UNIVERSITY OF CALIFORNIA, SAN DIEGO

Trehalose, an mTOR-independent Inducer of Autophagy, Inhibits HIV Infection in Primary Human Macrophages

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by

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The thesis of Simson Hon is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Trehalose, an mTOR-independent Inducer of Autophagy, Inhibits HIV Infection in Primary Human Macrophages

by

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While tremendous progress has been made in terms of prevention, detection, and treatment of human immunodeficiency virus type-1 (HIV), the agent that causes acquired immunodeficiency syndrome (AIDS), concerns remain as strains of HIV that are resistant to antiretroviral therapy (ART) have begun to emerge. In the face of this new threat, other methods must be considered in combination with medical treatment. Autophagy, a highly conserved pathway that enable cells to recycle cytoplasmic content to promote survival during periods of stress, has been receiving renewed attention for its role in neurodegenerative diseases and immune response to pathogen challenge. In

fact, chemically induced autophagy has been shown to inhibit HIV replication in human primary macrophages. However, autophagy has been classically studied as a pathway regulated by the mammalian target of rapamycin complex 1 (mTORC1), which recently, has been shown to have other modulatory effects beyond autophagy.

As such, we determined if trehalose, small-molecule enhancer of rapamycin 28 (SMER28), and spermidine, which are compounds that have been shown to induce autophagy in an mTOR-independent fashion, are able to inhibit HIV replication in human primary macrophages. Here, I show that all three mTOR-independent inducers of autophagy have an inhibitory effect on HIV replication. Furthermore, I demonstrate that trehalose can induce autophagic flux in human primary macrophages. Yet, perhaps the most striking result was that trehalose downregulates CD4 and chemokine CC receptor 5 (CCR5) expression, both of which are key receptors for HIV entry. In support of this, I observed decreased HIV entry into human primary macrophages following trehalose treatment. Taken together, these results support further investigation into the beneficial effects that trehalose may have as part of standard HIV treatment.

I. INTRODUCTION

In the early 1980s, during the peak of the AIDS epidemic, an AIDS diagnosis was considered a death sentence as most individuals diagnosed had a one year life expectancy. However, the advent of ART has greatly improved disease outcome by decreasing viral load and slowing disease progression. The different stages of the HIV lifecycle, including attachment, fusion, entry, reverse transcription, integration, maturation, and release are targets of ART [1]. There are several FDA approved drugs that target the reverse transcription, integration, and maturation steps, but there is only one FDA approved drug that targets either fusion or entry, enfuvirtide and maraviroc, respectively [2].

HIV attachment and fusion requires two receptors: the primary receptor, CD4, and a co-receptor, chemokine CXC receptor 4, CXCR4, or CCR5. Two important components of the HIV envelope are extracellular glycoprotein (gp) 120 and transmembrane gp41 that facilitate attachment and fusion, respectively, with target cell. HIV gp120 binds to CD4, which leads to a conformational change in gp120, which then binds to a co-receptor, such as CCR5, in the case of macrophages. Next, gp41 inserts itself into the target cell and folds over on itself to bring the viral membrane and host membrane in close contact to enable fusion and release of viral nucleocapsid into the cytoplasm [3]. Maraviroc prevents the entry of R5-tropic strains of HIV (strains of HIV that use CCR5 as the co-receptor) by blocking gp120 from binding to CCR5. It was approved for use as a component of ART by the FDA in 2007 [4]. While tremendous progress has been made, drug resistance remains an important concern as HIV has a high mutation rate and error prone replication during reverse transcription [5, 6]. Unfortunately, HIV is a lifelong infection that requires strict, lifelong adherence to therapy

in order to maintain control of viral load. As such, other methods of treating HIV infection should be considered.

One process that has been receiving renewed attention and interest is macroautophagy, referred to hereafter as autophagy. This process is responsible for recycling amino acids from degraded long-lived proteins or damaged organelles, which promotes cell survival during times of stress or starvation [7]. Autophagy has also been shown to have a role in clearing misfolded or aggregated proteins in neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease [8, 9]. Furthermore, autophagy plays an important role in aging, cancer prevention, modulating inflammation, immune activation, and immune response to infectious diseases [10-12]. Given the importance of autophagy, much effort has been dedicated to studying the underlying mechanism and regulation of autophagy. In fact, the groundbreaking research in the characterization of autophagy-related (*Atg*) genes in yeast was awarded the Nobel Prize in Physiology or Medicine in 2016. Information from yeast provided insight into the steps of autophagy and the identification of mammalian homologs of *Atg* genes.

The autophagy pathway can be broken down into six stages: initiation, nucleation, elongation, maturation, fusion, and degradation [13]. Initiation of autophagy is mediated by a complex consisting of Unc-51-like kinase 1 or 2 (ULK1/2), Atg13, and focal adhesion kinase family interacting protein of 200 kDa (FIP200) [13]. Upstream of this ULK complex is mTORC1, which is a known and well-studied modulator of autophagy. Under nutrient-rich conditions, mTORC1 is associated with the ULK complex and ULK1/2 and Atg13 is inactivated through phosphorylation, thus inhibiting autophagy initiation. However, under starvation conditions or rapamycin, also known as sirolimus, treatment, mTORC1 is dissociated and the ULK complex is hypophosphorylated, leading

to initiation of autophagy [14]. Following initiation, the nucleation complex, which includes p150, Atg14, and Beclin1, is recruited and serves as the site of formation for the *de novo* phagophore, a double-membrane structure [15]. Subsequently, the Atg12-Atg5-Atg16L complex is recruited to the phagophore and facilitates several reactions that help elongate the phagophore. Concurrently, microtubule-associated protein 1 light chain 3 beta (LC3B-I, cytosolic) is conjugated to phosphatidylethanolamine (PE) through a process involving Atg4, Atg7, and Atg3, to form LC3B-II, which tethers to the growing phagophore and is used as a marker for the number of autophagosomes [15]. As the phagophore grows, it matures until it eventually sequesters a portion of the cytosol and cargo within a double-membrane vesicle, now termed the autophagosome [15]. The outer membrane of the autophagosome then fuses with membrane of a lysosome to form the autolysosome, where the internal compartment is degraded and contents may be released back into the cytosol for reuse [15, 16].

Interestingly, the relationship between HIV and autophagy is far more complicated than what one would expect. It has been revealed that HIV infection induces autophagy and that certain autophagic proteins, such as Atg7 and Beclin1, are necessary for optimal viral production [17, 18]. Another group showed that HIV uses autophagy for its own purposes. They found that the early autophagic proteins promoted Gag processing, while HIV Nef inhibited the maturation and acidification of autophagosomes, thus protecting HIV from degradation [19]. The same group showed that the use of sirolimus, to induce autophagy, through inhibition of mTOR, actually increased viral production [19]. In stark contrast, other groups have demonstrated that sirolimus was able to inhibit HIV replication [20, 21]. Other mTOR-dependent inducers of autophagy have also been studied beyond sirolimus. Torin1, another inhibitor of mTOR, inhibits HIV extracellular release [22]. Likewise, our group has found that histone

deacetylase inhibitors decrease HIV release and promote HIV degradation through autophagy, induced by inhibition of mTOR [23]. Lastly, our group has also shown HIV replication can be inhibited by vitamin D induced autophagy [20, 24].

Autophagy has been regularly studied as an mTOR-dependent process, but greater efforts have been dedicated to understanding mTOR-independent pathways of inducing autophagy [25]. One reason to look beyond mTOR-dependent autophagy is due to the increasing complexity associated with mTOR modulation [26]. Unsurprisingly, as autophagy intersects with immunity, pathogens have evolved ways to modulate mTOR function [27-29]. As such, two recently studied examples of mTOR-independent autophagy include the inositol pathway and calcium/calpain pathway [30-32]. In terms of chemicals inducers, several compounds including SMER28, spermidine, and trehalose have been shown to induce autophagy in an mTOR-independent fashion. SMER28 decreased the levels of mutant proteins associated with several neurodegenerative diseases [33, 34]. Likewise, spermidine also protects against neurodegenerative diseases and has been given significant attention for its anti-aging properties [35-37]. Though the mechanism of SMER28 is still to be determined, spermidine is suspected to induce epigenetic changes that promote autophagy [33, 38].

Of the three inducers of mTOR-independent autophagy, trehalose has been the most studied. Trehalose is a natural sugar composed of two glucose joined by an alpha, alpha-1, 1 linkage that can act as an energy source or cryoprotectant, to name a few functions [39]. In insects, it is the most common sugar found in the blood circulation [40]. On the other hand, humans are unable to synthesis trehalose, but possess trehalase, which can hydrolyze trehalose during digestion in the small intestine [41]. Like SMER28 and spermidine, trehalose promotes the clearance of mutant proteins associated with Alzheimer’s, Huntington’s, and prion diseases [42-45]. In addition, trehalose is able to

inhibit viral infection by human cytomegalovirus (HCMV) [46]. The exact mechanism of trehalose induced autophagy requires further study. One group has proposed that trehalose inhibits several cellular glucose transporters (GLUT), which leads to a decrease in cellular ATP leading to a nutrient-poor state [47]. In response, adenosine monophosphate-activated protein kinase (AMPK) becomes activated and directly phosphorylates ULK1, leading to the induction of autophagy [47, 48]. Currently, trehalose is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA), which makes it an attractive therapeutic agent to explore further [39]. Given that no studies have been done in terms of the effects of mTOR-independent inducers of autophagy on HIV replication, I have examined all three aforementioned inducers and found that trehalose has the most profound effects during HIV infection in macrophages.

II. MATERIALS AND METHODS

Ethics Statement

Protocol for obtaining venous blood from HIV seronegative subjects is outlined and approved by the Human Research Protections Program of the University of California, San Diego (Project 08-1613). Procedures and related material are in accordance with requirements provided by the Code of Federal Regulations on the Protection of Human Subjects (45 CFR 46 and 21 CFR 50 and 56). All donors have provided written and informed consent prior to their participation. Each donor was reminded that their participation is voluntary and that they could withdraw at any time.

Cell Isolation and Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat by Ficoll-Pacque PLUS (17-1440-03; General Electric) density gradient centrifugation. Next, monocytes were purified from PBMCs during positive selection using CD14 microbeads (130-050-021; Miltenyi Biotec) and were seeded at densities suggested by manufacturers. We then generated human monocyte derived macrophages (MDMs) by culturing monocytes in RPMI 1640 Medium with L-glutamine (11875-093; Gibco) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (15140; Gibco), 10% (v/v) heat-inactivated fetal bovine serum (FBS, F0926; Sigma), and 10 ng/mL of macrophage colony stimulating factor (MCSF, 216-MC; R&D Systems). This media is hereafter referred to as MDM media. After overnight incubation, the non-adherent cells were removed and the remaining, adherent cells were cultured in MDM media for three to ten days at 5% CO_2 and at 37^{o}C, with complete

media changes every three days. Treatment conditions were prepared using MDM media in all experiments.

Reagents and cytotoxicity

Both sirolimus (R8781; Sigma) and bafilomycin A_1 (BML-CM110-0100; Enzo Life Sciences) were reconstituted in DMSO and diluted in MDM media and used at a final concentration of 100 nM and 50 nM, respectively. Maraviroc (M193000, Toronto Research Chemicals) was reconstituted in methanol and was diluted using MDM media and used at a final concentration of 30 nM. Trehalose (T9531, Sigma) was dissolved in MDM media at a concentration of 200 mM, filtered, and diluted using MDM media to various final concentrations. SMER28 (BML-EI397-0005; Enzo Life Sciences) was obtained and reconstituted in DMSO and diluted with MDM media and used at various final concentrations. Spermidine (05292-1ML-F; Sigma) was obtained as a solution and diluted in MDM media and used at various final concentrations. When assessing autophagic flux, macrophages were treated with bafilomycin A1 for two hours before cells were lysed. Prior to any cell lysate preparation, cells are washed with Dulbecco's phosphate buffered saline (DPBS, 14190250; Gibco),

Cytotoxicity was determined by measuring the extracellular presence of lactate dehydrogenase (LDH) in cell culture supernatant using a LDH assay (88953, Thermo Scientific). Measurements were obtained spectrophotometrically using instructions provided by the manufacturer.

HIV infection

The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH: $HIV_{Ba\text{-}L}$ from Dr. Suzanne Gartner, Dr. Mikulas

Popovic, and Dr. Robert Gallo [49, 50]. $HIV_{Ba\text{-}L}$ stock preparation was previously described [51].

Human monocyte derived macrophages were pretreated with vehicle, 100 nM sirolimus, or varying concentrations of trehalose, SMER28, or spermidine for 24 hours prior to HIV infection. In experiments assessing HIV entry, cells were also pretreated with 30 nM maraviroc for 24 hours prior to HIV infection. After 24 hours, cells were then infected at a multiplicity of infection (MOI) of 0.04 for eight hours. After eight hours, macrophages were then either used to measure intracellular HIV p24 or to assess the inhibition of HIV replication by drug treatment. Intracellular HIV p24 was collected and measured as previously described [20]. Macrophages used to assess the effects of treatment on HIV replication during infection were washed 2x with DPBS and cultured in MDM media with drug treatment for ten days. Approximately 50% of cell culture media was collected on days three, five, seven, and ten post-infection and replenished with fresh MDM media supplemented with drug treatment. Released HIV p24 in collected days five and ten cell culture supernatant was measured using a HIV p24 antigen ELISA (NEK050A001KT; Perkin Elmer) as a way to quantify HIV replication.

Western and Immunoblotting

After 24 hours of drug treatment, macrophages were lysed using a solution prepared as previously described [23]. Gel electrophoresis was performed using precast 12% polyacrylamide gels buffered with 2-[bis(2-hyxroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol buffered 12% polyacrylamide gels (Thermo Scientific) and then transferred to 0.45 μm polyvinylidene difluoride membranes (PVDF, 88518; Thermo Scientific).

The proteins of interest were β-actin (ACTB, A5316; Sigma) and LC3B (NBP2-46892, Novus Biologicals), with each monoclonal antibody incubated overnight at 4ºC. The corresponding secondary antibody was used and protein expression was detected using a chemiluminescent alkaline phosphatase substrate (T2147; Thermo Scientific). Densitometric analysis was done using ImageJ software (NIH) and densitometric values of LC3B were normalized to their respective ACTB densitometric values, before finally normalizing drug treatment densitometric values with vehicle treated densitometric values. This was done in three independent donors.

Flow cytometry

Following 24 hours of trehalose treatment, human macrophages were collected for evaluating the expression of surface receptors, CD4 and CCR5, by fluorescence activated cell sorting (FACS). Macrophages were stained by incubating cells with an aqua blue viability dye (L34957; Thermo Scientific), allophycocyanin anti-CD4 (APC, 17-0048-42; eBioscience), and phycoerythrin-Cyanine7-anti-CCR5 (PE-Cy7, 25-1956-42; eBioscience) according to manufacturer's instructions. The stained cells were then fixed using fixation buffer containing 4.21% (w/w) formaldehyde (554655; BD Biosciences). The cells were then resuspended in DPBS supplemented with 1% FBS (v/v). Flow cytometry was performed using a BD FACSCanto RUO-ORANGE analyzer. Expression levels of both CD4 and CCR5 was normalized to the expression levels of untreated cells.

In addition to using an ELISA, we also assessed HIV entry by measuring the levels of intracellular levels of HIV p24 by FACS. Macrophages were pretreated with 30 nM maraviroc, 100 mM trehalose, or 150 mM trehalose for 24 hours before being infected with HIV for 8 hours. These cells were then washed extensively before being collected for FACS. Macrophages were stained using an aqua blue viability dye and then

fixed and permeabilized using diluted fixation and permeabilization concentrate (00-5123-43; eBioscience) at a final concentration of 1X with instructions and diluent specified by manufacturers (00-5223-56; eBioscience). Following fixation and permeabilization, intracellular HIV p24 was stained using fluorescein isothiocyanate (FITC)-anti-p24 (6604665; Beckman Coulter). Expression levels of intracellular p24 was assessed as described above.

Statistical analysis

Data are presented as mean of at least three independent experiments ± standard deviation (SD). Statistical significance was determined by calculating p-values using Student's *t*-test. Differences were considered statistically significant between groups when $P < 0.05$.

III. RESULTS

Trehalose inhibits HIV replication in human primary macrophages

HIV manipulates autophagy to its advantage by upregulating the early stages of autophagy, such as autophagosome maturation, while inhibiting the degradative power of autophagy during permissive infection [17-19]. The Spector laboratory has shown that chemical inducers of autophagy, for example sirolimus, vitamin D, and HDACi, are able to overcome this blockage and inhibit HIV replication through autophagy [20, 23, 52]. In light of this, we decided to evaluate the inhibitory effects of mTOR-independent inducers of autophagy on HIV replication in HIV infected human primary macrophages. This was determined by quantifying released HIV p24 antigen in cell culture supernatant of infected cells.

Cell culture supernatant was collected on days five and ten of HIV infection in the continued presence of mTOR-independent inducers of autophagy. Sirolimus 100 nM which has been shown previously to inhibit HIV was used as a positive control and confirmed in our initial experiments (Figure 1A-C) [20, 21]. In the same experiment, 100 mM and 150 mM of trehalose inhibited HIV infection of macrophages by approximately 97% and 98% in HIV p24 release, respectively, on day five (Figure 1A). This striking decrease in HIV p24 release was not observed with any of our other drug treatments. By day ten of HIV infection, we continued to see a reduction of 85% and 88% in HIV p24 release in macrophages treated with 100 mM and 150 mM of trehalose, respectively (Figure 1A).

Although we saw a decrease in HIV p24 release with both SMER28 and spermidine treatment at varying concentrations, the reduction in HIV p24 release was not as effective as trehalose treated macrophages (Figure 1A-C). By day 10, we only

saw a 53% and 70% reduction in HIV p24 release in macrophages treated with 100 nM and 200 nM of SMER28, respectively (Figure 1B). Whereas, macrophages treated with 500 nM and 1 μM of spermidine, resulted in only 37% and 61% decrease in HIV p24 release, respectively, on day ten (Figure 1C).

While we concluded that HIV replication in human primary macrophages can be inhibited by mTOR-independent inducers of autophagy, we wanted to confirm that this effect was not due to significant cell death.

Trehalose, SMER28, and spermidine are non-cytotoxic to human primary macrophages

Trehalose is considered a GRAS substance and has been approved for human consumption up to 50 grams/day [39]. In human diets, trehalose can be consumed in everyday products such as honey and baker's yeast. Despite these findings, it is unknown if trehalose treatment is cytotoxic to human primary macrophages. Likewise, the cytotoxic effects of SMER28 and spermidine on human primary macrophages have not been studied. The cytotoxic profile of these drugs were important to explore further because we wanted to ensure that the reduction in HIV p24 release was not simply attributed to decreased cell viability. To assess the possible cytotoxic effects of these mTOR-independent inducers of autophagy, we measured the extracellular release of LDH in cell culture supernatant as a marker of cellular damage [53].

Cell culture supernatants were collected after treating cells with varying concentrations of trehalose, SMER28, and spermidine for 24 hours, 10 days, and 10 days during HIV infection. These supernatants were analyzed using a LDH cytotoxicity assay. Spectrophotometric measurements did not show any statistically significant increase in extracellular LDH release after trehalose, SMER28, or spermidine treatment at any of the aforementioned concentrations or time points (Figure 2A-I). As such, these

mTOR-independent inducers of autophagy were deemed non-cytotoxic to human primary macrophages at any of the tested concentrations before and after HIV infection. Although all three drugs were able to inhibit HIV replication and were non-cytotoxic, we only further evaluated the effects of trehalose because it was able to significantly decrease HIV p24 release during the course of infection.

Trehalose induces autophagy flux in human primary macrophages

Trehalose can induce mTOR-independent autophagy in several cell types [43, 45, 54]. Furthermore, trehalose induced autophagy has been associated with increased clearance of mutant neurodegenerative proteins and HCMV [44-46]. The effects of trehalose treatment on autophagic flux in human primary macrophages have not been studied to date. For that reason, we cultured monocyte derived macrophages with 100 mM and 150 mM of trehalose for 24 hours. We included 100 nM sirolimus in our experiment, because it is a known inducer of mTOR-dependent autophagy [54]. In addition to trehalose and sirolimus, we added 50 nM of bafilomycin A1, an inhibitor of autophagosome and lysosomal fusion, for two hours.

The cells were then washed and lysed. Protein expression was analyzed by polyacrylamide gel electrophoresis and Western blotting to assess autophagic flux. The initial steps of autophagy includes the formation of autophagosomes, which involves the conjugation of cytosolic LC3B-1 with PE through a process involving Atg4, Atg7, and Atg3 [15]. This process forms LC3B-II, which is bound to the autophagosome membrane and is used to track autophagosome formation [15]. Once the mature autophagosome forms, it fuses with lysosomes, where the internal compartment is then degraded [15, 16].

To measure the induction of autophagosome formation, we calculated the LC3B-II to ACTB protein ratio and normalized the values to the untreated cells to determine the effects of each treatment condition [55]. Upon treatment with 100 mM and 150 mM trehalose, we saw on average, a 1.7 and 2.5 fold increase in LC3B-II, respectively (Figure 3B). However, an increase in LC3B lipidation does not, alone, indicate an induction of autophagic flux. Increased expression of LC3B-II can be due to an increase in LC3B transcription or accumulation of autophagosomes as a result of blockage at the autophagosome and lysosome fusion step. A marker of autophagic flux is SQSTM1, which is a protein that binds to the contents of autophagosome. The degradation of SQSTM1 is used to measure the completion of autophagic flux [56]. In spite of that, several groups have found that SQSTM1 degradation may not be directly correlated with increased LC3B lipidation, with one group looking at the effects of trehalose in keratinocytes [43, 57, 58].

As such, we included bafilomycin A1 in our treatment conditions to identify if there is an accumulation of LC3B-II, which would indicate the induction of autophagic flux [46, 58-61]. Our results indicate that there was further increase in LC3B-II expression following bafilomycin A1 treatment for two hours in addition to 24 hour treatment with 100 mM and 150 mM trehalose, 2.4 fold and 2.7 fold, respectively (Figure 3B). These results indicate that trehalose at both 100 mM and 150 mM trehalose induce autophagic flux. In our cells treated with bafilomycin A1 alone, we see further increase in LC3B-II, which indicates blockage of basal autophagy [61].

Trehalose decreases CD4 and CCR5 expression in human primary macrophages

Having identified that trehalose induces autophagy in human primary macrophages, we further explored if trehalose had any additional effects, given the

significant decrease in released HIV p24 at day 5 (Figure 1A). The early steps in the HIV lifecycle includes attachment and entry, which is mediated by the primary receptor, CD4, and a co-receptor, CCR5 or CXCR4 [3]. To evaluate if trehalose altered the expression of these surface receptors, we cultured human primary macrophages with 100 mM and 150 mM of trehalose for 24 hours. We then analyzed the expression of surface receptor, CD4 and CCR5, by flow cytometry. Our results show that relative to untreated cells, there was approximately 60% and 55% reduction in surface expression of CD4 after 24 hours of treatment with 100 mM and 150 mM of trehalose, respectively (Figure 4C). Similarly, we saw a 28% and 40% decrease in surface expression of CCR5 in macrophages after 24 hours of treatment with 100 mM and 150 mM of trehalose, respectively (Figure 4D). The decrease in these two receptors, which are vital to HIV entry into macrophages, prompted us to evaluate if HIV entry into trehalose treated macrophages was compromised.

Trehalose inhibits HIV entry in human primary macrophages

We determined if the initial entry of HIV into trehalose treated macrophages was reduced by analyzing the amount of intracellular p24 after the initial eight hour infection by two different methods. First, we treated macrophages with 30 nM maraviroc or 100 mM or 150 mM trehalose for 24 hours. Maraviroc is used here because it is an effective CCR5 antagonist and is known to inhibit HIV entry [4]. The cells were then infected with HIV for eight hours. After infection, the cells were collected and analyzed for intracellular p24 by flow cytometry and p24 ELISA using cell lysates. Our flow cytometry data was obtained by fixing, permeabilizing, and staining the collected cells using an intracellular HIV p24 antibody that indicates the number of infected cells. Relative to our untreated macrophages, we saw approximately an 83% and 93% decrease in the number of p24-

positive cells in macrophages that were pretreated with 100 mM and 150 mM of trehalose, respectively (Figure 5B). For cells that were lysed, we measured the levels of intracellular p24 by p24 ELISA and found that there was approximately a 65% and 85% less intracellular p24 in 100 mM and 150 mM trehalose treated cells, respectively, when compared to untreated, infected cells (Figure 5C). These results, taken together, show that trehalose effectively inhibits HIV entry into human primary macrophages.

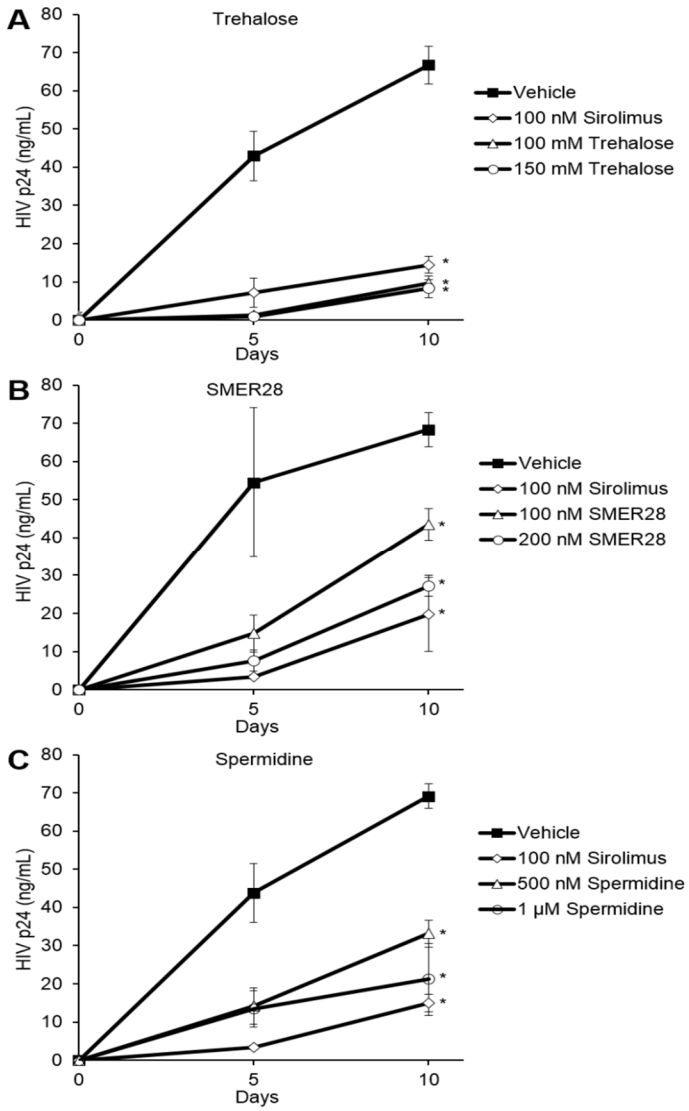

Figure 1. Inducers of mTOR-independent autophagy are effective inhibitors of HIV replication in human primary macrophages. Macrophages were pretreated with vehicle, 100 nM sirolimus, (A) 100 mM or 150 mM trehalose, (B) 100 nM or 200 nM SMER28, or (C) 500 nM or 1 μM spermidine for 24 hours prior to infection with HIV for eight hours. Macrophages were then washed and kept in MDM media supplemented with drug treatments with 50% media changes every two to three days. Cell culture supernatant was collected on days 5 and 10 and extracellular HIV p24 was measured by ELISA. Both sirolimus and mTOR-independent inducers of autophagy effectively inhibit HIV p24 release. For trehalose experiments, data are presented as mean of four independent experiments ± SD. For SMER28 and spermidine experiments, data are presented as mean of three independent experiments ± SD. *P ≤ 0.05

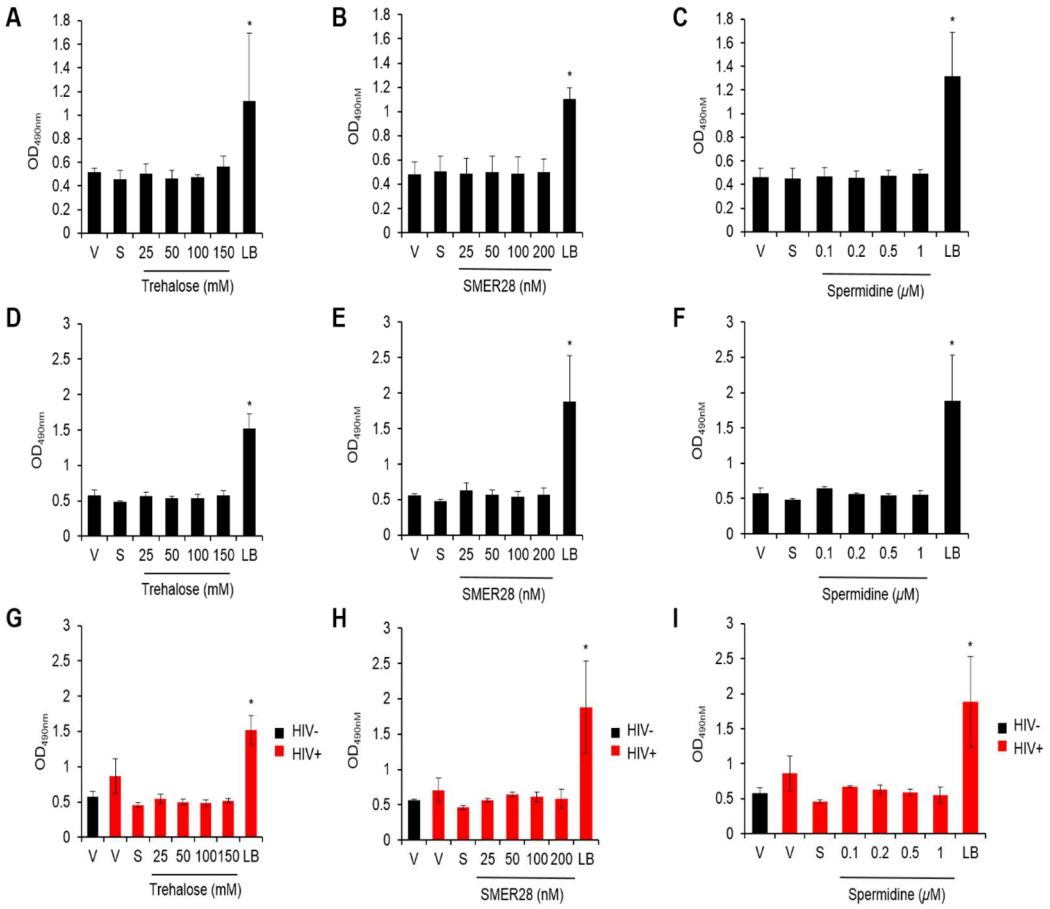

Figure 2. Trehalose, SMER28, and spermidine are non-cytotoxic to human primary macrophages. Macrophages were treated with vehicle (V), 100 nM sirolimus (S) or varying concentrations of trehalose, SMER28, or spermidine for (A-C) 24 hours, (D-F) 10 days, or (G-I) 10 days during HIV infection. As a positive control, 1x lysis buffer (LB) was added to untreated cells for two hours. Cell culture supernatant was collected at the aforementioned times. Spectrophotometric measurements of extracellular LDH was used to determine cellular cytotoxicity. Both sirolimus and trehalose were non-cytotoxic to macrophages after 24 hours of treatment.
Data presented as mean of three independent experiments ± SD. *P ≤ 0.05

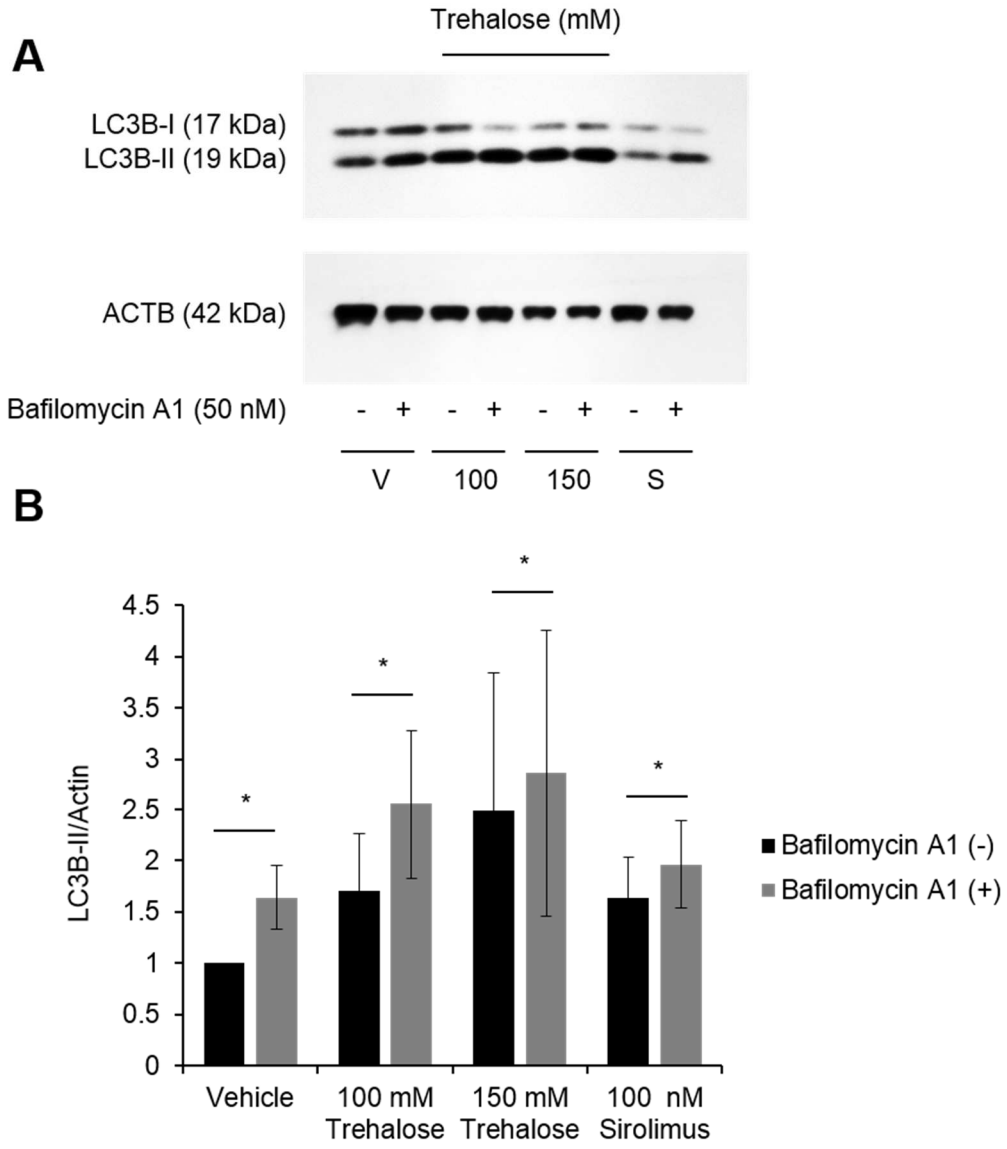

Figure 3. Trehalose induces autophagic flux in human primary macrophages. Macrophages were treated with vehicle (V), 100 nM sirolimus (S), or with trehalose for 24 hours with or without 50 nM bafilomycin A1 for 2 hours. Induction of autophagic flux was analyzed by Western blot. (A) Representative Western blot of LC3B isoforms and ACTB. (B) Densitometric analysis of LC3B-II accumulation (LC3B-II normalized to ACTB) after bafilomycin A1 treatment as means to measure induction of autophagic flux. Both sirolimus and trehalose induce autophagic flux as indicated by the accumulation of LC3B-II after bafilomycin A1 treatment. Data presented as mean of three independent experiments ± SD. *P ≤ 0.05

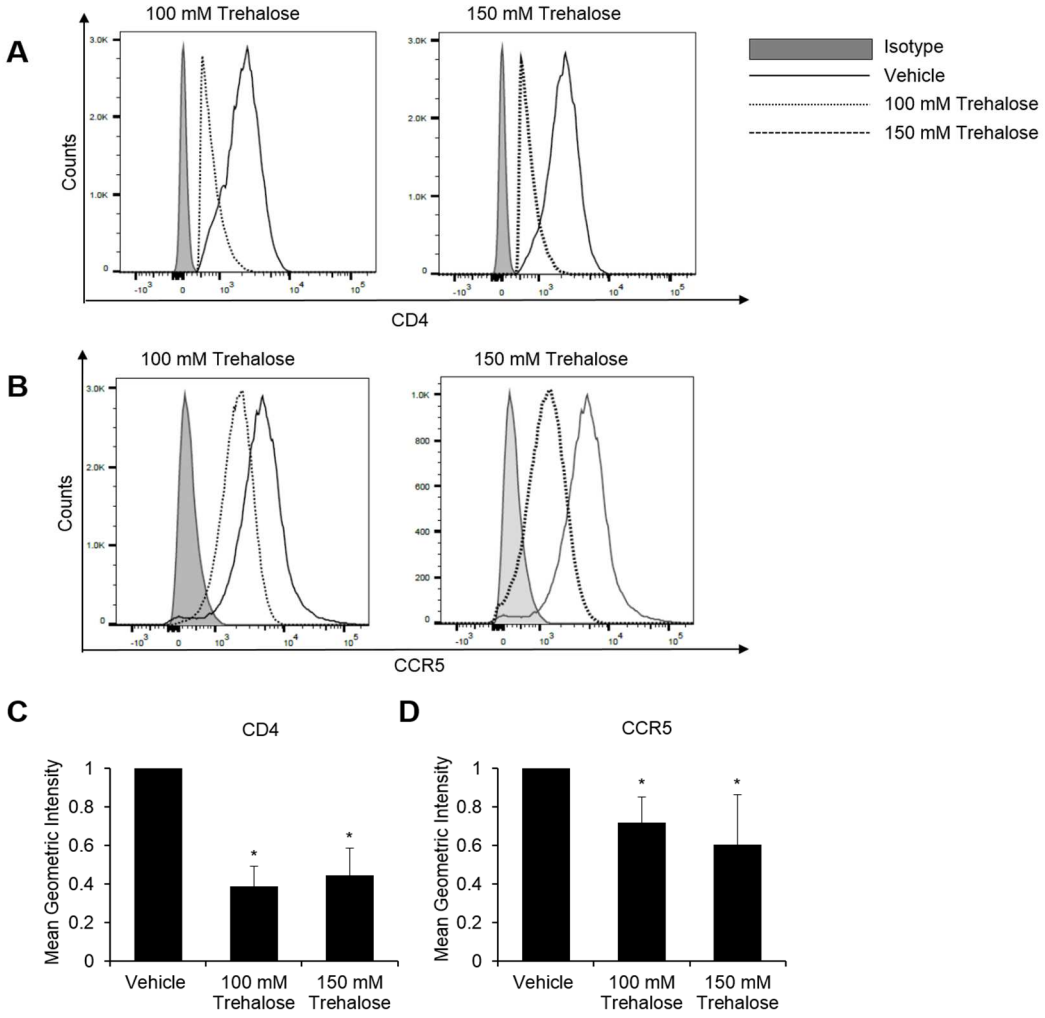

Figure 4. Trehalose reduces surface expression of CD4 and CCR5 on human primary macrophages. Macrophages were treated with trehalose for 24 hours. CD4 and CCR5 surface expression was analyzed by flow cytometry. (A) Representative histogram of surface receptor (A) CD4 and (B) CCR5 after 24 hours of trehalose treatment. Trehalose reduced the expression of surface receptor (C) CD4 and (D) CCR5. Data presented as mean of five independent experiments ± SD. *P ≤ 0.05

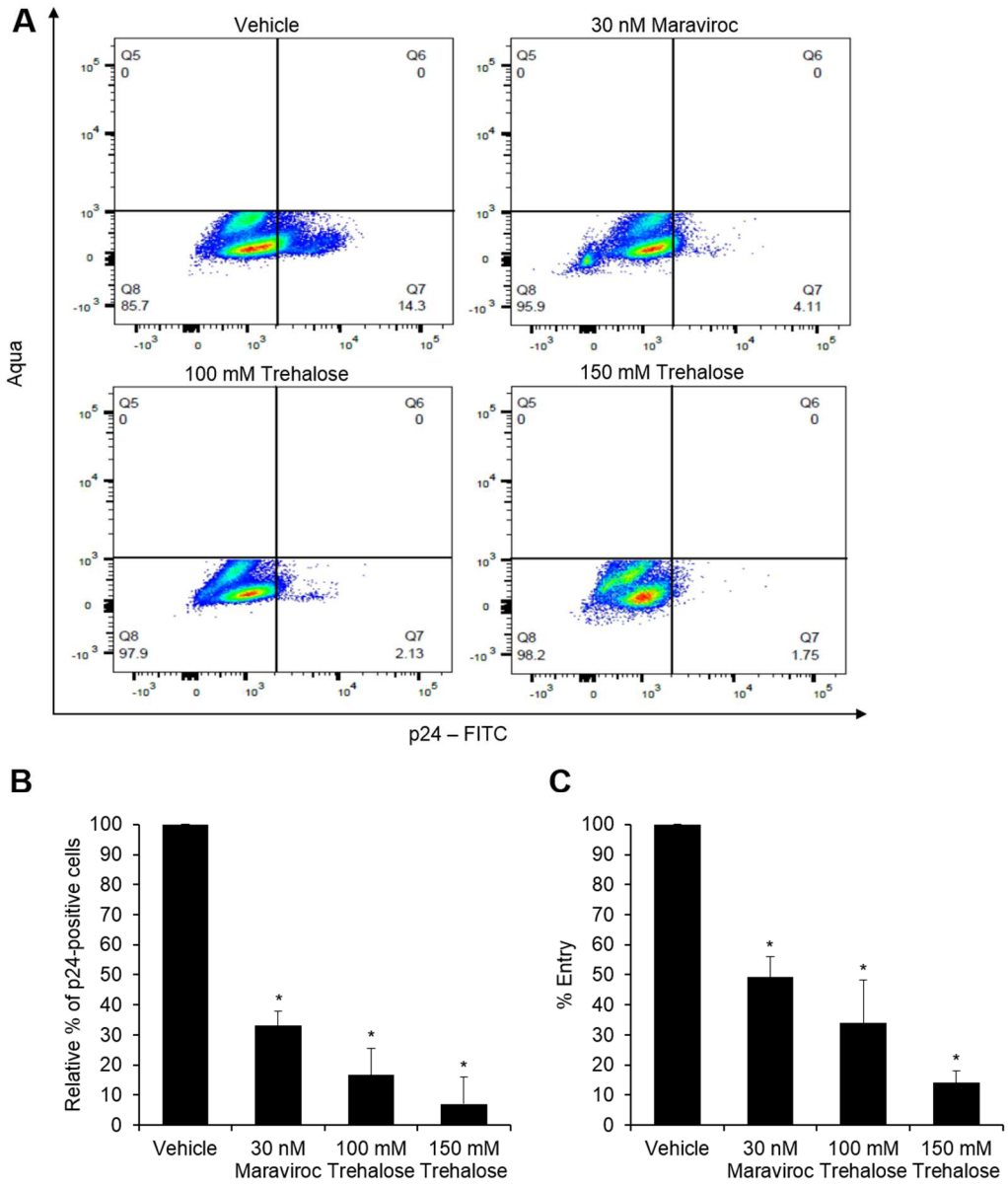

Figure 5. Trehalose effectively inhibits HIV entry into human primary macrophages. Macrophages were pretreated with either 30 nM maraviroc or trehalose for 24 hours prior to infection with HIV for eight hours. After eight hours, cells were (A-B) collected for detection of intracellular HIV p24 by flow cytometry or (C) washed extensively, trypsinized, and lysed to measure intracellular HIV p24 by ELISA. Both maraviroc and trehalose effectively inhibit HIV entry when analyzed by both methods. Data presented as mean of three independent experiments ± SD. *P ≤ 0.05

IV. DISCUSSION

While HIV has largely subsided from the headlines, it remains a pressing concern because 1.2 million people are infected in the United States with this lifelong disease [62]. Of those infected, approximately one in eight are unaware that they are infected and of those infected, almost half are not receiving proper care. Combined, these two groups account for about 90% of the approximately 45,000 new infections in the U.S. [62]. In terms of treatment, ART has helped patients increase their CD4+ cell count, decrease HIV viral load, and improve life expectancies [63]. Despite these advances, ART, much like any other treatment, is only effective as long as the virus remains susceptible to these drugs. As resistance, both acquired (resistance arising from mutations that counter selective pressures) and transmitted (resistant strains of HIV transferred between individuals), continue to pose a threat, additional strategies of inhibiting HIV replication must be considered in conjunction to ART [64-67].

Chemically induced autophagy, both mTOR-dependent and mTOR-independent, is an evolving strategy for combatting different neurodegenerative diseases, cancers and pathogen challenge [33, 35, 46, 52]. Our lab has extensively studied the effects of mTOR-dependent inducers of autophagy and has shown that HIV replication can be inhibited through this pathway. Similarly, the present data support the strategy of using inducers of autophagy to inhibit HIV as we saw decreased extracellular HIV p24 release when macrophages were treated with both a previously studied mTOR-dependent inducer of autophagy (sirolimus) and several mTOR-independent inducers of autophagy (SMER28, spermidine, and trehalose). Furthermore, we show that trehalose, in particular, is not only non-cytotoxic to human primary macrophages, but also induces autophagic flux, as demonstrated by the increase in LC3B-II and accumulation of LC3B-

II when autophagic flux is blocked during bafilomycin A1 treatment. Just as important, we show that trehalose decreases both CD4 and CCR5 expression, both of which facilitate HIV entry. This finding was supported by the decrease in intracellular HIV p24 during the early stages of infection, which was determined by FACS and ELISA.

Taken together, these findings suggest that trehalose should be explored further as an adjunctive therapy agent for HIV infection. As an inducer of mTOR-independent autophagy, trehalose bolsters a highly conserved pathway that has demonstrated protective properties against diseases. Unlike certain classes of drugs that target a specific HIV protein, trehalose induces autophagy, a host mechanism, which makes resistance less likely to occur. Another reason to further examine the effects of trehalose is because currently, there is only one FDA approved drug, maraviroc (a CCR5 antagonist), that specifically blocks HIV entry. Given that trehalose effectively decreases both CD4 and CCR5 expression, it may prove useful as a HIV entry inhibitor that can be taken in tandem with current medication. Trehalose is a sugar that is recognized as a GRAS substance and approved for human consumption, which may make ART less toxic and easier to tolerate, a major concern for HIV-infected individuals that can lead to medical non-compliance, which fuels the development of HIV strains resistant to ART. One last point to consider is that trehalose is in FDA trials for its effect on arterial aging, which may facilitate repurposing trehalose as a component for ART.

Even with all these possibilities, it remains important to consider some of the limitations presented in this research and possible additional experiments. Given that trehalose not only induces autophagy, but also prevents HIV entry through decreasing CD4 and CCR5 expression, it is worthwhile to determine which process, if not both, are contributing to the inhibitory effects of trehalose on HIV replication. Determining the effects of autophagy can be accomplished by inhibiting autophagic flux with an

autophagy inhibitor and observing the effects on both extracellular p24 release and intracellular p24 levels during HIV infection in the presence of trehalose. An alternative experiment could include silencing of key autophagy proteins and observing the effects on HIV replication. Furthermore, because trehalose decreases both CD4 and CCR5 expression, it is important to study the effects trehalose may have on immune function as CD4 plays an integral role in facilitating communication between certain T-cells and antigen presenting cells, while the exact function of CCR5 remains unclear. The exact mechanism behind the decrease in CD4 and CCR5 expression also requires further examination. Lastly, it would be interesting to determine if SMER28 and spermidine have any non-autophagy related effects that contribute to their inhibitory effects on HIV replication or if the HIV inhibition is autophagy dependent. Ultimately, these mTOR-independent inducers of autophagy may provide a new tool in our battle against HIV. Of these, the simple sugar, trehalose, seems to be the most promising.

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