INHIBITING SERUM AMYLOID P - FCγR1 INTERACTIONS ON HUMAN MACROPHAGES DECREASES NUMBERS OF INTRACELLULAR *MYCOBACTERIA*

A Thesis

by

WANG XIANG

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MASTER OF SCIENCE

Chair of Committee,	Richard Gomer
Committee Members,	James Smith
	Paul Straight
	Chaodong Wu
Head of Department,	Tom Mcknight

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ABSTRACT

Macrophages are a heterogeneous population of cells and, include classically activated macrophages (M1) and alternatively activated macrophages (M2). Macrophages can change from M1 to M2 and vice versa in response to environmental stimuli. Serum Amyloid P (SAP) is a constitutive plasma protein that polarizes macrophages to an M2 phenotype, and part of this effect is mediated through FcγRI receptors. In an effort to find ways to alter macrophage phenotypes, we screened for compounds that can block the SAP-FcγRI interaction. From a screen of 3000 compounds, we found 12 compounds that reduced the ability of fluorescently labeled human SAP to bind cells expressing human FcγRI. Based on cell surface marker expression, 8 of the compounds inhibited the effect of SAP on skewing human macrophages to an M2 phenotype and in the presence of SAP polarized macrophages to an M1 phenotype. In diseases such as tuberculosis, M1 macrophages are more effective at killing bacteria than M2 macrophages. SAP potentiated the numbers of the mycobacterial strains *M. smegmatis* and *M. tuberculosis* in macrophages. When added along with SAP, 2 of the compounds reduced intracellular mycobacterium numbers. Together, these results indicate that blocking SAP effects on macrophages can skew these cells toward a M1 phenotype, and this may be useful in treating diseases such as tuberculosis.

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Dr. Inna Krieger, Thomas Snavely assisted in mycobacteria culture. Dr. Nehemiah Cox assisted in identification of macrophage markers.All other work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

FCS	Fetal calf serum
GFP	Green fluorescent protein
GMCSF	Granulocyte macrophage colony stimulating factor
HEK293	Human embryonic kidney cells 293
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon-γ
IL4	Interleukin 4
MOI	Multiplicity of infection
NCI	National Cancer Institute
NOX2	Nicotinamide adenine dinucleotide phosphate-oxidase 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
SAP	Serum amyloid P
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

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CHAPTER I

INTRODUCTION

1.1 The development of tuberculosis

Tuberculosis is an airborne disease, which is only surpassed by AIDS in global mortality rates [1]. Although tuberculosis is decreasing in parts of the world, in Africa, it is still responsible for over 2 million deaths each year. Billions of people infected with latent mycobacterium can serve as reservoirs for new active infections [2]. The most effective treatment for tuberculosis continues to be antibiotics. However, antibiotic treatment is leading to the development of drug-resistant bacteria which are becoming more prevalent. The unresolved multidrug-resistant tuberculosis has become one the biggest global challenges that people face today [3]. Tuberculosis starts with a *Mycobacterium tuberculosis* infection in the lungs, which then triggers a strong inflammatory response and leads to the recruitment of immune cells, including neutrophils [4]. In the lungs, alveolar macrophages are among the first host cells to encounter mycobacteria upon infection, and thus the predominant cell-type infected by the bacteria [5]. Once infected, mycobacteria are sequestered inside alveolar macrophages and become inaccessible to clearing by effector T cells during the early stages of infection. Even with an external transfer of T cells prior to infection, the protective effect will not start until several weeks after the initial infection [6] . Upon infection, the lung tissue undergoes extensive remodeling to generate a macrophage-

enriched granuloma, a structure where immune cells and bacteria colocalize. For unknown reasons, some granulomas control bacteria whereas others permit bacteria dissemination [7].

The adaptive immune response can show up as late as 8 weeks after infection, which indicates that the innate immune systems responsible for controlling the early stages of the infection [8]. This delay in the adaptive immune response is due to the prolonged stay of mycobacteria in mononuclear phagocytes. After infecting a macrophage for 10 days, the mycobacteria start to grow significantly and spread to other myeloid cells[9]. Alveolar macrophages appear to provide a favorable niche for mycobacteria to grow. The reasoning has been largely attributed to the inability of the lysosome to fuse with the mycobacteria phagosome [10]. Alveolar macrophages are critical in eliminating mycobacteria at early stages of tuberculosis infection and are also needed to provoke an adaptive immune response[11].

1.2 Macrophage polarization and mycobacteria infection

Macrophage polarization is a key component controlling the microenvironment of macrophages. Several cytokines that drive macrophage polarization have been identified. Cytokine signals translate into a status change for the macrophages, which in turn, modulates its cellular function and affects their antimicrobial capabilities. During the infection, the interactions between the mycobacteria and the macrophages are

constantly changing the microenvironment, and thus it is difficult to generalize the pattern as disease progresses. Studies have shown that pushing M1 macrophage polarization can improve the general outcome of *M.tuberculosis* infection [12]. Mycobacteria interfere with M1 polarization by secreting virulence factors. Early secreted antigenic target protein-6 (ESAT-6) directly inhibits the activation of NF-κB and IFN-γ regulatory factors downstream of Toll-like receptor 2 (TLR2) [13]. The inhibited responses to IFN by mycobacteria also includes FcγR1[14]. Infected macrophages also fail to express type 1 helper T cells (Th1) promoting cytokine Interleukin 12 (IL12) [15]. However, M1 responsive components INF-γ and IL12 are generally essential in protecting from TB infection [16, 17].

Some patients also showed a profound increase in M2 macrophage gene expression after tuberculosis infection [18]. The reduction of pro-inflammatory signals together with the increase of anti-inflammatory signals leads to unrestrained mycobacteria replication. Although it is widely accepted that a protective immune response to tuberculosis infection depends on the host's ability to initiate an M1 cellular response, it is not clear how well cells respond to extracellular priming signals during infection, and how to maintain stable cell phenotypes after stimulation.

Macrophages have remarkable heterogeneity. Marked changes in physiology and gene expression can occur when macrophages come into contact with invading microorganisms and damaged tissues. There is a complex interplay between

microenvironmental signals and differentiation signaling that determines macrophage identity. During infection, blood monocytes are recruited to the tissues and differentiate into macrophages. The cells acquire a distinct functional phenotype depending on the microenvironment.

The progression of tuberculosis is closely related to the macrophage profile in tuberculosis-containing granulomas. Studies have shown that a mycobacteria infection will be alleviated with activated macrophages. Mycobacteria can manipulate macrophage phenotype or preferentially infect M2 macrophage as a strategy to survive [19]. It is reasonable to assume that a group of M1 dominant macrophages will outperform a group of M2 dominant macrophages when facing mycobacteria. There is evidence that the presence of M2 macrophages is part of the reason for the unresolved mycobacterial infections and high levels of M2 macrophage-derived IL-10 are found in early ulcerative lesions of Buruli disease [20]. Thus, a potential way to promote microbicidal activity would be to induce M1 macrophage cytokine profiles where there are decreased levels of IL10 and increased levels of IFN- γ. Also, it has been shown that FcγR plays a key part in macrophages responding to mycobacteria infection. Uptake of IgG-opsonized mycobacteria mediated by FcγR will induce the generation of reactive oxygen intermediates (ROI), which promotes the formation of phagolysosomes [13]. C57BL/6 mice lacking the γ-chain shared by activating FcγR had increased susceptibility and exacerbated immunopathology upon *M. tuberculosis* challenge, which is associated with the production of the immunosuppressive cytokine IL-10 [21].

1.3 Mechanism of IFN- γ on boosting microbicidal activity

Mycobacteria block the acidification of the vacuole, which inactivates the majority of lysosome hydrolases. Blocking phagosome maturation depends on the activation status of host macrophages. Activating cells with cytokines such as IFN- γ can reverse this blockage, bringing vacuole pH down from 6.4 to 5.2[22, 23]. Another predominant mechanism responsible for restricting M. tuberculosis growth inside the cell is through inducible nitric oxide synthase [24]. Oxygen radicals play a significant role in killing M. tuberculosis [25]. The level of reactive oxygen intermediates (ROI) will increase when stimulated by IFN- γ or LPS [26]. NOS2-/- mice are as susceptible to M. tuberculosis infection as are IFN-/- mice [24, 27, 28]. Increased expression level of Nitric oxide synthases (NOS) and production of Nitric oxide (NO) has also been shown in M. tuberculosis infected lungs in human patients, indicating the relevance of NO in controlling disease progression[29, 30].

Unlike more common bacterial infections, mycobacteria express the surface lipid phthiocerol dimycoceroserate (PDIM) which masks the PAMPs. Therefore, they are not "seen" by the host innate immune system and the macrophages are not primed to be microbicidal. Another surface lipid, phenolic glycolipid (PGL), is used to induce the macrophage chemokine CCL2 to recruit and infect macrophages that are growth-permissive [31]. These suggest that mycobacteria employ various strategies to avoid infecting M1 macrophages.

CHAPTER II

SAP- FCγ RECEPTOR INTERACTION AND ITS EFFECT ON *MYCOBACTERIUM* INFECTION[*]

2.1 Introduction

Macrophages are a group of heterogeneous cells. Two well-established polarized phenotypes are referred to as classically activated macrophage M1, and alternatively activated macrophage M2[32]. The classification is largely aligned with Th1 and Th2 immune responses, however, M1 and M2 cells are not stably differentiated as Th1 and Th2 cells[33]. Earlier studies performed in our lab have found that when differentiated macrophages are treated with SAP, they will change their expression profile and phenotype, acquiring M2 macrophage features[34]. The effect of SAP on macrophage polarization was identified through the different expression levels of macrophage markers: IRF5, ICAM-1(M1) and IRF4, CD206(M2)[35]. Since SAP pushes macrophages to become M2 macrophages, it is important to find out if the skewed macrophages could fight mycobacteria efficiently when infected.

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2.2 Materials and methods

Macrophage polarization

Human blood was collected from adult volunteers who gave written consent and with specific approval from the Texas A&M University human subjects Institutional Review Board. The only criterion for donor selection was that they described themselves as being healthy. Blood collection and isolation of PBMCs was done as described previously[36]. The PBMCs were resuspended to 1 x 10^6 cells/ml in RPMI-1640 (Lonza, Allendale, NJ), and 250 µl were placed in each well of a type 89626 µ-Plate 96 well plate (Ibidi, Madison, WI). After 2 hours at 37 ˚C in a humidified 5% CO_2 incubator, gentle pipetting was used to remove non-adherent cells [37, 38]. The medium was replaced with 250 µl of serum medium (10% FCS (Seradigm, VWR, Radnor, PA), 100 U/ml Penicillin/Streptomycin (Lonza), and 2 mM glutamine (Lonza) in RPMI). At day 7, the medium was replaced with serum-free medium (10 mM HEPES (Lonza), 2 mM glutamine, 100 U/ml penicillin/streptomycin, and 1 x ITS-3 (Sigma-Aldrich, St. Louis, MO), 1mM sodium pyruvate (Lonza), and 1 x non-essential amino acids (Lonza) in RPMI). Human SAP (Calbiochem, San Diego, CA) was buffer exchanged to 20 mM sodium phosphate pH 7.4 using a 0.5 ml 10 kDa cutoff Amicon Ultra centrifugal concentrator (Sigma). Compounds (stored in 100% DMSO), were diluted in RPMI to 100 µM. Where indicated, SAP and/or compounds were added at day 7 to the cells to 5 µg/ml and 1µM final concentration, respectively. After an additional 3 days (day 10 after isolating the PBMC), cells were air-dried, fixed, and stained by immunocytochemistry

for CD54 or CD206 as previously described [39]. Approximately 100 to 200 macrophages were examined, and the number of macrophages and the number of the macrophages that showed staining was recorded. All experiments used PBMCs from at least 3 different blood donors, and 2 technical replicates for each donor. The average of the two technical replicates was then calculated, and the results are expressed as the mean ± SEM of the three or more averages, with each average from a different donor. We never used the same donor twice for a given experiment.

Bacterial cultures

M. smegmatis Mc^2-155 [40] (a gift from Jim Sacchettini, BioBio, TAMU) was grown at 37˚C on 7H10 (Becton Dickinson, Franklin Lakes, NJ) plates supplemented with 0.2% glucose and 0.083% NaCl, or in shaking culture in 3 ml 7H9 (Becton Dickinson) medium supplemented with 0.2% glucose, 0.083% NaCl, and 0.07% Tween 80 (Sigma) [41]. M-cherry-labeled *M. tuberculosis* Mc^2-7000 transformed with a mCherry hygroR plasmid [42, 43] (a gift from Jim Sacchettini) were grown as previously described [44].

Mtb infection assay, M. smegmatis infection assay

PBMCs were isolated, plated, and non-adherent cells were removed as described above. The enriched monocytes in each well were cultured in 200 µl of serum medium supplemented with 10 ng/ml GMCSF (Biolegend, San Diego, CA) for 7 days. The medium was then changed to serum-free medium with or without 5 µg/ml SAP and/or 1

µM compound. 3 days later (day 10 after isolating PBMC), cells from an overnight *M. smegmatis* culture at an OD 600 of ~1 were vortexed with ColiRoller Plating beads (Thermo Fisher) at high for 10 minutes with an analog vortex mixer (VWR, Radnor, PA). After allowing the beads to settle out, the supernatant was decanted and sonicated at power 6 for 30 seconds 6 times using a Sonic Dismembrator 60 (Fisher, Waltham, MA). Clumped bacteria were removed by centrifugation at 50 x g for 6 minutes at room temperature. The OD 600 of the supernatant containing non-clumped bacteria was measured to estimate the bacterial concentration, with an OD600 of 1 equivalent to 3.13×10^7 cfu/ml [45]. Before infection, cells in one well were washed with PBS, treated with Trypsin-versene Mixture (Lonza) at 37 ˚C for 5 minutes to detach cells, and cells were counted. *M. smegmatis* was diluted in RPMI/ 10% FCS/ 2 mM glutamine (and no antibiotics), and incubated with macrophages for 4 hours using a MOI of 10 with respect to the cell count described above. After incubation, cells were gently washed with 37 ˚C PBS 3 times, and cultured with RPMI/ 10% FCS/ 2 mM glutamine/ 20ug/ml gentamicin (Thermo Fisher Scientific) to kill bacteria outside the cells [46, 47]. At 48 hours after the infection, the cells were lysed with ice-cold 0.5% Tween 20 in water. Serial dilutions of the cell lysates were plated on 7H10 plates supplemented as described above, and colonies were counted after 3 days at 37 ˚C.

M-cherry expressing *M. tuberculosis* infection was done as described above with the exception that the *M. tuberculosis* was incubated with macrophages for 4 hours in RPMI/ 10% FCS/ 25 µg/ml pantothenic acid (Sigma). After infection, the macrophages

were washed as above and cultured in RPMI/ 10% FCS/ 20 µg/ml gentamicin/ 25 µg/ml pantothenic acid. After 4 days, cells were imaged using an IN Cell Analyzer 2000 (GE, Fairfield, CT) in 6 randomly selected fields of view from each well with a 20 x objective in the Texas Red channel and a 0.05 second exposure. Phase images were also taken. Pairs of images were analyzed using IN Cell Developer Toolbox 1.8 (GE), with the segmentation and sieve parameters adjusted to count macrophages detected in the phase images with internalized fluorescent bacteria. Phagocytosis of bacteria was measured as the amounts of bacteria inside macrophages at 2 hours after the cells were changed back into gentamicin medium.

2.3 Results

SAP polarized macrophages become Mtb-tolerant

M2 macrophages appear to be more associated with tuberculosis than M1 macrophages [48-52]. In agreement with this, we observed that compared to unpolarized human macrophages, human macrophages polarized to a M1 phenotype with interferon–γ (IFN-γ) [53] showed decreased numbers of intracellular *M. smegmatis* at 48 hours after infection, and a decreased percentage of cells infected with *M. tuberculosis* at 5 days after infection (Figure 1A). In addition to M1 and Mreg, macrophages can also be polarized to a pro-fibrotic M2a phenotype with interleukin 4 (IL-4) [54, 55], and we observed that compared to unpolarized macrophages, polarization of macrophages to a M2a phenotype with IL4 resulted in increased numbers of intracellular *M. smegmatis*

and an increased percentage of cells infected with *M. tuberculosis* (Figure 1A). The survival of mycobacteria within macrophages over infection was measured by comparing the 0 hour time point after infection to 48 hours (*M. smegmatis*) or 5 days (*M. tuberculosis*) after infection. In the absence of added factors, the number of *M. smegmatis* cells decreased, while the number of *M. tuberculosis* cells increased (Figure 11).

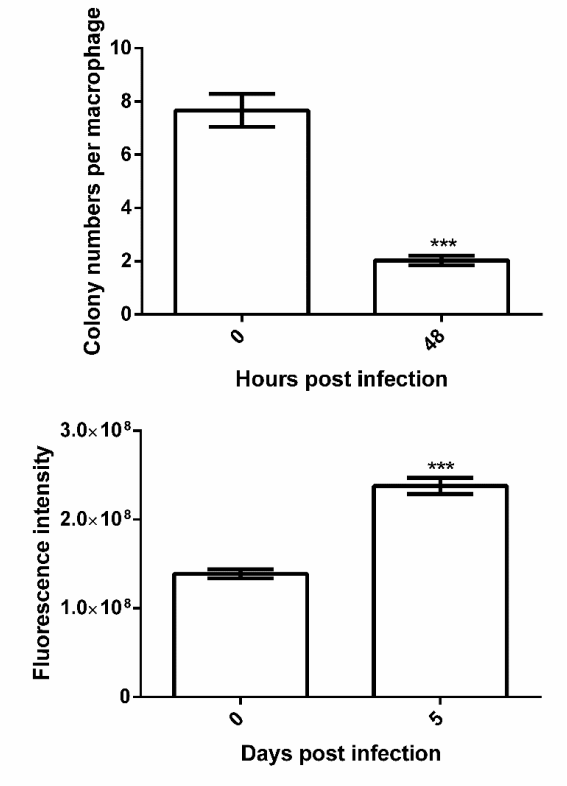

Figure 1. Changes in the numbers of intracellular *M. smegmatis* and *M.tuberculosis* during infection. PBMCs were cultured as in Figure 6. **A)** *M. smegmatis* counts are shown as colony numbers per macrophage. **B)** *M. tuberculosis* numbers were measured as total fluorescence intensity per well. Values are mean ± SEM, n=3 different donors. *** indicates $p < 0.001$ (t test).

Mycobacterium infection appears to potentiate M2 macrophages [56]. SAP also potentiates M2 macrophages and inhibits M1 macrophages [34, 57]. To test the prediction that SAP potentiates *Mycobacterium* infection of macrophages, we added *Mycobacterium* bacteria to macrophages in the presence or absence of SAP. SAP potentiated the number of intracellular *M. smegmatis* at 48 hours after infection, and potentiated the percentage of macrophages infected with *M. tuberculosis* at 5 days after infection (Figure 1B).

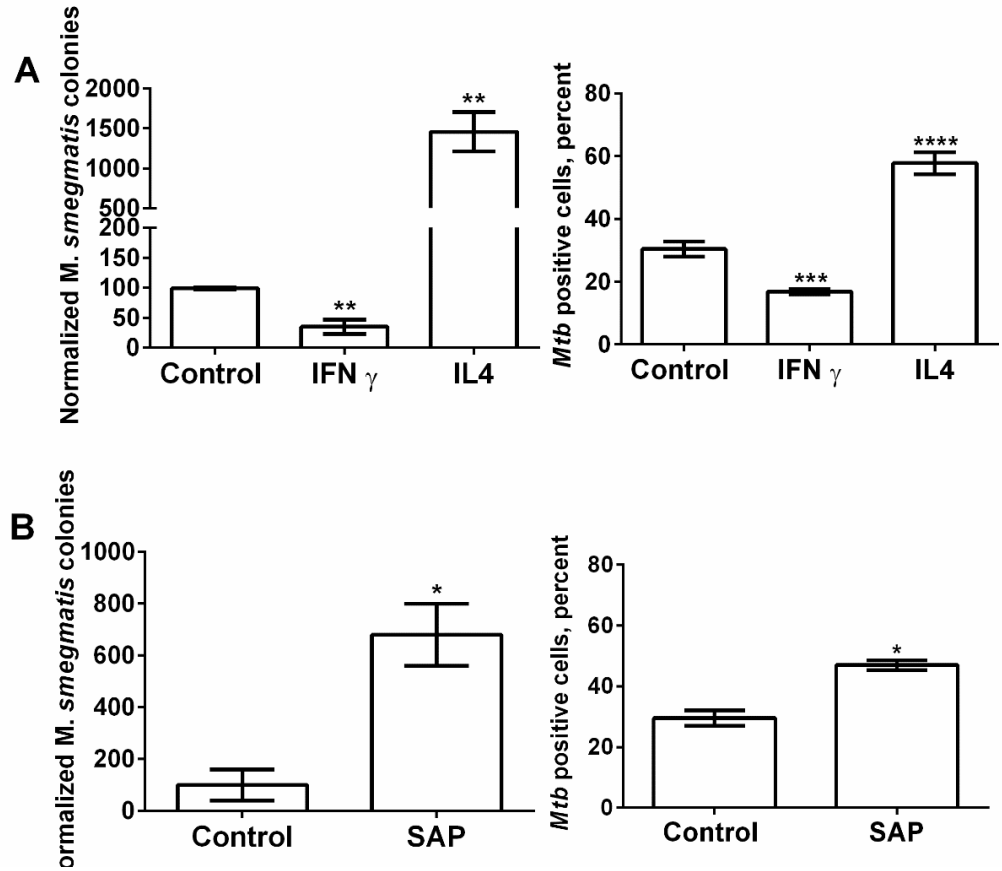

Figure 2. SAP potentiates the number of intracellular mycobacteria. A) Human PBMCs were cultured in 10% FCS/ RPMI with 10 µg/ml GMCSF for 8 days and, treated with IFN-γ or IL4 for an additional 2 days. Cells were then infected with *M. smegmatis* or recombinant *M. tuberculosis* expressing a fluorescent protein. *M. smegmatis* infected cells were lysed and bacteria were plated at 48 hours post infection, and colonies were counted. *M. tuberculosis* fluorescence was imaged at 5 days post infection. **B)** PBMCs were similarly cultured with GMCSF for 7 days, and then cultured for an additional 3 days in the presence or absence of SAP. Mycobacteria were measured as in panel A. Values are mean ± SEM, n=3 different donors. * indicates $p < 0.05$ (t test).

2.4 Discussion

SAP is a constitutive component of plasma [58], and SAP also appears to be present in the extracellular space of tissues [59]. We and others found that SAP regulates monocyte and macrophage phenotypes at least in part through its binding to FcγRI [60, 61].

Under conditions such as SAP with 27H8, the number of cells expressing CD54 increased and the number of cells expressing CD206 also increased, suggesting that some cells may have been expressing both markers. Under other conditions, the percent of $CD54^+$ cells and the percent of $CD206^+$ cells did not add up to 100, indicating that some cells may not have expressed either marker. As has been observed by others [62, 63], both results suggest that in the presence of some of the compounds, some macrophages develop a phenotype that is not perfectly M1 or perfectly M2.

CHAPTER III

SCREENING FOR COMPOUND INHIBITORS OF SAP-FC RECEPTOR INTERACTION*

3.1 Introduction

When performing preliminary experiments, we were able to show which receptor was involved in this polarizing effect of SAP. Human monocytes express FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa [64]. All corresponding orthologs in mice are: FcγRI, FcγRII, FcγRIIb, FcγRIIIa. All Fcγ receptors showed different levels of binding affinity to SAP, among which FcγRI has the highest binding affinity[60]. Both Fc receptor knockout mice and siRNA knockdowns of human receptor were used to demonstrate the necessity of certain Fcγ receptors. The results suggest that SAP binds to FcγRI on monocytes to inhibit fibrocyte differentiation[60]. We show that SAP is partially acting through FcγRI when polarizing macrophages. Thus, if the interaction between SAP and cell surface receptor FcγRI is interrupted by a chemical compound, it can slow or stop disease progression by manipulating and skewing the macrophage phenotype. As a component of human blood and a marker in tuberculosis[65], SAP is related to

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tuberculosis infection progression, and it is critical to come up with a strategy to block the effect.

3.2 Materials and methods

SAP binding to FcγRI

The binding of SAP labeled with Dylight 650 NHS Ester (Thermo Fisher Scientific, Waltham, MA) to HEK293 cells transfected with plasmids driving expression of FcγRI-GFP and Fcε common γ-chain was done following [60] with the exception that cells were transfected using a 4D-Nucleofector (Lonza, Allendale, NJ) with the preset parameters for HEK293 cells. The SAP binding was measured as median fluorescence intensity. The fluorescence intensity from SAP binding was normalized as 100 and all compounds group were compared to SAP. Libraries containing 10 mM or 1 mM of compounds were obtained from the National Cancer Institute (Diversity and Mechanistic Diversity Sets). To reduce assay size, compounds were initially pooled (Kainkaryam and Woolf 2009) into groups of 10 compounds immediately before doing the assays (Elkin, Harden et al. 2015). The NCI compound collections are sent out in 96 well plates, with 10 compounds per row. Pools 1A to 9H corresponded to rows from compound plates 4762/62-4776/29 from the Diversity set IV library, pools 17A to 23H corresponded to compound plates 4784/29-4790/18 from the Diversity set IV library, and pools 24A to 32H corresponded to compound plates 4791/18-4800/18 from the Mechanistic Diversity set II library. 1 µl of drug pool containing 100 µM of each drug in 100% DMSO (VWR,

Radnor, PA) was mixed with 50 µl of binding buffer containing labeled SAP, and this was then mixed with 50 µl of cells. For follow-up of active pools, the 12 compounds that inhibited SAP binding were ordered as specific compounds from the NCI repository, and these were used for subsequent studies, including the rescreening of these compounds for their ability to inhibit SAP binding. None of the 12 compounds have been used in clinical trials.

3.3 Results

Identification of compounds that decrease SAP binding to FcγR1

To identify compounds that can block SAP binding to FcγR1, we used Fluor647 to label SAP, and transfected HEK293 cells with GFP-tagged FcγR1. The binding of labeled SAP to the FcγR1 expressed on HEK293 cells was measured by flow cytometry. Using this method, we previously observed a KD of 4.6 nM for SAP binding to FcγR1 [60]. Due to transfection efficiency, only some cells expressed FcγR1. Since the FcγR1 was tagged with GFP, we were able to identify $Fc\gamma R1^{+}$ and $Fc\gamma R1^{-}$ cells by flow cytometry. The binding of SAP to the $Fc\gamma R1^{-}$ cells was used to measure nonspecific binding, which was subtracted from the SAP binding to $Fc\gamma R1^{+}$ cells to obtain SAP binding to FcγR1. Pools of 10 compounds, containing 1 µM final concentration of compound in the assay, were assayed for their ability to reduce the binding of 80 nM SAP (10 µg/ml) to FcγR1. From a screen of 200 pools, 9 pools decreased SAP binding and 11 pools increased SAP binding (Figure 1).

We retested the 9 compound pools that significantly reduced the binding of SAP to FcγR1, and additionally retested 15 pools that appeared to decrease SAP binding although without achieving statistical significance (Figure 2). 6 of the pools again showed an inhibition of SAP binding. The 60 individual compounds from these 6 pools were then assayed for inhibition of SAP binding at a final compound concentration of 1 μM, and 12 compounds decreased SAP binding (Figure 3). When re-tested at a variety of compound concentrations, all 12 compounds significantly decreased SAP binding (Figure 4). The structures of the compounds are shown in Table 1.

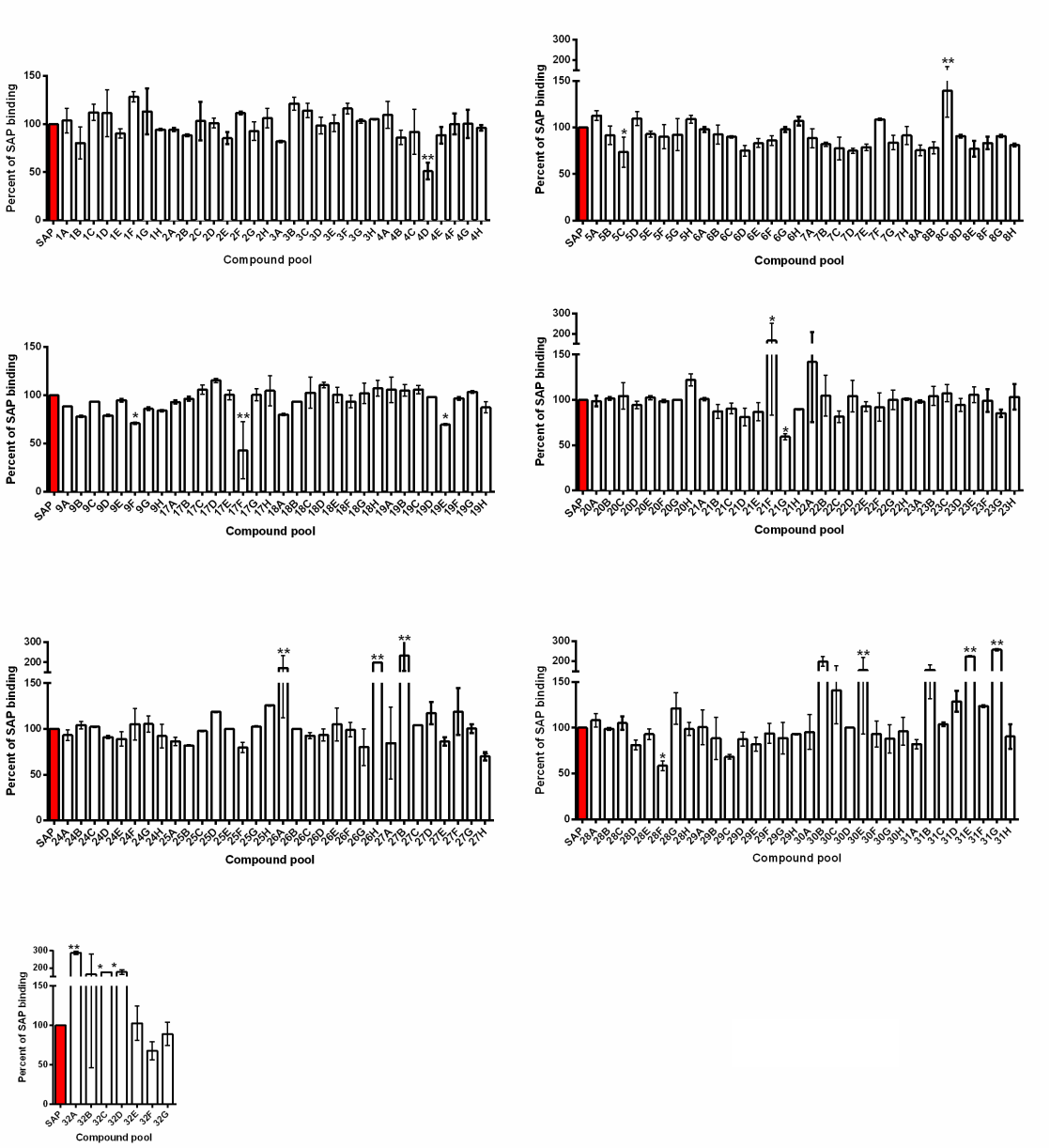


Figure 3. Identification of compound pools which decrease SAP binding to HEK293 cells expressing FcγR1. HEK293 cells expressing FcγR1 were incubated with 10 µg/ml fluorescently-labeled SAP and the indicated compound pools. Flow cytometry was then used to measure SAP binding to the cells. The binding was measured as median fluorescence intensity. The binding of the labeled SAP to FcγR1 negative cells was used to estimate non-specific binding, and this was subtracted from total binding. Values are mean ± SEM, n=3 independent experiments. * indicates $p < 0.05$, ** $p < 0.01$ (1-way ANOVA, Fisher's test compared to SAP and no compound).

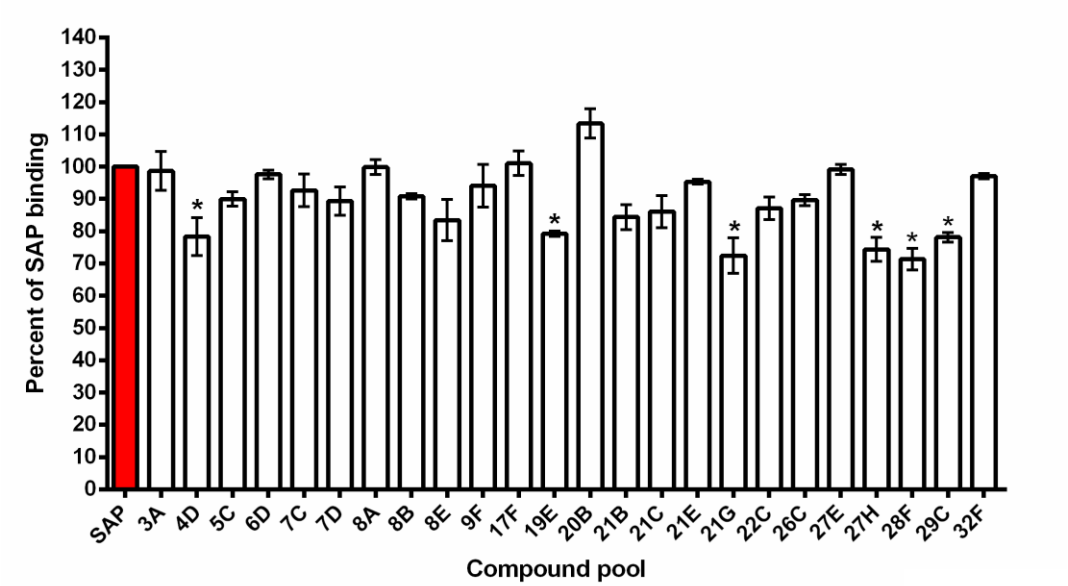


Figure 4. Second round of screening. Compound pools that significantly decreased SAP binding, along with pools that potentially decreased binding, were re-screened as in Figure 1. Values are mean ± SEM, n=3 independent experiments. * indicates $p < 0.05$ (1-way ANOVA, Dunnett's test compared to SAP and no compound).

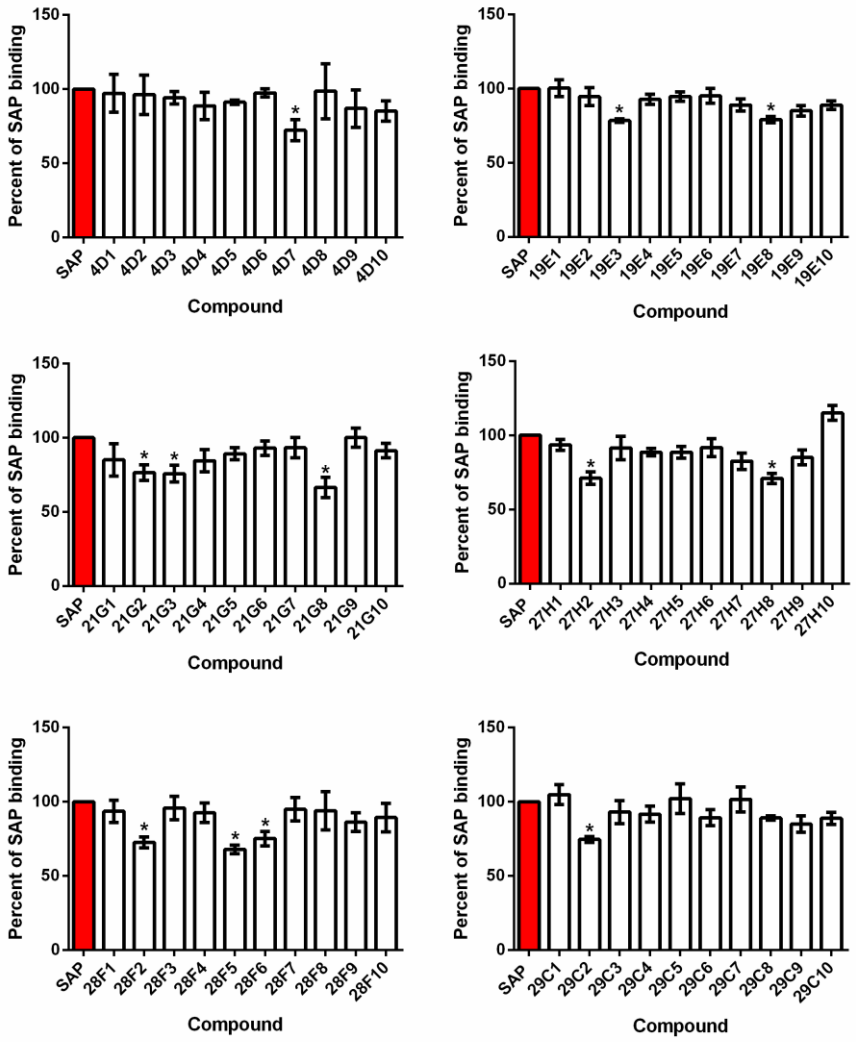


Figure 5. Screening of individual compounds for ability to decrease SAP binding. Individual compounds from the pools that significantly decreased SAP binding in Figure 2 were screened as in Figure 1. Values are mean ± SEM, n=3 independent experiments. * indicates $p < 0.05$, ** $p < 0.01$ (1-way ANOVA, Dunnett's test compared to SAP and no compound).

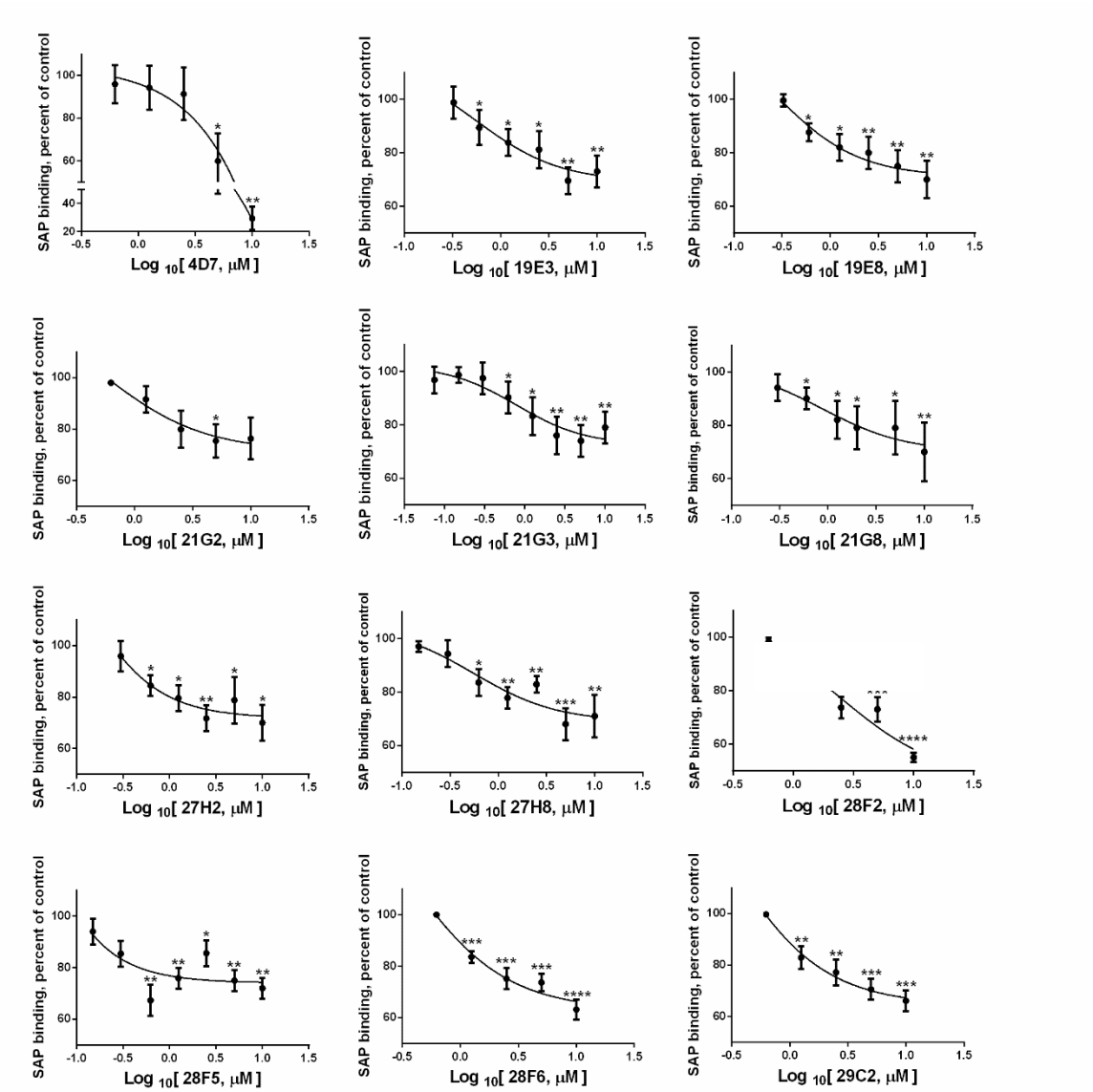


Figure 6. The reduction of SAP binding as a function of compound concentration. Compounds that significantly decreased SAP binding in Figure 3 were tested at the indicated concentrations for their ability to decrease the binding of fluorescently-labeled SAP to HEK293 cells expressing FcγR1 as in Figure 1. Values are mean ± SEM, n=3 independent experiments. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (1-way ANOVA, Dunnett's test compared to SAP and no compound). Lines are nonlinear regression fits to a one-site competitive binding curve, using a SAP concentration of 80 nM, and 4.6 nM for the K_D of SAP binding to FcγR1.

Table 1. Compounds and their effects on macrophage phenotype and mycobacteria infection. This table lists the compounds that decreased SAP binding. Arrows pointing up indicate that the compound significantly increased marker expression (Figure 5) compared to the SAP only group; arrows pointing down indicate that the compound significantly decreased marker expression or mycobacterial growth (Figure 7) compared to SAP only group. Structures were obtained from the NCI compounds library database. The structure of 28F6 is incorrect in this database, the 28F6 structure shown here is from PubChem.

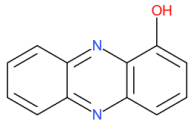
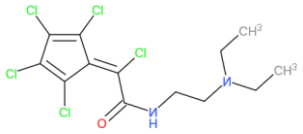
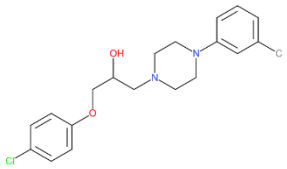
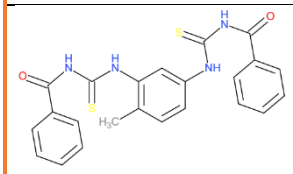
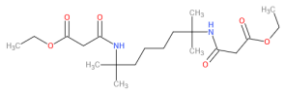
Code	NSC number	Structure	CD54	CD206	*M. smegmatis* infection	*M. tuberculosis* infection
4D7	88882					
19E3	7419		↑		↓	
19E8	91356		↑			
21G2	5856					
21G3	57608					

Table 1 Continued

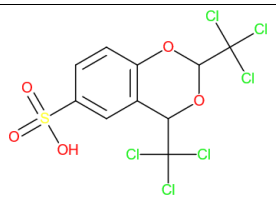
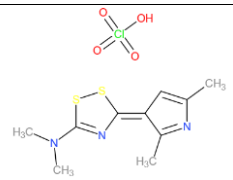
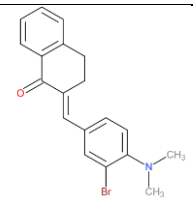
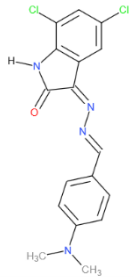
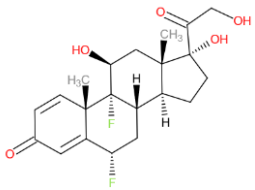
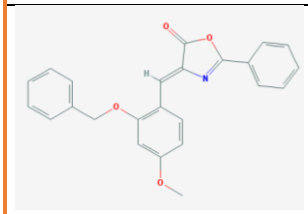
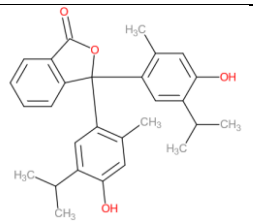
21G8	15910					
27H2	622690		↑	↓	↓	
27H8	643162		↑		↓	↓
28F2	327697		↑	↓	↓	↓
28F5	77021			↓	↓	
28F6	262665			↓	↓	↓

Table 1 Continued

29C2	2186		↓	↓

3.4 Discussion

We screened a small compound library and found 12 compounds that inhibit human SAP binding to cells expressing human FcγRI. Eight of the compounds blocked the ability of human SAP to increase the expression of an M2 marker or decrease the expression of an M1 marker. The 12 compounds identified in the initial screen were only able to inhibit SAP binding to cells expressing FcγRI by ~20%, indicating that the identified compounds are far from optimal binding inhibitors.

How the compounds inhibit SAP binding is unknown, as they could directly interfere at the SAP-FcγRI binding site, allosterically alter the extracellular conformation of either of the two proteins, or act inside cells to allosterically alter the conformation of FcγRI. If a compound binds to FcγRI in such a way that it interferes with the ability of FcγRI to bind the Fc domain of an antibody that is pathogen-bound a pathogen, that compound could possibly have the undesired side effect of decreasing the ability of cells to uptake antibody-bound pathogens. Although 27H2, 27H8, and 28F2 all have

dimethylamines on an aromatic ring, 27H8 and 28F6 have α, β unsaturated carbonyl groups, and 19E3, 27H2, 27H8, 28F2, 28F5, and 28F6 all have electrophilic centers. There does not appear to be any consistent structural feature in the 12 compounds, nor do the compounds that have a specific effect on a marker or numbers of mycobacterium in macrophages have a structural consistency(Table 1). This diversity supports the idea that the compounds could affect macrophages at a variety of binding sites.

CHAPTER IV

INVESTIGATE THE EFFECT OF COMPOUND INHIBITOR ON MACROPHAGE PHENOTYPE AND MYCOBACTERIA INFECTION*

4.1 Introduction

Since the M1 and M2 macrophage ratio is the key to resolving mycobacteria infection at early stage[12], and compound inhibitors can block SAP binding to FcγRI to some extent. It is important to investigate the ability of the lead compound to alleviate the outcome of a mycobacteria infection. To confirm whether the compounds skewed the macrophage phenotype specifically through FcγRI, the infection result will be compared in presence or absence of SAP.

4.2 Materials and methods

Macrophage viability

Macrophage viability assays were done as described for the infection assay, except that at 48 hours after *M. smegmatis* infection or 4 days after *M. tuberculosis* infection, macrophages were washed with Hanks' buffer (Sigma), and then incubated for

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15 minutes at 37°C with 165 nM Sytox green (Life Technologies, Carlsbad, CA) in Hanks' buffer. The medium was then removed and replaced with Hanks' buffer, and cells were imaged with a 20 x objective on an IN Cell 2000 in the FITC channel with an exposure time of 0.05 seconds.

M. smegmatis viability

M. smegmatis were grown in liquid culture to an OD 600 of ~1 and were then diluted in 7H9 medium supplemented as described above to an OD 600 of 0.005. In a well of 80040LE 0910 clear flat-bottom 96 well plate (Thermo Fisher), 196 µl of diluted bacteria were mixed with 4 µl of compound (diluted in DMSO) and incubated at 37 ˚C. After 24 hours, 20 µl of Deep Blue cell viability compound (resasurin; Biolegend) was added to each well, and incubated at 37 ˚C for 2 hours. Fluorescence was measured on a Synergy Mx plate reader (Biotek, Winooski, VT) following the manufacturer's directions.

Statistics

Statistical analysis with t tests or 1-way ANOVA with appropriate post test, and curve fits, were done using GraphPad Prism 4 (GraphPad, San Diego, CA). Fisher's Least Significant Difference test was used for the ANOVA post test during the initial screening stage to improve the sensitivity of the screening. Subsequently, the hit compounds were tested again to rule out potential false positives, and comparisons were done using Dunnett's post test. Significance was defined as $p < 0.05$.

4.3 Results

Some compounds that decrease SAP binding to FcγR1 decrease SAP effects on macrophages

SAP potentiates the polarization of macrophages to an anti-inflammatory Mreg (M2c) phenotype [34, 66]. To determine if the compounds that reduce SAP binding to FcγR1 can reduce the ability of SAP to potentiate M2c macrophages, we examined the effects of the compounds in the presence or absence of SAP on macrophage phenotype. Human PBMCs were cultured for 6 days to allow the monocytes to differentiate into macrophages, and the cells were then washed and cultured in the presence or absence of SAP and compounds for 3 days. The cells were then fixed and stained, and the percent of the macrophages that stained for a given marker was measured. Representative images for the control group are shown in Figure 12. ICAM-1 (CD54) is a proinflammatory M1 macrophage marker [67-69]. In the absence of SAP, 4D7, 21G2, 27H2 and 28F6 significantly decreased the percent of macrophages showing ICAM-1 staining (Figure 5A). SAP also decreased the percent of ICAM-1^+ macrophages, and compared to SAP alone, 19E3, 19E8, 27H2, 27H8 and 28F2 in the presence of SAP significantly increased the percent of ICAM-1^+ macrophages (Figure 5A). CD206 is an Mreg (M2c) macrophage marker [70]. In the absence of SAP, 4D7, 21G3, 28F5 and 28F6 significantly increased the percent of macrophages that were $CD206^+$ (Figure 5B). SAP also increased the percent of $CD206^+$ macrophages, and compared to SAP alone,

27H2, 28F2, 28F5, 28F6 and 29C2 in the presence of SAP significantly decreased $CD206^+$ macrophages (Figure 5B).

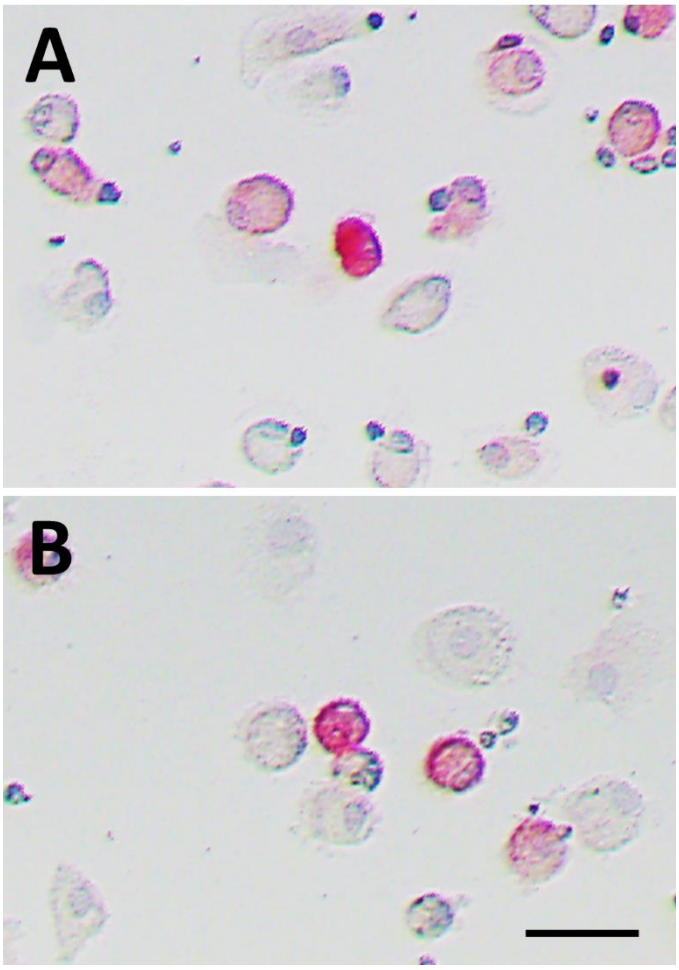

Figure 7. Immunocytochemistry staining of the macrophage markers CD54 and CD206. PBMC were cultured as in Figure 5. **A)** Cells were fixed and stained for the M1 marker ICAM-1 (CD54) or **B)** the M2 marker CD206. Cells were then counterstained with hematoxylin. Positive staining was identified by red staining, with nuclei counterstained blue. Photos show representative results from 3 different donors. Bar is 50 µm.

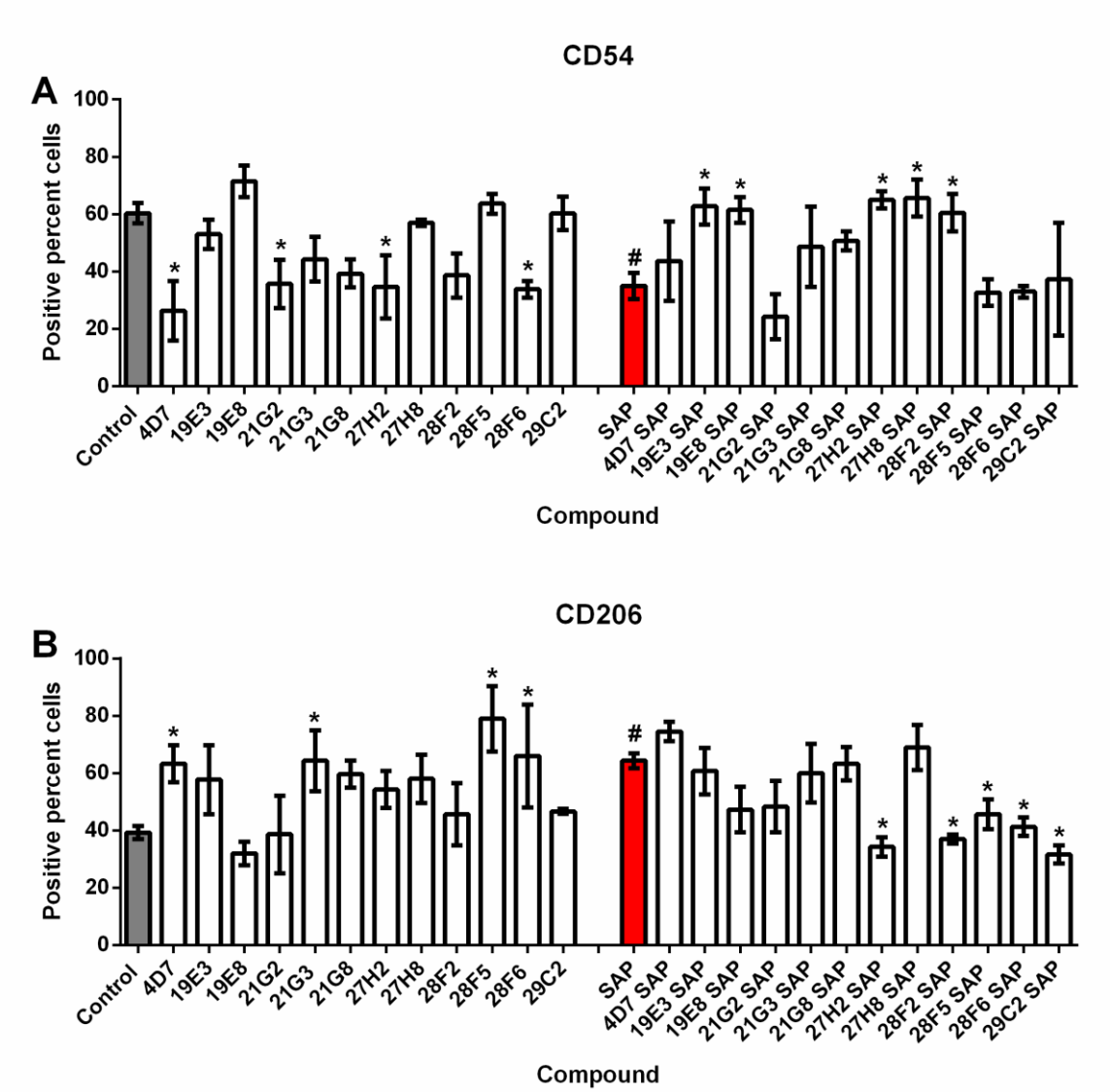

Figure 8. Some compounds which decrease SAP binding affect macrophage marker expression. Human PBMCs were cultured for 6 days to allow macrophage differentiation, and were then cultured for an additional 3 days in the presence or absence of SAP and the indicated compounds. **A)** Cells were fixed and stained for the M1 marker ICAM-1 (CD54) or **B)** the M2 marker CD206. The number of morphologically identifiable macrophages that stained for the indicated marker were then counted and expressed as a percentage of macrophages. Values are mean ± SEM, n=3 different donors. # indicates $p < 0.05$ (t test compared to control). * indicates $p<0.05$ (1-way ANOVA, Dunnett's test comparing compounds in the absence of SAP to the control, and compounds incubated with SAP to SAP alone).

Some SAP- FcγR1 inhibitors decrease mycobacteria numbers in infected macrophages

Since some of the compounds that decrease SAP binding to FcγR1 also inhibit the effect of SAP on macrophages, we determined whether any of these compounds might affect the number of *Mycobacterium* in macrophages. Human PBMCs were cultured for 6 days to allow the monocytes to differentiate into macrophages, and the cells were then washed and cultured in the presence or absence of SAP and compounds for 3 days. The medium was then removed and medium containing *Mycobacterium* was then added. After 4 hours, the macrophages were washed to remove non-internalized bacteria and cultured for 2 or 4 days. The level of internal *Mycobacterium* was then measured. In the absence of SAP, 27H2 increased the number of intracellular *M. smegmatis* at 48 hours after infection (Figure 7A). SAP also increased the number of intracellular *M. smegmatis*, and compared to SAP alone, in the presence of SAP, 19E3, 27H2, 27H8, 28F2, 28F5 and 28F6 decreased the number of intracellular *M. smegmatis* (Figure 7A). In the absence of SAP, 21G8 and 28F5 increased the percentage of macrophages infected with *M. tuberculosis* at 4 days after infection (Figure 7B), and compared to SAP alone, in the presence of SAP 27H8, 28F2, 28F6 and 29C2 decreased whereas 21G8 increased the percentage of macrophages infected with *M. tuberculosis* (Figure 7B).

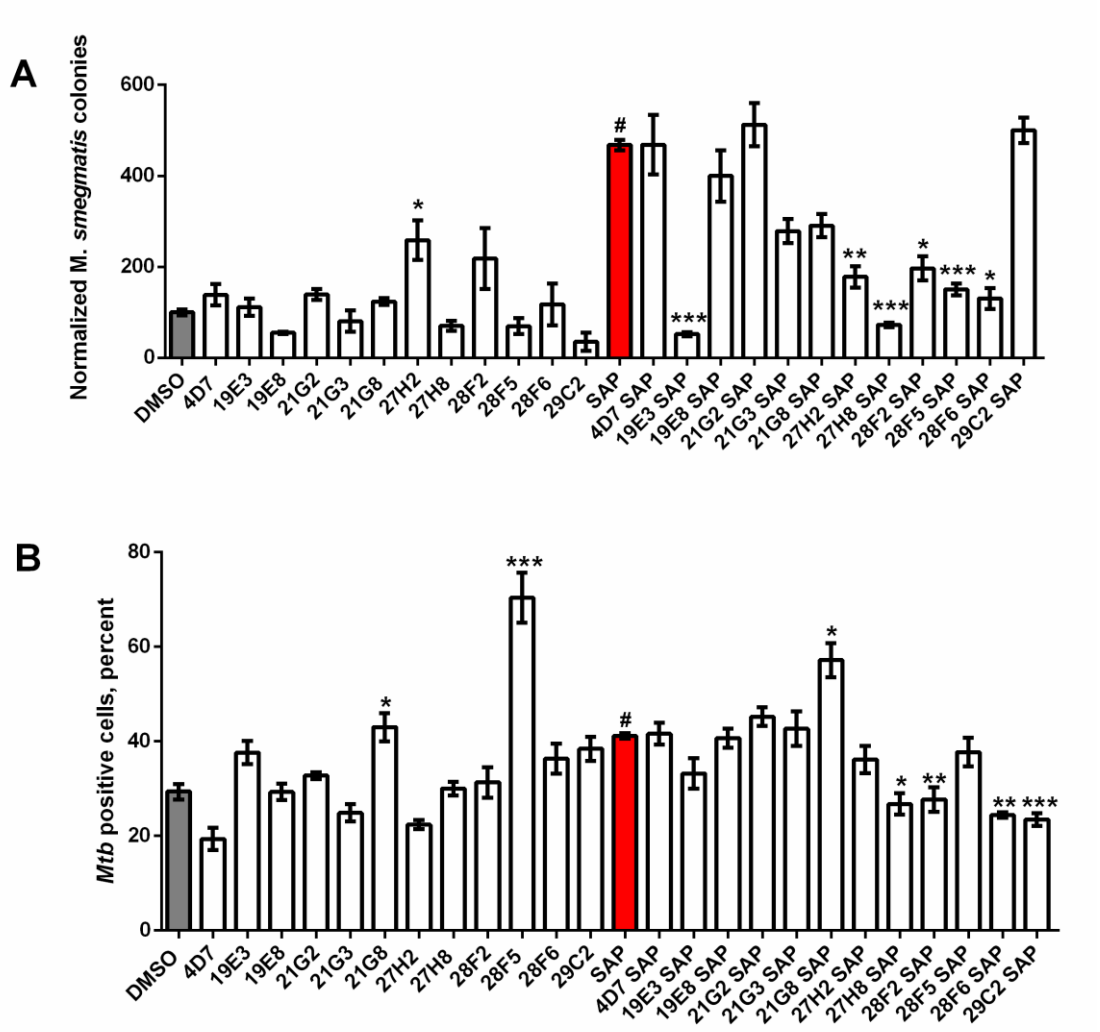

Figure 9. Some compounds inhibit the number of intracellular mycobacteria in the presence of SAP. PBMCs were cultured as in Figure 6B, with the exception that the indicated compounds were added in the presence or absence of SAP for the 3 days before bacterial infection. **A)** *M. smegmatis* were measured as in Figure 6. **B)** The percent of macrophages infected with *M. tuberculosis* was measured as in Figure 6. Values are mean ± SEM, n=3 different donors. # indicates $p < 0.05$ (t test compared to control). * indicates $p < 0.05$ (1-way ANOVA, Dunnett's test).

Confirmation of lead compounds' activity on macrophage infection

We found that in the presence of SAP, 7 compounds inhibited either *M. smegmatis* or *M. tuberculosis* numbers inside macrophages. To determine if these

compounds are directly inhibiting *Mycobacterium* growth and/or proliferation, we added these compounds to *M. smegmatis* for 24 hours. We then quantified the amounts of live bacteria with the fluorescent dye resazurin. Compound 27H2 killed bacteria at concentrations of 4 µM and above, similar to the killing ability of the antibiotic rifampicin (Figure 8). However, in the mycobacteria infection assay, compound 27H2 did not kill bacteria directly because the concentration used was 1 µM, and the compounds were washed off before adding in bacteria. The other 6 compounds did not significantly affect *M. smegmatis* viability (Figure 8). These results indicate that 6 of the compounds we identified improve the response of SAP-exposed macrophages to mycobacteria infection.

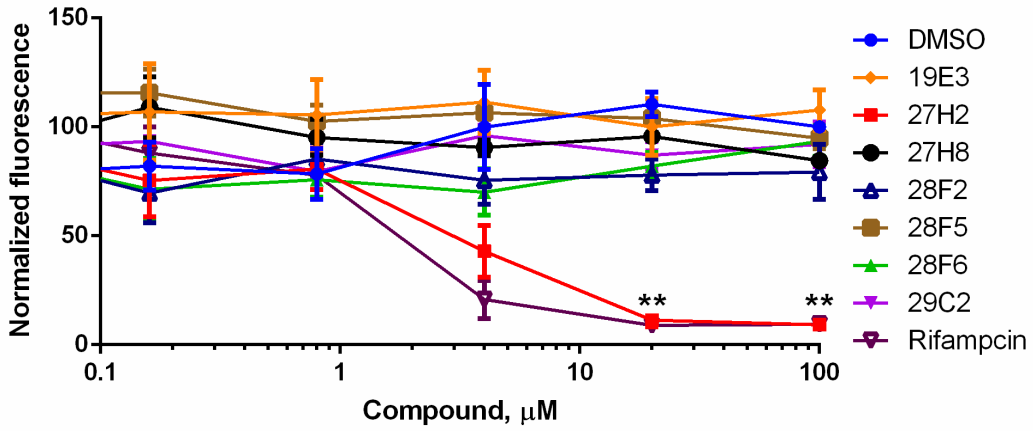

Figure 10. The direct effect of compounds on *M. smegmatis* proliferation and growth. *M. smegmatis* was grown for 24 hours in the presence of the indicated concentrations of compounds, and then incubated with resazurin for 2 hours. Growth was measured as the fluorescence intensity of reduced resazurin. Values are mean ± SEM, n=3 independent experiments. * indicates $p < 0.05$ (t test compared to DMSO control).

Another possible reason that the compounds might appear to decrease the numbers of bacteria inside macrophages is that the compounds decrease macrophage viability. To test this possibility, we did compound treatments and *Mycobacterium* infections as described for Figure 7, and measured macrophage viability using Sytox green to stain for dead macrophages. In the *M. smegmatis* model, we observed that approximately 10% of the macrophages were dead at 48 hours after adding bacteria to macrophages, and that SAP did not significantly affect this percentage (Figure 9A-F). Of the 6 compounds that, in the presence of SAP, decreased the number of intracellular *M. smegmatis* in macrophages, only 5 μM 28F6 increased the percentage of dead macrophages (Figure 9F). At 4 days after *M. tuberculosis* infection, SAP did not significantly increase the number of dead macrophages (Figure 9G). Of the 4 compounds that, in the presence of SAP, decreased the percentage of macrophages infected with *M. tuberculosis* at 4 days after infection, 29C2 increased the percentage of dead macrophages in the absence of SAP, but in the presence of SAP, none of the 4 compounds had a significant effect on macrophage viability (Figure 9G). Together, these results suggest that 5 μM 28F6 decreases macrophage viability in the presence of SAP, while the other compounds appear to potentiate the response of macrophages to *Mycobacterium* infection in the presence of SAP rather than inhibiting macrophage viability.

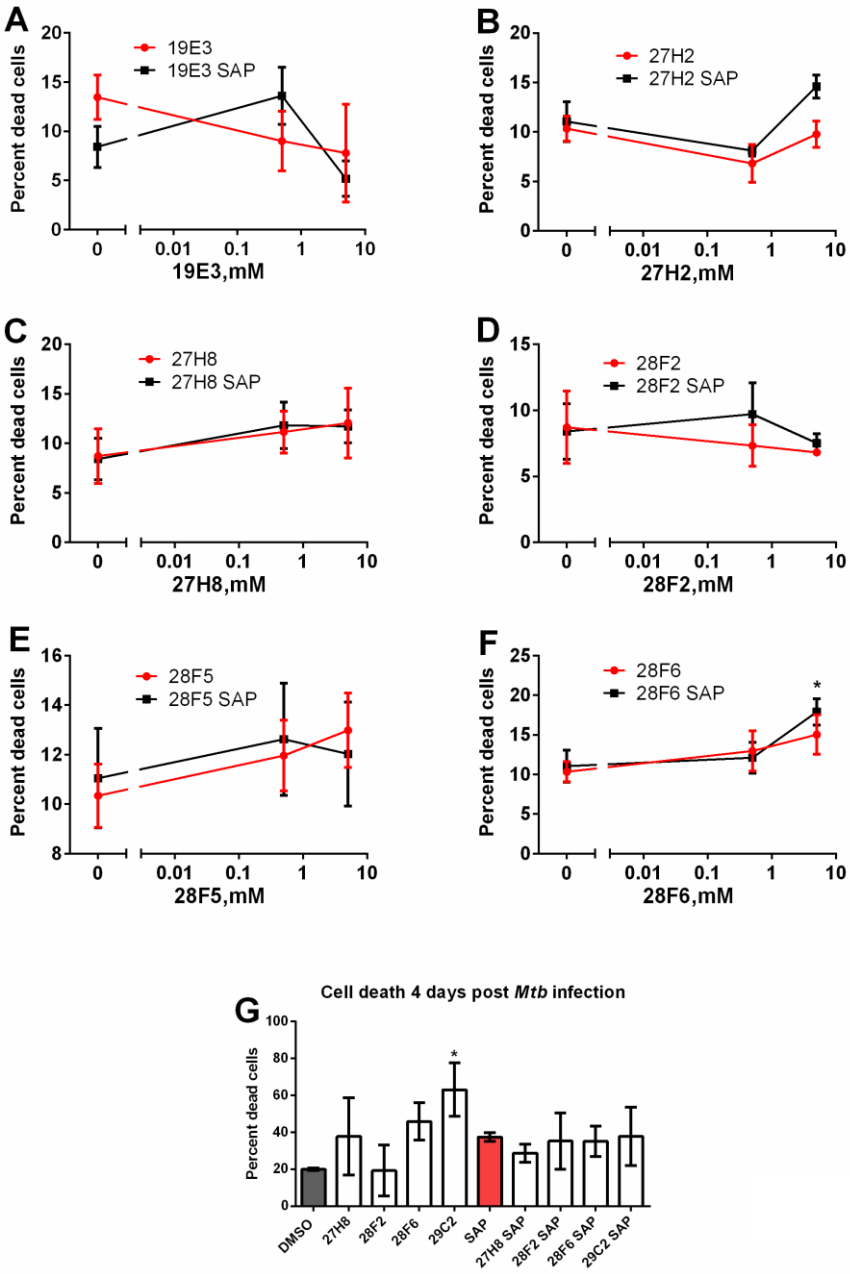

Figure 11. The effect of compounds on macrophage viability. Macrophages were cultured and infected as in Figure 6. **A-F)** Macrophage viability was measured by staining with Sytox Green at 48 hours after *M. smegmatis* infection, and the percent of dead cells in images was calculated. * in F indicates $p < 0.05$ for 5 µg/ml SAP (t test compared to control). **G)** Macrophage viability was measured at 5 days after *M. tuberculosis* infection as in panels A-F. Values in A-G are mean ± SEM, n=3 different donors. * indicates $p < 0.05$ compared to DMSO control (1-way ANOVA, Dunnett's test).

SAP and the compounds could potentially alter the ability of macrophages to bind to and/or internalize bacteria. To determine if SAP and/or the identified compounds affect these processes, we infected macrophages with bacteria as described for Figure 7, and then lysed macrophages immediately after washing off bacteria that had not been internalized. Neither SAP nor the 6 compounds that decreased *M. smegmatis* affected the phagocytosis of *M. smegmatis* (Figure 10A), and neither SAP nor the compounds that decreased *M. tuberculosis* affected the phagocytosis of *M. tuberculosis* (Figure 10B).

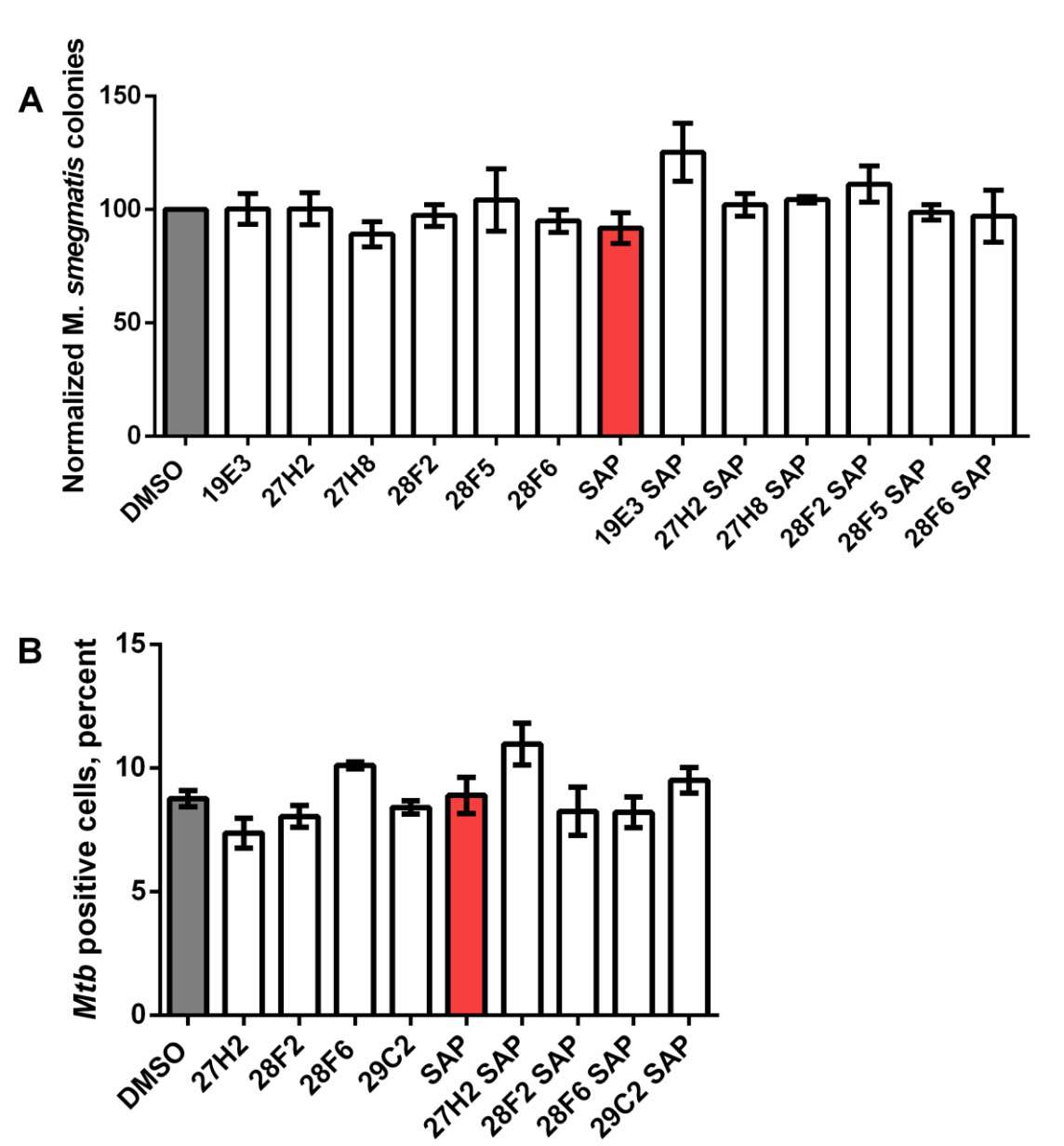

Figure 12. The effect of compounds on phagocytosis of bacteria by macrophages. Macrophages were cultured and infected as in Figure 6. After infection, macrophages were treated with gentamycin for 2 hours to remove extracellular bacteria. **A)** *M. smegmatis* infected cells were then lysed immediately after the 2-hour gentamycin treatment, bacteria were plated, and colonies were counted. **B)** *M. tuberculosis* fluorescence was imaged immediately after the 2-hour gentamycin treatment. Values are mean ±SEM, n = 3 different donors. No values were significantly different from the DMSO controls (1-way ANOVA, Dunnett's test).

4.4 Discussion

As predicted, pre-exposure of human macrophages to human SAP potentiated the number of mycobacteria in the macrophages. Some but not all of the compounds reduced the effect of SAP. Compounds 27H8, 28F2 and 28F6 effectively ameliorate both *M. tuberculosis* and *M. smegmatis* infection, although 28F6 may have a moderate cytotoxic effect at high concentration. Together, these results indicate that blocking the human SAP – human FcγRI interaction could be a potential method to decrease the ability of mycobacteria to proliferate in human macrophages. This contrasts with the effect of mouse SAP on mouse alveolar macrophages, where mouse SAP decreases the uptake and number of *M. tuberculosis* bacteria in mouse alveolar macrophages [71]. This difference may be due either to differences between mice and humans, or as a result of a different response between circulating monocyte-derived macrophages and alveolar macrophages.

Some compounds that inhibited SAP binding had no significant effect on the ability of SAP to decrease expression of the M1 marker CD54 and increase expression of the M2 marker CD206, suggesting that they may have partially activated FcγRI while inhibiting SAP binding. Several of the compounds mimicked the effect of SAP on CD54 and/or CD206 expression (Figure 5), indicating that they may interact directly with FcγRI. Compound 27H2 decreased expression of the M1 marker CD54 in the absence of SAP, but increased CD 54 expression in the presence of SAP, while compounds 28F5

and 28F6 increased expression of the M2 marker CD 206 in the absence of SAP, but decreased CD206 expression in the presence of SAP. This indicates that these compounds can act as SAP agonists or SAP antagonists depending on whether SAP is present.

In the presence or absence of SAP, compounds 27H8 and 28F2 did not affect the initial uptake of mycobacteria by macrophages, the viability of mycobacteria in the absence of macrophages, or the viability of macrophages. However, in the presence of SAP, these two compounds reduced the number of *M. smegmatis* bacteria in macrophages by two days after infection and reduced the percentage of macrophages containing *M. tuberculosis* by five days after infection. Together, this indicates that in the presence of SAP, 27H8 and 28F2 may have induced some macrophages to kill their ingested bacteria. By comparing figures 10B and 7B, indications are that over the four days of *M. tuberculosis* infection, there is an increase in the percentage of infected macrophages. An alternative possibility is that in the presence of SAP, 27H8 and 28F2 may have inhibited the spread of *M. tuberculosis* from one macrophage to another. In addition to helping macrophages kill the internalized mycobacteria, altering the macrophage phenotype may be useful for the treatment of diseases such as cancer and obesity [72, 73]. Together with our results, this suggests that blocking SAP effects on macrophages may be useful for a variety of diseases. However, SAP also appears to prevent the development of fibrosis [74], so use of this approach would need to proceed with some caution.

CHAPTER V

SUMMARY

Macrophages are a group of heterogeneous cells. When macrophage cell-typess are imbalanced, it can indicate different diseases. During the progression of tuberculosis infection, M2 macrophages are predominant. Protein serum amyloid P is also involved in the development of the disease and used as a diagnostic marker. We reasoned that SAP promoted mycobacteria growth by converting macrophages from one type to the other. Macrophage plasticity allow them to respond to environmental signal changes accordingly. Therefore, the project focused on employing the versatility of macrophages to affect the outcome of mycobacteria infection. Earlier research has shown that macrophages experience a phenotype conversion in presence of Serum amyloid P. The work started by searching for compound inhibitors of SAP-Fcγ receptor. These potential lead compounds were tested on their ability to reverse macrophage phenotypes and create a functional conversion.

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