2+} is the first indicator of cell activation [36]. Compared with the RPMI-1640 medium control, fMLF, f-MLFRSQD, f-MLFHQVQ and NH2-MLFRSQD all showed free Ca^{2+} levels that exceeded the baseline (Fig 6A). However, when normalized to the medium control, only the fMLF, f-MLFRSQD and f-MLFHQVQ peptides induced Ca^{2+} levels above the baseline control (Fig 6B). These results suggested that the formylation of the peptides played a key role in neutrophil activation. This conclusion was supported by the finding that non-formylated peptide did not induce Ca^{2+} levels above baseline. However, the CF of H37Rv (7H9) did not induce Ca^{2+} levels significantly above baseline. This is probably due to: 1) multiple chemoattractant components present in the CF might have competed for neutrophil receptor activation, thus slowing the Ca^{2+} mobilization process, 2) neutrophil activation might have occurred very quickly, and the Ca^{2+} mobilization might have been missed because it occurred before the reading, or 3) the concentration of the formylated peptides in the culture filtrate might not have been as high as the chosen concentration of the synthetic mimetic used. Overall, we deduced that Ca^{2+} mobilization occurred in response to stimulation of neutrophils with formylated peptides of *Mtb*.

In conclusion, our results showed that two formylated peptides identified in CF of *Mtb* via mass spectrometry analysis showed biological chemotactic activity toward primary neutrophils of both mouse and human origin. Our findings add two potential N-formylated peptides to the list of other chemotactic factors identified to be of *Mtb* origin, such as monocyte chemoattractant protein (MCP)-1 like protein of *Mtb* [37] and ESAT-6 and glutamine synthetase derived N-formylated peptides [38], thus lending support to the existence of chemotactic proteins and peptides of *Mtb* origin.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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