Sphingosine-1-phosphate receptors 2 and 3 reprogram resting human macrophages into M1 phenotype following mycobacteria infection

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A B S T R A C T

Mycobacterium tuberculosis (M.tb) the causative agent for tuberculosis has been accredited for a high rate of morbidity and mortality worldwide. The rise in MDR and XDR cases has further created new obstacles in achieving the “End TB Strategy”, which is aimed for 2035. In this article, we have demonstrated the potential of sphingosine-1-phosphate (S1P) analogs in providing an anti-mycobacterial effector response by altering macrophage polarity into M1. Among S1PR1 and S1PR3 analogs, S1PR2 analogs proficiently favor selective polarization of infected human macrophages into M1 phenotypes, marked by increased expression of M1 markers and decreased M2 markers. Furthermore, S1PR1-3 analogs treated macrophages were also able to decrease the secretion of anti-inflammatory cytokine IL-10 and can induce NO secretion in infected macrophages. Lastly, only S1PR2-3 analogs were able to restrict the growth of mycobacteria in human macrophages. Taken together our study reflects the potential of S1PR2-3 analogs in providing host defenses following mycobacterial infection by favoring M1 macrophage polarization.

1. Introduction

Mycobacterium tuberculosis (M.tb), an etiological agent for tuberculosis (TB), claimed about 1.3 million deaths in 2019 worldwide, crowning M.tb as the leading cause of mortality by a single pathogen (WHO, 2019). Despite having effective antibiotics, the present situation is further complicated by the emergence of antibiotic-resistant mycobacteria. According to World Health Organization (WHO), 186 million cases of multidrug-resistant TB (MDR-TB) and an additional 13 million cases of extensively drug resistance-TB (XDR-TB) were registered in 2019 (WHO, 2019). Although the bacillus Calmette–Guérin (BCG) vaccine is effective in infants and adolescents but has variable efficacy in adults (Dockrell and Smith, 2017). In addition, non-tubercular mycobacterial infections in humans by Mycobacterium avium spp. are growing (Kaczmarkowska et al., 2022). All these shortcomings of the present TB therapy and increased Mycobacterium avium complex (MAC) infections have complied with the search for alternative therapies. In this regard, it is highly anticipated that the repolarization of activated macrophages into M1 phenotype can be utilized as novel therapeutics for TB (Arish and Naz, 2022). Macrophages are highly dynamic and plastic cells of the immune system. These immune cells offer protection during infection by differential activation and polarization into inflammatory macrophages (M1), however, mycobacteria have evolved strategies to favor anti-inflammatory (M2) macrophages phenotypes over M1 polarization (Arish and Naz, 2022).

Sphingosine-1-phosphate (S1P), a bioactive lipid mediator, has been previously shown to influence macrophage polarization (Yang et al., 2018). S1P is a natural ligand for five S1P receptors subtypes, a family of G-protein coupled receptors, which are classified as S1PR1-5. There is differential expression of S1PR on M1 and M2 macrophages. Furthermore, S1P induced proinflammatory cytokines in resting and M2 polarized mouse bone marrow-derived macrophages (M ü ller et al.,

** Abbreviations: M.tb, Mycobacterium tuberculosis; W.H.O, World Health Organization; MDR, Multidrug resistant; MAC, Mycobacterium avium complex; S1P, Sphingosine-1-phosphate; MAPKs, Mitogen-activated protein kinases; STAT, Signal transducer and activator of transcription; NO, Nitric oxide; ROS, Reactive oxygen species.

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2017). Particularly, S1PR2-3, not S1PR1, regulates M1 polarization in mouse bone marrow-derived macrophages (Yang et al., 2018). S1P mediated signaling regulates macrophage effector functions and plays a protective role in several diseases and infections, including TB (Nadella et al., 2019; Naz and Arish, 2020a; Arish et al., 2016, 2018). Therefore, S1P may be regarded as an attractive therapy for various infectious diseases. However, due to its short half-life as it is readily dephosphorylated by sphingosine lyase (Serra and Saba, 2010), using S1P as a therapeutic could not be considered a feasible option. Alternatively, S1PR analogs may be utilized that mimic the S1P functioning by binding to a particular receptor subtype, minimizing the off-target effects of S1P. Hence, in this study, we studied the role of S1PR analogs as a therapeutic effect upon mycobacterial infection.

Firstly, we checked the expression of S1PR1-3 in both Mtb and M. avium infected macrophages. We also checked for the expression of prominent M1 markers such as nos2, cd40, cd80, cd86, il-1β, and il-12p40 at mRNA level in S1PR analogs treated and untreated mycobacterial infected macrophages. We further looked for the change in the expression level of some M2 markers genes also, including arginase (arg), cd163, and il-10. For the confirmation of our results, we checked for the activation of MAPKs and STAT3 pathways in infected macrophages. In addition, we also revealed the change in the Nitric oxide (NO), Reactive oxygen species (ROS), and cytokine levels such as IL-10, IL-6 and TNF-alpha secretion in S1PR treated and untreated infected macrophages. Finally, we demonstrated the effects of S1PR analogs on the intracellular mycobacterial burden.

2. Methodology

2.1. THP-1 monocytes culturing and differentiation

The human THP-1 monocytes were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies) and 1% Antibiotic-Antimycotic (Life Technologies) at 37 °C in 5% CO₂, 10 ng/ml PMA (phorbol 12-myristate 13-acetate; Sigma) was used to differentiate THP-1 monocytes into macrophages for 24 h at 37 °C in 5% CO₂ in flat-bottom 12-well tissue culture plates (BD Biosciences). The differentiated macrophages were then allowed to rest for 24 h before infection studies.

2.2. Mycobacteria culturing

Mycobacterial spp. (H37Ra and M. avium) were grown in Middlebrook 7H9 broth supplemented with albumin dextrose catalase (OADC, BD). Mycobacteria were then harvested, suspended in sterile PBS (pH 7.2), aliquoted, and stored at −80 °C until use. M. avium was obtained through BEI Resources, NIAID, NIH: M. avium (DJO-44271), NR-49092. The purity of the mycobacteria was evaluated using acid-fast staining.

2.3. Infection and evaluation of mycobacterial growth after in-vitro infection

Differentiated THP-1 macrophages were infected for 4 h with M.tb H37Ra or M. avium at a multiplicity of infection (MOI) of 10 in an antibiotic-free media. After 4h, non-phagocytosed bacilli were removed by gentle washing with PBS and the infected macrophages were treated with S1PR analogs and further cultured for an additional 24 h or 48 h in complete media supplemented with gentamicin.

2.4. Colony forming unit (CFU) assay

To quantify Mycobacterium CFU, macrophages were infected at 24 h and 48 h; infected cells were washed with PBS to remove any extracellular bacilli. The infected macrophages were lysed with sterile water for 10 min and plated after serial dilution on Middlebrook 7H11 agar plates supplemented with OADC. Colonies were counted after 4 weeks of growth at 37 °C.

2.5. Treatments

S1PR1-3 analogs such as CMY5442 (S1PR1 agonist), CMY5520 (S1PR2 agonist), and CMY5541 (S1PR3 agonist) respectively were dissolved in DMSO and stock solutions were stored at −20 °C. All of the analogs were purchased from cayman chemicals. The stock solutions of analogs were further diluted in serum-free media. The macrophages were treated with a 10 μM concentration of S1PR analogs.

2.6. cDNA synthesis and real-time PCR

RNA was extracted by Trizol method (Sigma) and RNA concentration was measured on NanoDrop (Thermo). For cDNA synthesis, 1 μg of total RNA from each sample was used to transcribe cDNA using the iScript cDNA synthesis kit (Bio-rad) as per the manufacturer’s protocol. Levels of arg, nos2, cd40, cd80, cd86, cd163, il-1β, il-10, and il-12p40 expressions were determined in the treated and untreated infected macrophages (IM) by quantitative PCR (qPCR), with β-actin as an endogenous control (Primer sequences in supplementary table no. 1). Subsequently, qPCR was carried out in 10 μl reaction mixture containing 2X SYBR green iTaq (Bio-Rad, USA), using CX96 (Biorad). Relative gene expression was analyzed by the Livak method.

2.7. Protein extraction and western blotting

After treatment, infected macrophages were washed twice with PBS and lysed with ice-cold lysis buffer (50 mM Tris-HCl, [pH 7.4], 150 mM NaCl, 1% Triton-X, 1 mM Sodium Orthovanadate, 10 mM Sodium Fluoride). 1X protease inhibitor cocktail (Sigma) was added freshly to the lysis buffer. Lysates were centrifuged at 14,000 × g at 4 °C for 15 min, and the resulting supernatants were transferred to fresh tubes and stored at −80 °C until required.

2.8. Cytokine measurement

IL-10, IL-6, and TNF-α were quantified in cell supernatants using specific ELISA kits according to the manufacturer’s instructions (BD Bioscience).

2.9. Nitric oxide assay

Nitrite concentrations were determined using Nitric oxide assay kit (Thermo). NO levels in the culture supernatants in the mycobacteria infected macrophages treated and untreated with S1PR1-3 analogs were quantified according to the manufacturer’s protocol.

2.10. CellROX assay

The generation of reactive oxygen species was measured by CellRox green reagent (Thermo). The ROS was measured in the mycobacteria infected macrophages, treated and untreated with S1P analogs according to the manufacturer’s protocol.

2.11. Auramine-O staining

THP-1 derived macrophages were seeded on sterile coverslips placed in 12 well culture plates. Infection was given at MOI 1:10 by M. avium for 4h and infected cells were treated with or without S1PR2-3 analogs and cultured for the next 24h. Auramine-O staining was performed as described previously (Jung et al., 2013).
2.12. Statistical analysis

The results are a representation of a minimum of three reproducible experiments or otherwise stated. The statistical analysis was performed using GraphPad Prism, version 6.0 (GraphPad, San Diego, CA, USA). p-value of less than 0.05 was considered significant. The error bars represent the value from the replicates ± SD. One-way ANOVA and Student t-test were performed to ascertain the significance of the differences between the means of the control and the experimental groups. *p < 0.05; **p < 0.01; ***; p < 0.001; ****, p < 0.0001; ns non-significant.

3. Results

3.1. Differential expression of S1PRs in mycobacterial infected macrophages

As THP-1 derived macrophages mainly express three isotypes of S1PR 1–3 (Arish et al., 2018), we checked the real-time PCR-based expression of these receptors upon infection with M.tb H37Ra and M. avium in THP-1 derived macrophages at 24 and 48h post-infection (pi). M.tb infection resulted in a non-significant change in mRNA level of S1PR1 at both 24h and 48h infection (Fig. 1A and B), however, S1PR2 showed up-regulation at 24h pi but down-regulation at 48h pi (Fig. 1A and B). On the other hand, S1PR3 showed an opposite expression pattern. S1PR3 showed down-regulation at 24h pi and up-regulation at 48h pi (Fig. 1A and B). In the case of M. avium infection, there wasn’t any significant change at 24h (Fig. 1C). However, the expression of S1PR1-3 were upregulated at 48h pi non-significantly (Fig. 1D).

3.2. S1PR analogs favor M1 polarization and block M2 polarization

S1P signaling has been previously known to regulate macrophage polarization (Yang et al., 2018). Hence we checked the mRNA expression of M1 and M2 markers by real-time PCR in infected, treated, and untreated, THP-1 derived macrophages. As M.tb infection programs macrophages into pro-bacterial M2 phenotype, we checked whether the decrease in bacterial burden is due to reprogramming of these macrophages into the M1 phenotype which is more effective in clearing the infection. In this study, S1PR analogs drive selective activation of M1 markers in M.tb infection of THP-1 derived macrophages. As compared to S1PR1 and S1PR3 analogs, S1PR2 analog was more effective in increasing M1 markers in infected macrophages. S1PR2 treatment resulted in an increase in the expression of prominent M1 markers such as nos2, cd80, il-1beta, and il-12 at mRNA levels (Fig. 2B–F). However, S1PR3 treatment only increased the expression of cd40, nos2, and il-1beta (Fig. 2 A, D, E), and on the other hand, S1PR1 agonist treatment resulted in the increased expression of cd80, il-1beta, and il-12 (Fig. 2 B, E and F). Only S1PR1 analog treatment was showed a substantial increment in the expression of cytokines il-1beta and il-12p40 at mRNA level (Fig. 2E and F).

For further confirmation of our results, we checked whether S1PR1-3 analogs could also regulate the expression of M2 markers such as arg, cd163, and il-10 (Fig. 2H–I). In this study, we observed that S1PR2-3 analogs inhibited the expression of arg and only S1PR2 analog inhibited il-10 expression in infected macrophages (Fig. 2G and H). To our surprise, we observed a strongly increased expression of arg in S1PR1 analog-treated infected macrophages. Also, there was no significant change in the expression pattern of cd163 upon treatment with S1PR1-3 analogs (Fig. 2I).

3.3. S1PR regulates MAPKs and STAT3 in infected macrophages

We next observed the activation of mitogen-activated protein kinases (MAPKs) and Signal Transducer and Activator of Transcription 3 (STAT3) signaling in S1PR analogs treated and untreated mycobacterial infected macrophages. Here we found almost similar results with both M.tb and M. avium infection (Fig. 3A and B). In our study both M.tb and M. avium infected macrophages treated with S1PR1-3 analogs showed decreased phosphorylation of ERK1/2 (Fig. 3A and B). On the other hand, there was increased phosphorylation of p38 in S1PR1 analog and...
S1PR2 analog treated infected macrophages (Fig. 3A and B). In the case of STAT3, S1PR1-2 analogs result in increased phosphorylation of STAT3 following *M. avium* infection. Intriguingly, S1PR3 analog treatment results in decreased STAT3 phosphorylation. However, STAT3 phosphorylation was mildly increased in S1PR2-3 analog treatment upon *M. tb* infection (Fig. 3B).

**Fig. 3.** S1PR regulates MAPKs and STAT3 in infected macrophages: Western Blot showing the change in the phosphorylation status of MAPKs (p38 and ERK) and STAT3 following (A) *M. avium* and (B) *M. tb* H37Ra infection. GAPDH was used as a loading control. The western blot image data is the representation of two independent experiments.
3.4. S1PR analogs induce NO, ROS, and cytokine secretion in infected macrophages

We further checked for the NO and cytokine secretion in S1PR1-3 analog treated M.tb and M. avium infected human macrophages (Fig. 4A, B, and F). In the case of M. avium infection, we observed that S1PR2-3 analogs were capable of inducing NO in infected macrophages 24h pi (Fig. 4A). However, at 48h pi only S1PR2 was able to induce the NO secretion and the change was highly significant as compared to infected untreated macrophages (Fig. 4B). S1PR1 and S1PR3 were not able to induce NO beyond 24h (Fig. 4B). Furthermore, all the S1PR analogs were also able to increase the secretion of NO in M.tb infected human macrophages 24h pi (Fig. 4F). However, at 48h of infection there weren’t significant changes in the NO in infected and infected S1PR analogs treated macrophages (data not shown). To this end, S1PR analogs were also able to induce ROS production in infected treated macrophages as evident by CellRox assay in live macrophages (Fig. 4G).

For cytokines, we looked for IL-10, IL-6 and TNF-alpha secretion in S1PR analog treated and untreated, mycobacteria infected and untreated macrophages. Treatment of S1PR analogs in uninfected macrophages doesn’t alter the cytokine level (Fig. 4C, D, and E). However, S1PR analogs were able to modulate cytokine secretion in M.tb and M. avium infected macrophages (Fig. 4C-E, H-J). In context to IL-10, all the analogs were capable to decrease the secretion very significantly in M.tb and M. avium infected macrophages (Fig. 4 D and I). IL-6 secretion was only increased in M. avium infected macrophages treated with S1PR1 and S1PR2 analogs (Fig. 4C). Surprisingly, S1PR3 analog treatment was showing inhibition in the secretion of IL-6 in M. avium infected macrophages (Fig. 4C). In contrast, M.tb infected macrophages have shown a nonsignificant increase in the IL-6 levels upon S1PR1-3 treatment (Fig. 4H). M.tb infected macrophages treated with S1PR2 also revealed slightly increased TNF-alpha levels (Fig. 4J). Surprisingly, S1PR analogs have shown inhibition of TNF-alpha in M. avium infected macrophages (Fig. 4E). However, in the case of S1PR2 the change wasn’t significant, but in S1PR1 and S1PR3 analog treated infected macrophages showed a significant decrease in the secretion of TNF-alpha (Fig. 4E).

3.5. S1PR2-3 analogs restrict intracellular infection

As S1PR analogs can alter macrophage polarity and cytokine secretion, we further checked for the intracellular bacterial load in the presence of S1PR1 analogs. THP-1 derived macrophages were infected with M.tb and M. avium and treated with S1PR1-3 analogs, S1P, and sphingosine. In the case of M.tb, there was low CFU in S1PR2 and S1PR3 analogs treated infected macrophages, however, these changes were not significant at 24h pi (Fig. 5A and B). Furthermore, at 48h pi the changes were more significant as S1PR2 analog, S1PR3, S1P, and sphingosine (sph) treatment were able to restrict the growth of M.tb (Fig. 5A). With M. avium, we observed that at 24 pi, only S1PR2 analog and S1P treatment were able to restrict the intracellular bacterial growth (Fig. 5B). At 48h pi, S1PR2, S1PR3, and S1P treatment were found to be effective in restricting infection significantly (Fig. 5B). For further confirmation, we stained the S1PR2 and S1PR3 analogs of treated and untreated M. avium infected macrophages with auramine-o staining and observed them under a fluorescence microscope (Fig. 5C). Here we conclude that S1PR2-3 analog treated infected macrophages were able to control intracellular infection as evident with low bacilli inside macrophages (Fig. 5C).

4. Discussion

S1P has been established as a potent regulator of the immune response. In the context of TB, S1P has been previously known to exert anti-mycobacterial responses (Nadella et al., 2019; Garg et al., 2004; Santucci et al., 2007). However, the role of S1PRs in the regulation of anti-mycobacterial response was still elusive (Nadish and Arish, 2020h). Therefore, to study the role of the exact S1PR subtype following mycobacterial infection we utilized S1PR analogs such as CYM5442, CYM5520, and CYM5541, which are agonists for S1PR1, S1PR2, and S1PR3, respectively. As S1P can be readily bound to S1PR1-5, analogs with selective binding to a particular S1PR is a more approachable method as it minimizes the off-target effects of S1P, with the same binding efficacy (Xiao et al., 2016). Hence, in our present study, we examined the role of exact S1PR in the regulation of macrophage polarization and mycobacterial infection.

IL-6 has been known to be essential for the control of mycobacterial infection (Ladel et al., 1997). Earlier studies have shown that virulent M. tb and its proteins induce IL-6 production via p38 and STAT3 phosphorylation (Natarajan and Narayanan, 2007; Jung et al., 2017). In our study, we revealed that following M. avium infection, treatment with S1PR1-2 analogs resulted in increased phosphorylation of p38 and STAT3, and IL-6 secretion. Here we suggest that IL-6 secretion may be p38 or STAT3 dependent. Also as S1PR3 analog didn’t increase p38/STAT3 phosphorylation, we observed a decreased IL-6 secretion. However, a similar trend wasn’t observed in M.tb infected macrophages, which also showed enhanced p38/STAT3 phosphorylation, but no significant changes in IL-6 levels. In addition, S1PR3 analog also increased p38/STAT3 phosphorylation, but no change in IL-6 secretion. On the other hand, ERK phosphorylation, which is essential for mycobacterial entry (Yang et al., 2016) and enhanced IL-10 secretion following mycobacterial infection, which further blocks phagosomal maturation for intracellular survival (O’Leary et al., 2011). Previous clinical studies also showed that increased IL-10 levels have been associated with active TB (Gerosa et al., 1999; Verbon et al., 1999). Furthermore, in-vivo studies showed that IL-10 blockade clears the intracellular infection and improves the survivability of mice (Pitt et al., 2012; Moreira-Teixeira et al., 2017). In our study, S1PR3-1 analog treatment in infected macrophages revealed decreased ERK phosphorylation and decreased production of IL-10 in these macrophages. TNF-alpha is also regarded as a critical cytokine to control the dissemination of mycobacterial infection as a result of enhanced phagosomal maturation and enhanced T-cell response (Harris et al., 2008; Olsen et al., 2016). Surprisingly, in our study TNF-alpha levels were decreased in M. avium infected macrophages treated with S1PR1-3 analogs, however, there was a non-significant decrease in the macrophages treated with S1PR2 analog. In contrast, TNF-alpha levels were found to be slightly increased in M.tb infected macrophages upon S1PR2 treatment. S1PR1 and S1PR3 analog treatments don’t alter TNF-alpha production in M.tb infected macrophages.

Our study showed that S1PR2 analog was able to effectively polarize macrophages into M1 phenotype, which was demonstrated by the marked increased expression of M1 markers, enhanced NO and ROS secretion in infected macrophages. S1PR2 analogs hence can effectively program rest macrophages into M1 phenotype, which is critical to suppress intracellular bacterial load. In addition, S1PR3 analog treatment, and not S1PR1 analog, also resulted in enhanced expression and secretion of NO and increases ROS levels in the infected macrophages together with decreased IL-10 secretion, which could explain the lower CFU counts in S1PR3 treated macrophages. Although S1PR1 analog treatment also results in an increase in IL-6 and a decrease in IL-10, it couldn’t control the intracellular mycobacteria infection. This might be because S1PR1 wasn’t able to effectively programs macrophages to M1 phenotype, evident by unchanged NO secretion and unchanged TNF-alpha expression. Furthermore, pro-inflammatory cytokine secretion solely couldn’t be able to control intracellular mycobacterial infection. In summary, we have identified the S1PR2-3 analog as a critical regulator of macrophage polarization and effector response following mycobacterial infection. Both S1PR2 and S1PR3 analogs were able to program rest THP-1 into M1 phenotype that is critical for pathogen killing Fig. 6. Taken together, this could lead to open a whole new paradigm of targeted host-directed therapy against intracellular pathogens such as mycobacteria.
Fig. 4. S1PR agonist induces NO, ROS, and cytokine secretion in infected macrophages: The graphic indicates NO secretion in mycobacteria infected macrophages with or without S1PR analogs treatment (A) and (F) 24h and (B) 48h pi. (G) ROS was measured in the live macrophages infected with M.tb 24 h pi. Cell-free supernatants were collected in M.avium infected macrophages and uninfected macrophages with or without S1PR analogs treatment and (C), IL-6, (D) IL-10, (E) TNF-alpha secretion was measured by ELISA. (F) The graphic indicates NO secretion in M.tb infected macrophages with or without S1PR analogs treatment 24 pi. Cell-free supernatants were collected from M.tb infected macrophages and uninfected macrophages with or without S1PR analogs treatment and (C), IL-6, (D) IL-10, (E) TNF-alpha secretion was measured by ELISA. The data is a representation of mean ± SD from three independent experiments**, p < 0.01 ***, p < 0.001.
Nevertheless, there is some limitation of our study. For further characterization of M1 and M2 phenotype, a detailed expression pattern of cluster of differentiation (CD), both at mRNA and protein level could help explain which CDs are upregulated in S1PR analogs treated infected and uninfected macrophages. Although we provided much evidence that S1PR2-3 analogs can suppress intracellular infection by selective polarisation of macrophages, more in-depth in-vivo studies are required in this direction to further dissect the role of S1PR analogs in mycobacteria infection.

CRediT authorship contribution statement

Mohd Arish: Conceptualization, Methodology, Data curation, Writing – original draft preparation, Writing – review & editing, Data interpretation. Farha Naz: Conceptualization, Methodology, Writing – review & editing, Data interpretation.
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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