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## Secreted factors induced by PKC modulators do not indirectly cause HIV latency reversal

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### Abstract

HIV can establish a long-lived latent infection in cells harboring integrated non-expressing proviruses. Latency reversing agents (LRAs), including protein kinase C (PKC) modulators, can induce expression of latent HIV, thereby reducing the latent reservoir in animal models. However, PKC modulators such as bryostatin-1 also cause cytokine upregulation in peripheral blood mononuclear cells (PBMCs), including cytokines that might independently reverse HIV latency. To determine whether cytokines induced by PKC modulators contribute to latency

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**Jose A. Moran:** Formal analysis, Data curation, Conceptualization, Funding acquisition, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Alok Ranjan:** Investigation, Resources, Writing - review & editing. **Rami Hourani:** Investigation, Resources, Writing - review & editing. **Jocelyn T. Kim:** Conceptualization, Funding acquisition, Writing - review & editing. **Paul A. Wender:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing. **Jerome A. Zack:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Matthew D. Marsden:** Formal analysis, Data curation, Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stanford University has filed patent applications on SUW133 and related technology, which has been licensed by Neurotrope BioScience and Bryologyx Inc. P.A.W. is an adviser to both companies and a cofounder of the latter. J.A.Z. is a cofounder of CDR3 Therapeutics and is on the SAB of Bryologyx.

reversal, primary human PBMCs were treated with bryostatin-1 or the bryostatin analog SUW133, a superior LRA, and supernatant was collected. As anticipated, LRA-treated cell supernatant contained increased levels of cytokines compared to untreated cell supernatant. However, exposure of latently-infected cells with this supernatant did not result in latency reactivation. These results indicate that PKC modulators do not have significant indirect effects on HIV latency reversal *in vitro* and thus are targeted in their latency reversing ability.

## Keywords

HIV; PKC modulators; Latency reversal; Bryostatin-1; SUW133; Kick-and-kill approach

## 1. Introduction

In 2020, approximately 38 million people globally were living with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). HIV primarily infects CD4<sup>+</sup> T cells and other immune cell types such as macrophages (Gottlieb et al., 1981; Gartner et al., 1986). A defining characteristic of retroviruses, including HIV, is their ability to convert their viral RNA genomes into proviral DNA and subsequently integrate this DNA into the host cell's chromosomes (Brown et al., 1997). HIV disease progression can be halted with antiretroviral therapy (ART) (Marsden and Zack, 2013). ART uses an antiretroviral drug cocktail, consisting of drug combinations that inhibit HIV replication and prevent the spread of virus to new host cells. However, ART does not cure HIV infection because some integrated HIV proviruses remain latent and are not directly targeted by currently available antiretroviral drugs. These latent proviruses express little or no viral RNA and no viral proteins, but can be induced to produce infectious virions if the host cell becomes stimulated (Marsden and Zack, 2013). Furthermore, recent studies indicate that even on ART, HIV expression occurs and is dynamic in a portion of the reservoir (Pinzone et al., 2019; Bachmann et al., 2019; Yeh and Ho, 2021). Hence, if ART treatment is discontinued, the virus can replicate at high levels again (Marsden and Zack, 2013; Wong et al., 1997; Finzi et al., 1997; Chun et al., 1997). Replication-competent HIV persists during ART in latently-infected CD4<sup>+</sup> T cells, which are naturally long-lived due to their key role in immunological memory (Blankson et al., 2002; Siliciano et al., 2003). Latently-infected CD4<sup>+</sup> T cells therefore have a long half-life of over 40 months during ART, effectively making HIV a lifelong infection with currently available therapies (Siliciano et al., 2003; Finzi et al., 1999). To cure HIV infection, all infected cells, including latently-infected cells, must be permanently silenced or eradicated.

Various approaches to an HIV cure have been proposed (Marsden and Zack, 2013, 2015). The “kick-and-kill” strategy, also known as “activation and elimination” or “induce and reduce”, works by inducing, or “kicking”, latently-infected cells to express viral proteins (Deeks et al., 2015; Archin and Margolis, 2014; Spivak and Planelles, 2018; Sengnpta and Siliciano, 2018). This allows these previously latent HIV-infected T cells to be “killed” by other immune cells or cytopathic effects (Marsden and Zack, 2015). To induce HIV viral protein expression from latency, reservoir cells can be treated with latency reversing agents (LRA), which include benzotriazoles, histone deacetylase (HDAC) and bromodomain

inhibitors, toll-like receptor agonists, a phosphatase and tensin homologue (PTEN) inhibitor (disulfiram), SMAC mimetics, cytokines, and protein kinase C (PKC) modulators such as bryostatin-1 (Marsden and Zack, 2013; Spivak and Planelles, 2018; McKernan et al., 2012; Sloane et al., 2020; Chun et al., 1999; Scripture-Adams et al., 2002; Marsden et al., 2017; Bullen et al., 2014; Archin et al., 2014; Barouch and Deeks, 2014; Laird et al., 2015; Nixon et al., 2020; Pache et al., 2020; Albert et al., 2017; Bosque et al., 2017). Of these, PKC modulators have proven capable of efficiently reversing HIV from latency in several preclinical models of HIV persistence and are thus promising leads (Marsden et al., 2017, 2020; Kim et al., 2022; Gama et al., 2017). PKC modulators diffuse into cells and bind to soluble cytosolic PKC forming a complex that in turn translocates to intracellular membranes and activates downstream kinases, ultimately activating NFkB, an important transcription factor for HIV expression (Marsden and Zack, 2013; McKernan et al., 2012; Jiang and Dandekar, 2015). Bryostatin-1, a natural PKC agonist produced by commensal bacteria in sea moss *Bugula neritina*, has been shown to be an effective LRA in HIV studies (Archin and Margolis, 2014; Schwartzmann et al., 2001; Kollár et al., 2014). Bryostatin-1 is produced only in small amounts by this natural marine source and is neither evolved nor optimized for HIV latency reversal. This motivated us to design, synthesize, and characterize new bryostatin analogs, some of which are superior to bryostatin-1 in latency reversal and *in vivo* tolerability (Marsden et al., 2017, 2018, 2020; Kim et al., 2022; DeChristopher et al., 2012). One particularly potent bryostatin analog termed SUW133 was found to reverse HIV from latency in cell line models, *ex vivo* in patient-derived cells, and *in vivo* using humanized BLT mice (Marsden et al., 2017, 2018, 2020; Kim et al., 2022; DeChristopher et al., 2012). Additional humanized mouse studies demonstrated that when administered during ART, this compound can induce HIV expression resulting in killing of latently-infected cells and a reduction in rebound-competent HIV reservoirs when stopping ART (Marsden et al., 2020). Administration of natural killer cells in addition to SUW133 further augmented the delay in rebound (Kim et al., 2022). Given these striking results, it is important to develop a better understanding of the effects that PKC modulators have on healthy and latently-infected cells and their mechanisms of action.

Bryostatin-1 and other PKC modulators induce effects in immune cells beyond HIV latency reversal. This includes transcriptional changes (Li et al., 2020) and production of cytokines (Sloane et al., 2020; Marsden et al., 2018). Cytokines and other secreted factors produced by cells exposed to LRAs might reverse HIV from latency in an indirect manner, as several cytokines including TNF $\alpha$ , Interleukin (IL)-2, IL-7 and combinations of cytokines (such as IL-6 + TNF $\alpha$  + IL-2 or IL-10 + TNF $\alpha$ ) have been shown to do (Chun et al., 1998, 1999; Scripture-Adams et al., 2002; Rabbi et al., 1998). Therefore, it is possible that some of the observed latency reversal induced by PKC modulators is via indirect effects mediated by secreted factors. To date, the contribution of these additional indirect effects to latency reversal has not been studied.

Here, we sought to characterize the differences in secreted proteins between bryostatin-1- or SUW133-treated and untreated human PBMCs. This was accomplished by first treating PBMCs with either bryostatin-1 or SUW133 *in vitro* and then removing the compounds prior to harvesting and filtering the supernatant, to produce 'conditioned media'. The conditioned media's cytokine composition was then measured, and the media was added to

different latently-infected cell lines, to evaluate whether it can reverse latency in the absence of PKC modulators. Remarkably, while we found that several cytokines are upregulated by PKC modulators in the culture supernatant, these conditioned media samples did not independently reactivate latent HIV in any of the cell line models tested. Together, these data indicate that direct intracellular effects of PKC modulators are responsible for their latency reversing capabilities rather than indirect effects mediated by secreted factors.

## 2. Materials & methods

### 2.1. Conditioned media synthesis in primary human PBMC

Peripheral blood mononuclear cells (PBMCs) from healthy HIV seronegative donors were collected by the UCLA Virology Core using UCLA Institutional Review Board approved protocols and provided for this study in a de-identified fashion. A total of  $5.0 \times 10^7$  cells were obtained per donor, which were plated a T-75 flask in 30 mL RF10 media consisting of RPMI media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. The following day, PBMCs were directly stimulated with 10 nM LRA (bryostatin-1 or SUW133) or untreated (media-only control) at  $5.0 \times 10^5$  cells/mL in 30 mL of RF10 media in a T-75 flask. Twenty-four hours post-stimulation, PBMCs were pelleted at 296 RCF for 5 min and resuspended in fresh RF10 before being pelleted again and resuspended with fresh RF10 media to remove the LRA. Twenty-four hours post-wash, the cells were pelleted at 296 RCF for 5 min, where the supernatant was collected and filtered with a 0.22- $\mu$ m filter. The filtered 24-h-conditioned media was frozen at  $-80^\circ\text{C}$ .

### 2.2. Human cytokine multiplex assay

Conditioned media samples (bryostatin-1-, SUW133-treated or untreated) were analyzed by the UCLA Immune Assessment Core for identification of potential differences between untreated, and LRA-treated conditioned media samples. This was achieved with a Luminex Multiplex assay and Human 38-plex Cytokine/Chemokine panel, which detects and quantifies proteins that were secreted by the PBMCs into the conditioned media. This assay runs standards and controls in parallel, and our untreated conditioned media served as the negative biological control for each respective donor. For the purposes of statistical analysis and comparison, undetectable values were reported as the mean between zero and the quantification limit, which varies by cytokine and soluble factor.

### 2.3. HIV latency reversal assay

The following reagents were obtained through the NIH HIV Reagent Program, Division of AIDS, NIA ID, NIH: Human T Lymphocyte J-Lat Full Length Cells 6.3 (cat# 9846); 8.4 (cat# 9847); 9.2 (cat# 9848); 10.6 (cat# 9849); & Human Immunodeficiency Virus J-Lat Tat-GFP Cells A2 (cat# 9854), all of which were contributed to the NIH HIV Reagent Program by Dr. Eric Verdin (Jordan et al., 2001, 2003). U1 cells (ARP-165) contributed by Dr. Thomas Folks were also obtained through the NIH HIV Reagent Program (Folks et al., 1987). J-Lat clones (6.3, 8.4, 9.2, 10.6, and A2) and U1 cells were plated at  $5 \times 10^5$  cells in 100  $\mu$ L of RF10 media in each well of 96-well U-bottom plates. Forty-eight-hour stimulation with conditioned media was performed by adding 100  $\mu$ L conditioned media to respective wells to study potential indirect effects of LRAs, bringing the total volume to 200

μL per well. J-Lat clones and U1 cells were also treated directly with 10 nM bryostatin-1 (Wender et al., 2017), 10 nM SUW133 (DeChristopher et al., 2012) or were untreated as positive and negative controls, respectively. In all J-Lat clones, each condition, including controls was performed in technical duplicates, in three independent biological replicates. In U1 cells, positive and negative controls were performed in technical triplicate in two independent biological replicates. The conditioned media samples were performed in two independent biological replicates, with each condition containing four donor samples, giving an n = 8. After 48 h-stimulation, each plate was centrifuged at 296 RCF for 5 min to pellet cells. The supernatant was carefully removed, and the pelleted J-Lat clone cells were resuspended in 200 μL freshly prepared 2% paraformaldehyde (PFA) and GFP expression was measured using the FITC channel on a LSR Fortessa (BD Biosciences) flow cytometer within 24-h post-fixation. At time of harvest U1 cells were also pelleted and resuspended in freshly prepared 2% PFA and incubated for 1 h at 4 °C. U1 cells were then washed with PBS before resuspension in 0.02% Tween 20 (in PBS) for 20 min at room temperature (RT). U1 cells were subsequently washed with PBS before being resuspended in 50% human AB serum (in PBS) containing KC57-RD1 (Beckman Coulter cat# 6604667) and incubated at 4 °C for 20 min, washed with PBS and resuspended in 2% PFA. HIV expression was measured via KC57-RD1 using the PE channel on a LSR Fortessa (BD Biosciences) flow cytometer. Data were analyzed with FlowJo software version 10.8.1.

### 3. Results

#### 3.1. Determining cytokine concentration differences in LRA-treated conditioned media

PBMC from 6 to 8 different healthy human donors were obtained and stimulated for 24 h with PKC modulating LRAs after which the LRA was removed, and cells were washed with fresh RF10 media. To remain consistent with previous studies that treated human primary cells with bryostatin-1, which ranged from 18 to 48 h, a 24-h timepoint was selected (Sloane et al., 2020; Marsden et al., 2018; Hany et al., 2022). After another 24 h, the new media containing secreted factors from the stimulated cells was obtained and this cell-free supernatant, or conditioned media (CM), analyzed (Fig. 1). We first determined cytokine composition differences between the bryostatin-1-treated, SUW133--treated, and untreated CM produced from PBMCs 24 h after LRA-removal. As expected, there were differences in cytokine concentrations between the three conditions with many cytokine concentrations trending higher in the stimulated cells (Fig. 2A) (Sloane et al., 2020; Marsden et al., 2018). Some donor-to-donor variation in LRA response and cytokine production was observed, but several broad patterns of responses were evident. Of the thirty-eight cytokines in the immunoassay, twenty-eight cytokines had an increased mean percent change in concentration, with ten being significantly increased in at least one stimulation condition. Of the remaining ten cytokines that showed a decreased mean percent change in concentration in at least one stimulation condition, only two were significantly decreased (Fig. 2A). Anti-inflammatory cytokine IL-10 was present at significantly lower concentrations in both the bryostatin-1- and SUW133-treated CM (−42.66% and −53.24% change, respectively) (Commins et al., 2010; Emilie and Galanaud, 1998). Pro-inflammatory chemokine IP-10 was also present at lower concentrations in both treatment groups, though only significantly decreased in bryostatin-1-treated group (−67.65% change) (Commins et

al., 2010). This decrease in both pro- and anti-inflammatory proteins is indicative of the complex environment that is created through PKC modulatory treatment.

There was a significant increase in anti-inflammatory IL-1RA and pro-inflammatory cytokines IL-12P70, IL-8, and TNF $\alpha$  in one or both PKC modulator-treated conditions (Fig. 2A & B) (Commins et al., 2010; Emilie and Galanaud, 1998; Vignali and Kuchroo, 2012). Other cytokines and chemokines with diverse functions were also significantly increased. Eosinophil-recruiter eotaxin and regulatory T cell-recruiter MDC (CCL22) were both increased 260-to-364% (Fig. 2A & B) (Faget et al., 2011; Garcia-Zepeda et al., 1996). Eosinophil recruitment typically indicates a pro-inflammatory response, while regulatory T cells are normally recruited to suppress further T cell activation, an anti-inflammatory response (Commins et al., 2010; Faget et al., 2011; Garcia-Zepeda et al., 1996). Both MIP $\alpha$  and MIP $\beta$ , ligands to HIV entry co-receptor CCR5, were elevated and play a role in T cell recruitment and development, and induction of pro-inflammatory cytokines in other cells as well (Commins et al., 2010). These chemokines can also inhibit infection with CCR5-tropic strains of HIV (Cocchi et al., 1995). MCP-3, a monocyte chemoattractant protein, was also significantly increased in both treatment groups (Fig. 2B) (Commins et al., 2010; Cocchi et al., 1995).

### 3.2. Evaluation of HIV latency reversal with conditioned media from LRA-treated PBMC

An array of different molecules can induce latency reversal to some extent, including cytokines IL-7, IL-2, TNF $\alpha$  and specific combinations of cytokines (Chun et al., 1998, 1999; Scripture-Adams et al., 2002; Rabbi et al., 1998). Once we determined that several cytokines were upregulated in response to PKC modulator stimulation, we then assessed whether these cytokines or other soluble factors could independently induce expression of latent HIV in the absence of LRA. Indirect latency reversal was measured *in vitro* using T lymphocyte Jurkat-Latency (J-Lat) cell clones and promonocytic U1 cells. J-Lat cells contain either a near-full length or “mini genome” of replication incompetent HIV DNA, while U1 cells contain two integrated HIV-1 DNA copies with a defective Tat gene that enables latency (Jordan et al., 2001, 2003; Folks et al., 1987; Emiliani et al., 1998). J-Lat clones also contain a GFP reporter gene, thus latency reversal can be measured as GFP $^{+}$  cell percentage. A panel of J-Lat clones containing the near-full length HIV genome consisting of clones 6.3, 8.4, 9.2, and 10.6 were used as they vary in integration site and responsiveness to different LRAs. J-Lat clone A2 was also included as it contains a “mini genome” of HIV, lacking most HIV genes except Tat, a regulatory protein that enhances viral transcription efficacy (Jordan et al., 2001, 2003). U1 cells do not encode reporter genes and thus latency reversal in these cells was assessed by conducting intracellular staining for HIV-1 p24 core antigen expression.

As expected from previous studies, we observed a significant increase in the percentage of GFP $^{+}$  cells (J-Lat) and HIV-1 core antigen $^{+}$  cells (U1) in direct LRA stimulation with either bryostatatin-1 or SUW133 at 10 nM concentrations in all latently-infected cell lines (3- to 40-fold range) relative to the media-only control (Fig. 3A & B) (Sloane et al., 2020; Marsden et al., 2018; DeChristopher et al., 2012; Mehla et al., 2010). However, strikingly, little or no latent HIV reactivation was observed in any of the latently-infected



cell clones treated with conditioned media. In J-Lat clones 6.3, 8.4, and A2, there were no significant differences between the untreated control group and either the bryostatin-1- or SUW133 treated conditioned media groups (Fig. 3A). In J-Lat clones 9.2 and 10.6, there was no significant latency reversal observed in bryostatin-1-treated CM relative to the untreated CM. However, there was a very small (less than 1%) but significant increase in the SUW133-treated CM (Fig. 3A). In U1 cells, there was no significant differences between the untreated CM group and either the PKC modulator-treated CM groups (Fig. 3B). Together these data indicate that despite containing an array of cytokines and other soluble factors, conditioned media from LRA-treated PBMC induces little or no latent HIV expression in these cell lines.

## 4. Discussion

PKC modulators, including bryostatin-1, represent important leads as HIV latency reversing agents and have been investigated as therapeutic agents in both cancer and Alzheimer's disease research (Kollár et al., 2014). The small amounts of bryostatin-1 that can be collected from natural sources coupled with the need to optimize its activity has led to a scaled laboratory synthesis of bryostatin-1 and has inspired the production of new designed, synthetic bryostatin analogs, or bryologs (Schwartzmann et al., 2001; DeChristopher et al., 2012; Wender et al., 2017). One particularly potent bryostatin analog termed SUW133 is better tolerated in mice than the parent compound and is capable of depleting latently-infected cells and delaying viral rebound in humanized mouse models of HIV persistence (Marsden et al., 2017,2018,2020; Kim et al., 2022; DeChristopher et al., 2012). However, PKC modulators can also upregulate cytokine production in treated cells (Sloane et al., 2020; Schwartzmann et al., 2001; Marsden et al., 2018). It was unknown whether these soluble factors contribute to or independently produce some of the latency reversal observed during PKC modulator stimulation.

Our first aim was to characterize the cytokine composition in conditioned media (CM) generated by bryostatin-1- or SUW133-treatment in primary human PBMCs. This was achieved by using a human cytokine 38-plex immunoassay. Overall, twenty-eight cytokines were present at increased concentrations in both LRA-treated CM, with ten showing a significant increase in concentration in at least one of the LRA-treated conditions (Fig. 2A). Seven cytokines were present at lower concentrations in both treatment conditions, with only one cytokine, IL-10 being significantly decreased in both LRA-treated PBMC conditions (Fig. 2A). Three cytokines (Flt-3L, IL-12P40, and MCP-1) showed an increase in one treatment condition, but a decrease in the other (Fig. 2A). Of interest is that IL-7 and TNF $\alpha$  were present at increased concentrations, but IL-2 showed a decrease in concentration in the LRA-treated CM conditions. We also did not observe increases in all individual cytokines that make up the combinations which have previously been shown induce HIV latency reversal *in vitro* (IL-6 + TNF $\alpha$  + IL-2 or IL-10 + TNF $\alpha$ ). As these were primary human PBMC samples that were treated with either bryostatin-1 or SUW133, some donor-to-donor variation was observed in cytokine induction and release into the conditioned media.

We next sought to determine whether there was indirect stimulation caused by the LRA-treated conditioned media in a five clone Jurkat-Latency cell panel. This was achieved using



J-Lat clones that vary in the site of integration of the proviral HIV DNA and in some cases, the sequence of the inserted viral vector. These J-Lat clones, however, all harbor a latent replication-incompetent HIV genome encoding GFP. We observed a significant increase in the percentage of GFP<sup>+</sup> cells in all J-Lat clones when directly stimulated with either 10 nM bryostatin-1 or SUW133 relative to the media only control. However, when these same J-Lat clones were treated with LRA-treated CM, we found little or no reactivation of HIV from latency. Similar results were obtained with a different U1 latency model (Fig. 3B). Notably, concentrations of cytokines IL-7, TNF $\alpha$ , IL-2, and IL-6 produced by these LRA-treated PBMCs were lower than those used for latency reversal in some previous studies (10 ng/mL, 2.5 ng/mL, 100 U/mL, and 1–100 U/mL respectively) (Chun et al., 1998,1999; Scripture-Adams et al., 2002; Poli et al., 1990). These data show that none of the secreted factors produced by PBMC (including those not specifically analyzed in this study) stimulated expression of latent HIV in the cells tested.

Our data thus indicate that bryostatin-1 and SUW133 have a targeted latency reversing ability mediated by direct effects of the compounds themselves, rather than via secondary indirect mechanisms mediated through secreted soluble factors and cytokines in chronically-infected cell lines. The experiments described here do not encompass all possible ways that indirect effects might influence HIV reservoirs, and future studies involving latently-infected primary human cells treated with PKC modulator-induced conditioned media may provide additional insights in this regard. For example, cytokines induced by PKC modulators may influence the differentiation of CD4<sup>+</sup> T cell subsets, which may alter their HIV expression or responsiveness to LRAs (Kulpa et al., 2019). Overall, however, these new data improve our understanding of how PKC modulators affect non-infected cells and define the cytokines induced in healthy human PBMC by SUW133 treatment. In addition, the data suggest that successful HIV eradication with these LRAs will require ubiquitous delivery of LRAs to each individual latently-infected cell rather than to only a few cells in a population that subsequently propagate the latency reversal process via secreted factors. These data also provide new insights into the mechanism of action that PKC modulators use to reverse HIV from latency and imply the further development of PKC modulators, including more efficacious analogs and prodrugs with reduced ability to induce proinflammatory cytokines would not hamper their capacity to reverse latency (Sloane et al., 2020; Marsden et al., 2018). Hence, development of PKC modulating LRAs that reverse latency but induce limited cytokine production is a promising area for future HIV cure research.

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Fig. 1 created with [Biorender.com](https://www.biorender.com).

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## Data availability

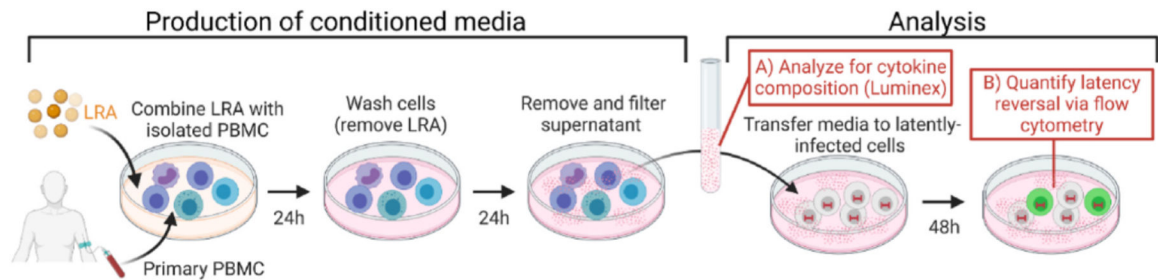
Data will be made available on request.

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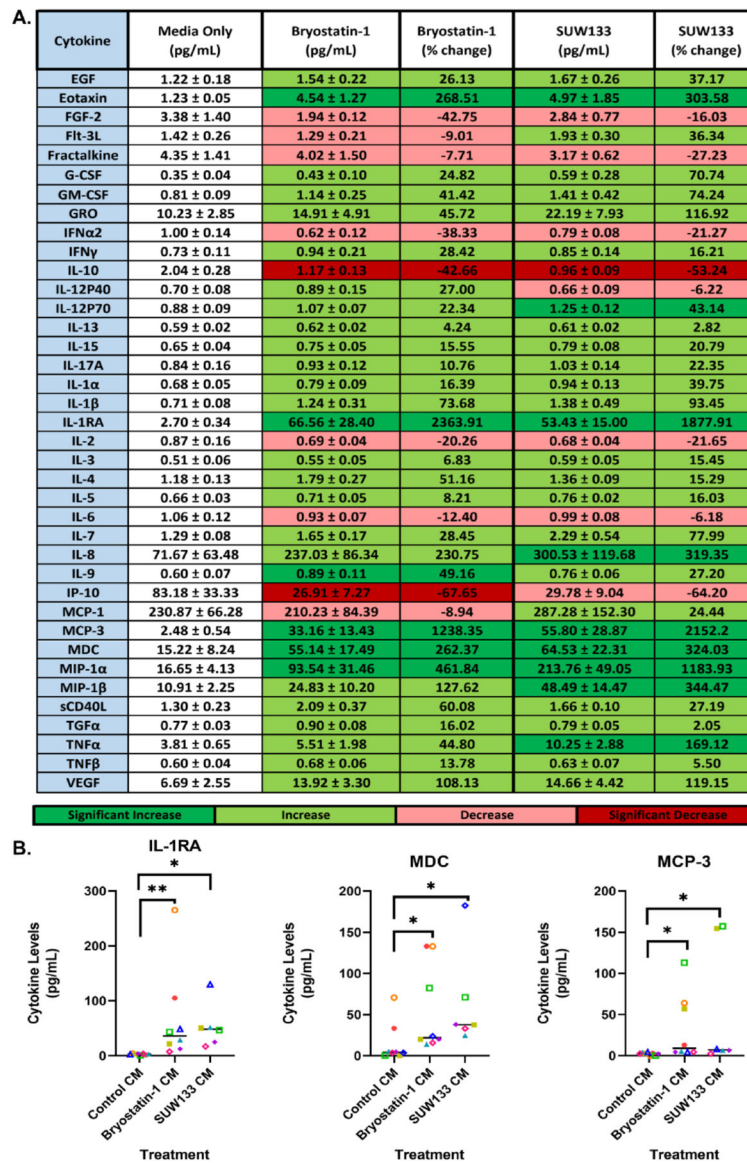
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**Fig. 1. Schematic representation of study.**

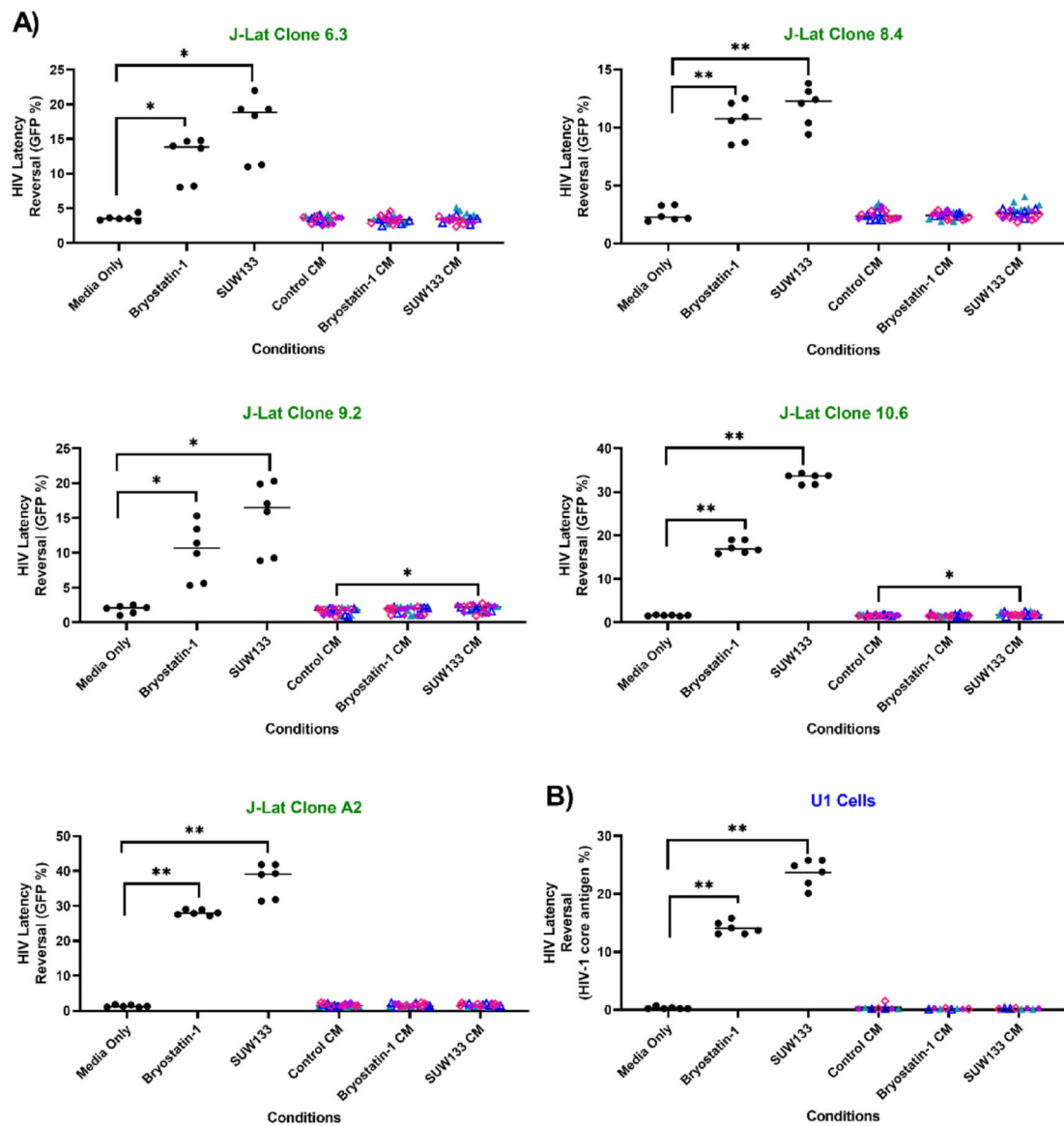
Primary human PBMC were isolated from 8 healthy human donors and combined with a latency reversing agent (LRA) in culture. Cells were washed after 24 h and incubated for a further 24 h then the supernatant was filtered and analyzed for cytokine composition (Fig. 2) and capacity to induce HIV latency reversal (Fig. 3).



**Fig. 2. Cytokines induced in primary human PBMC by PKC modulators.**

Conditioned media (CM) generated with peripheral blood mononuclear cells (PBMC) from 8 (control and bryostatin-1) or 6 (SUW133) different healthy human donors were analyzed using a human cytokine 38-plex Luminex immunoassay. **A)** A heat map showing results of all 38 human cytokines is provided and the mean concentration values in pg/mL along with the standard error of the mean (SEM). The mean percentage change relative to media only control are also shown. Dark green indicates a significant increase ( $P < 0.05$ ); light green indicating a non-significant increase; light red indicating a non-significant decrease; and dark red indicating a significant decrease (Mann-Whitney  $U$  test,  $p < 0.05$ ). **B)** Example cytokine profiles from data shown in part A, with each color and shape representing results using PBMC from a different human donor.





**Fig. 3. HIV Latency Reversal in select J-Lat clones stimulated with conditioned media (CM).**  
**A)** Various Jurkat-Latency (J-Lat) clones were treated with direct LRA or conditioned media (CM) for 48 h. All control conditions (media only and direct stimulations) were done in technical duplicates, in three independent biological replicates, resulting in an  $n = 6$ ; For conditioned media samples, technical duplicates in three independent biological replicates per donor (4 donors each) resulted in  $n = 24$ . **B)** Promonocytic U1 cells were treated with direct LRA or CM for 48 h. All control conditions were done in technical triplicate, in two independent biological replicates, resulting in an  $n = 6$ ; For conditioned media samples, technical duplicates in two independent biological replicates per donor (4 donors each) resulted in  $n = 8$ . A two-tailed, unpaired, unequal variance Student's  $t$ -Test was performed, with (\*) indicating  $p < 0.05$ ; and (\*\*) indicating  $p < 0.001$ . Each color and shape correspond to PBMC from a different human donor.