THE LATENT RESERVOIR IN ELITE CONTROLLERS AND FACTORS THAT AFFECT THE SUPPRESSIVE CAPACITIES OF IMMUNE EFFECTOR CELLS IN HIV POSITIVE INDIVIDUALS

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ABSTRACT

Our inability to efficiently target and eliminate the latent reservoir remains the biggest barrier to a cure to HIV/AIDS. Accordingly, there exist several ongoing efforts to not only understand the reservoir in varied patient groups, but also to determine an efficient means to achieve either a functional or a sterilizing cure. To this end, several trials are underway with the shock-and-kill strategy remaining the most clinically advanced. Shock-and-kill employs the use of small pharmacological molecules termed latency reversing agents (LRAs) to reverse latency with two goals in mind. First, to transcriptionally activate latent reservoir cells so that their subsequent production of viral proteins renders them visible to the immune system for targeted elimination and second to achieve this in the presence of antiretroviral therapy (ART) so as to prevent new rounds of infection.

Despite the allure of the shock-and-kill strategy, there has been no evidence to date of an ability of this strategy to reduce the size of the latent reservoir. One reason that shock-and-kill trials continue to fail to show significant reductions in the latent reservoir may be the inability of poorly functioning chronic progressor (CP- control HIV with ART) immune effector cells to effectively target and eliminate reactivated reservoir cells. While this may arise from pre-existing immune exhaustion, it is possible that the LRAs employed may themselves contribute to immune impairment. Consequently, enhancing the antiviral activity of effector cells might improve the effectiveness of shock-and-kill trials and may also enhance other therapeutic strategies aimed at eliminating the latent reservoir. Outside of shock-and-kill strategies however, other avenues exist to attain a functional cure of HIV/AIDS. In particular, given that elite suppressors (ES- control HIV without ART) control viral replication without therapy, they represent a model of a functional cure. Accordingly, coupled with a continued understanding of the immune factors that mediate

elite control, characterizing the frequency of latently infected CD4+ T cells in ES may shed light on the reservoir size needed to attain a functional cure, with or without shock-and-kill strategies.

We address each of these questions in this thesis write-up. Specifically, in chapter one, we assess the size of the inducible reservoir in ES to better understand what the latent reservoir in a functional cure might look like. Among other findings, our data demonstrates that despite the fact that most ES have markedly small reservoirs compared to CP, a small reservoir size may not be a requirement for attaining a functional cure. In chapter two, we tackle the shock-and-kill strategy to better understand if the LRA ingenol-B has any adverse effects on the HIV suppressive immune function of ES CD8+ T cells. We observe that while ingenol-B on its own has no adverse effects on ES CD8+ T cell HIV suppressive capacity, the combination of ingenol with another LRA, JQ1, inhibits the HIV specific suppressive capacity of CD8+ T cells in a dose dependent manner. Finally, in our effort to enhance the antiviral capacity of CP effector cells for the control of HIV infection, we explore the potential of using short course treatments of the type-1 interferon, IFN-α, to enhance NK cell immune function in ES and CP without inhibiting similar responses in patient CD8+ T cells. Our findings demonstrate that short course treatments of IFN-α not only enhance the HIV suppressive capacities of progressor and non-progressor NK cells, but achieve this while also enhancing the antiviral capacity of patient CD8+ T cells. Overall, our data have significant implications for the cure agenda.

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1 INTRODUCTION

1.1 A BRIEF INTRODUCTION TO HIV/AIDS

Human Immunodeficiency Virus (HIV) is the causative agent for the Acquired Immunodeficiency Syndrome (AIDS). There are two types: HIV-1 and HIV-2 (GAC, 2016). Although HIV-1 is clinically similar to HIV-2, the two viruses are genetically distinct with HIV-2 being predominant in West Africa and exhibiting a slower disease pathogenesis and progression to AIDS than HIV-1 (Visseaux et al, 2016). Despite these differences, both viruses infect target cells expressing the CD4 receptor (required for entry). These include, mainly, CD4+ T cells, as well as macrophages, dendritic cells and astrocytes (GAC, 2016). Furthermore, depending on the tropism of the virus, the co-receptors CCR5 (the natural receptor for several chemokines including MIP-1 beta and RANTES) or CXCR4 (the natural receptor for the chemokine SDF-1) are also utilized. Specifically, the virus is termed R5 tropic when the CCR5 receptor is used, and X4 tropic when CXCR4 is used (GAC, 2016). Upon initial infection with either virus type, the immune response attempts to control and suppress viral replication. However, for numerous reasons touched upon in subsequent paragraphs, this control is not complete and over time, the chronic presence of the infection leads to death of the (main) target cells. This death is mediated by both apoptotic viral cytopathic effects and the pyropoptosis of non-productively infected cells (Doitsh et al, 2014). Ultimately, large scale death, with disproportionate rates of replacement of target CD4+ T cells, leads to unhealthy reductions with less than < 200 cells/ul of blood leading to the acquired immune deficiency syndrome termed AIDS (Figure 1).

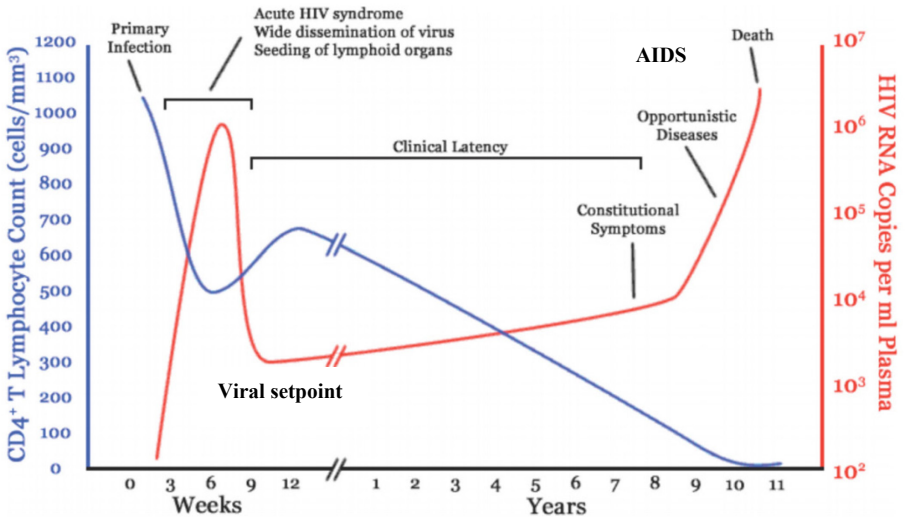

Figure 1. Viral load and CD4+ T cell counts from infection to AIDS. At primary infection, HIV viral loads rapidly rise within the first 3 weeks. In subsequent weeks, this replication is quickly controlled by antiviral immune responses leading to a drop in viremia to set point. Over time, and in the absence of ART, viral replication steadily continues, gradually depleting CD4+ T cells. While variable from patient to patient, ultimately the loss of CD4+ T cell allows susceptibility to opportunistic infections which, in the absence of a strong immune response leads to AIDS and if still untreated, death. Adapted from Tirado-Ramos et al, 2010.

It is worth noting that while all of this thesis work is focused on HIV-1 (clade and tropisms used will be clarified in subsequent methodologies), it is our understanding of both types of HIV that has fueled our progress in the HIV/AIDS therapeutics arena. In this regard, the most critical advancement has been the generation of several kinds of effective antiretroviral therapies (ART). Existing in 7 classes with several new drugs in the pipeline (Gravatt et al, 2017), combinatorial ART is capable of reducing viral loads to below limits of detection by standard assays (less than 20 copies of HIV mRNA per ml of plasma). However, because ART bars replication, but does not eliminate viral particles, it must be taken daily, for life. While this might come with challenges including adherence and (potentially) undesirable side effects, for most individuals (termed chronic progressors, CP), there are no other alternatives. In addition to CP, however, other groups of HIV infected individuals exist with varying degrees of ability to control viral replication. One

group, termed long term non-progressors (LTNP) is able to control HIV-1 infections without therapy and maintain CD4+ T cell counts above 500 cells/ul of blood for about 5-7 years before ultimately losing control (Grabar et al, 2017, Okulicz and Olivier, 2011). A different group of patients, called elite controllers or suppressors (ES), spontaneously control viral replication to below the limits of detection of clinical assays for extended periods of time without therapy or clinical signs of disease progression (Blankson, 2010).

1.2 HIV ELITE CONTROLLERS/SUPPRESSORS

As might be expected, ES are a very rare group of HIV infected individuals. Representing only about 1% of all HIV infected subjects (Okulicz and Olivier, 2011), their unique ability for spontaneous control without therapy has inspired many studies, conducted to understand how they achieve this and if this control can be recapitulated. To date, we have no single explanation for the phenomenon of elite suppression. While some studies have demonstrated that what might have been considered elite control was actually infection with defective viruses (Calugi et al, 2006, Yamada and Iwamoto, 2000, Deacon et al, 1995), many other studies have demonstrated infection with replication competent viruses (Salgado et al, 2014, Buckheit III et al, 2012, Blankson et al, 2007) and even transmission amongst discordant couples where a CP passes on their virus to a partner who then becomes an ES (Buckheit III et al, 2012, Bailey et al, 2008). To date, elite suppression has been associated with several factors, including immunogenetics such as having HLA-B*57 and HLA-B*27 (Martin and Carrington, 2014), cellular CD8+ T cell adaptive immune responses (Altfeld et al, 2006, Betts et al, 2006, Migueles et al, 2000), and innate immune responses (Walker-Sperling, 2017, Song et al, 2014, Soumelis et al, 2001).

Despite these unique characteristics, it is important to note that while viral replication is undetectable by standard assays in ES, it is not absent. Data have shown that there is in fact an evolution of plasma viremia amongst these patients- a phenomenon that cannot occur in the absence of active replication (O'Connell, 2010). As such, ES do not represent a sterilizing cure where virus is completely eliminated, but rather a functional one, where the virus is persistent, but does not cause disease, as in most cases of varicella zoster virus (VZV) or herpes simplex virus (HSV) infections. In the examples of VZV and HSV, the infection is considered 'functionally' cured because in the absence of any medication, a quiescent latent state of infection allows the virus to persist in its host without (for the most part) causing disease. Nevertheless, with the reactivation of HSV or VZV, cold sores or shingles, respectively, is possible (Kennedy et al, 2015).

HIV, like the afore mentioned examples, also establishes latency (Siliciano and Greene, 2011). A stochastic event, it is thought to occur when the infected CD4+ T cell transitions into a resting memory state with a virus stably integrated into its genome as a provirus (Murray 2016). Many resting memory cells also harbor defective viruses. As such, it is the inducible reservoir, harboring intact proviruses capable of yielding productive infections with latency reversal and virus reactivation (Figure 2) that serves as the barrier to an HIV cure (Bruner et al, 2019, Siliciano and Greene, 2011, Chun et al 1997). Usually, in the absence of CD4+ T cell activation, latent reservoir proviruses are mostly quiescent. When the reservoir cells are activated however, trace levels of viremia occur. This low level viremia does not lead to ongoing viral replication due to the presence of ART (Siliciano and Greene, 2011, Bailey et al, 2006). In the absence of ART however, for instance in the event of treatment interruption, the same residual viremia is capable of establishing new rounds of infection and, by extension, expanding the reservoir. This occurs irrespective of how early ART is initiated in humans (Heinrich, 2017) and animal models

(Pinkevych et al, 2019). In addition to ART interruption, the fact that these memory CD4+ T cell latent reservoirs can be expanded clonally, either by homeostatic proliferation (Hosmane et al, 2017) or possibly antigen stimulation (Mendoza et al, 2020, Wang et al, 2018, Simonetti et al, 2015), and that myeloid derived macrophages might also contribute as a latent reservoir of their own (Abreu et al, 2019, Wong et al, 2019), only serves to further complicate the challenge of potentially eliminating this reservoir.

If we can understand and eliminate latent HIV, then we could potentially cure HIV/AIDS. Barring this, the next best option is to understand how to control and/reduce the size of the reservoir, and to achieve this to the point where any viral rebound can be controlled by the immune system so that the virus does not cause disease in the absence therapy. If this can be achieved (particularly in CP), then a functional cure would have been achieved. Yet, this is actually quite a challenge because data suggest that in people destined to be progressors, no matter how early therapy is initiated and how small the initial reservoir is, disease inducing rebound is inevitable (Pinkevych et al, 2019, Heinrich, 2017, Persaud, 2013). Despite these data, the impetus for trying rests in two facts. First, mathematical modeling from existing case reports suggests that greater than 3.5 log reduction in viral reservoir size might be needed for an ART free remission with fewer logs extending, but not eliminating, the time to rebound (Hill et al, 2016). The second (and more likely impetus given a larger sample size than the afore described

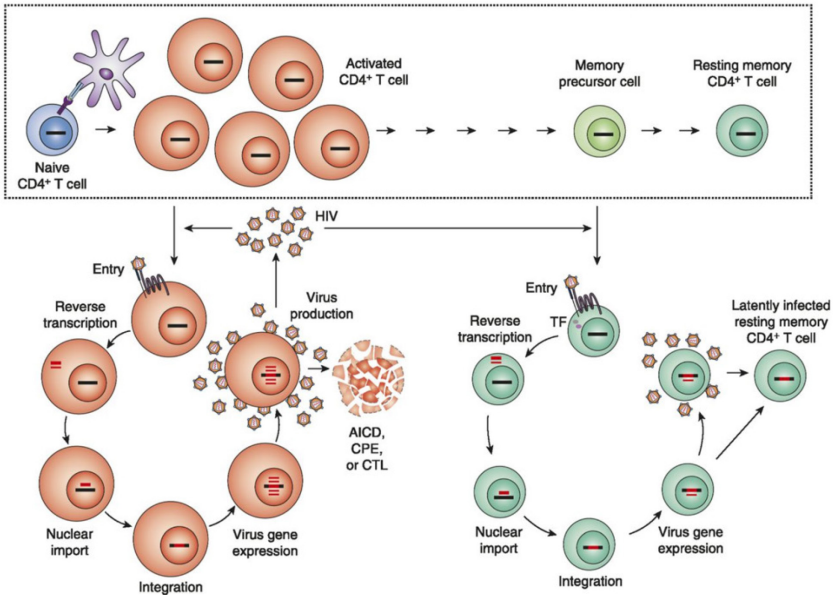

Figure 2. The establishment of latent reservoirs in resting CD4+ T cells. After antigen presentation, most activated CD4+ T cells die with a few remaining as memory sentinels (top boxed pane). In the event of HIV infection and HIV latency establishment, the activated, infected, CD4+ T cells also die (bottom left brown panel of cells). Some activated CD4+ T cells transitioning to a state of rest may be infected with the virus and transition with the provirus stably integrated into its genome (right hand green panel of cells). If this latent infection is with a provirus capable of being induced and producing new, replication competent virions, then this latently infected cell is considered part of a reservoir. These comprise a very small percentage of latently infected cells; however. For the most part, latently infected cells actually contain defective proviruses, which are of less concern for the cure agenda. Image from Murray et al, 2016.

case reports) is that ES seem to have smaller inducible reservoirs and maintain control without therapy. In this scenario however, it is unclear if this is cause or consequence: is a smaller reservoir really the cause of sustained elite suppression? Or is an overall strong immune response against infection the cause of a smaller reservoir and simultaneously elite control?

To better understand this from the perspective of the second impetus, it is imperative that we first appreciate the true size of the reservoir in model ES subjects. While there are currently no markers to correctly identify and delineate the reservoir, there is an assay that efficiently helps to

accurately quantify it. Recently identified, the intact proviral DNA assay (IPDA) uses unique primers and HIV proviral DNA from patient cells in a digital droplet PCR assay that distinguishes, intact proviruses from defective ones (Bruner et al, 2019). Using this assay, we quantified and confirmed the size and frequency of intact proviruses in ES in order to contribute to the growing data towards a functional cure. The associated findings are discussed in chapter one of this thesis.

1.3 THE SHOCK-AND-KILL AGENDA

As previously discussed, the majority of the inducible latent reservoir resides in resting CD4+ T cells (Siliciano and Greene, 2011). This quiescent state renders reservoir cells inaccessible to immune mediated elimination. One way to get around this challenge, therefore, is to reverse the latent state of reservoir cells. This would, theoretically, permit viral replication with subsequent inhibition of new rounds of infection by ART while reactivated cells are eliminated by the immune system.

The shock-and-kill agenda is geared towards attempting to achieve this feat. The most clinically explored cure strategy to date, it focuses on first reversing latency using small pharmacological molecules termed latency reversing agents (LRAs). This is termed a 'shock' and must be achieved without global T- cell activation (Prins et al, 1999). As shown in Figure 3, the activation of these cells should yield two results. First, the production of viral particles which are precluded from infecting new cells by ART and second, the production of viral proteins which mediate elimination of the host cell by both viral cytopathic effects and/immune effector cell cytotoxicity (Deeks, 2012). Thus far, several classes of LRAs, including histone deacetylase inhibitors, bromodomain inhibitors and PKC agonists, have been identified (Spivak and Planelles, 2017). Furthermore, existing *in vitro* data has demonstrated that while all of these are indeed

capable of reversing latency, specific LRA combinations tend to yield higher levels of latency reversal than single drugs alone (Laird et al, 2015).

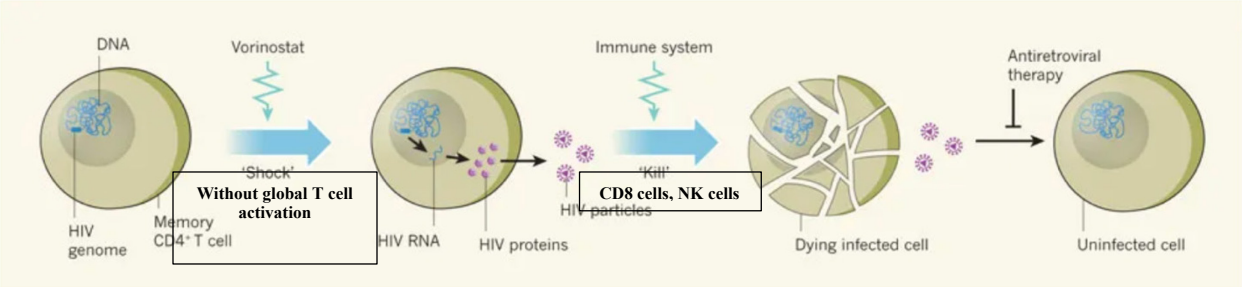

Figure 3. Proposed flow of events with shock-and-kill. Infected target cells contain intact provirus, which can be induced to produce viral proteins via a shock with an LRA. The production of viral proteins mediates the kill by immune sentinel cells while ART simultaneously blocks de novo infections. Adapted from Deeks et al, 2012.

Despite its conceptual appeal, there are at least three main challenges associated with the pursuit of shock-and-kill as an HIV cure strategy. First is the fact that there are many anatomical sites for the latent reservoir (Wong and Yukl, 2017), and when a site is privileged, like the brain, it may only permit, to varied degrees, the penetration of a therapeutic agent (Letendre et al, 2008) like an LRA or effector cells like sentinel CD8+ T cells. Second is that no properly controlled clinical trial with an LRA has, to date, demonstrated success in the main goal of shock-and-kill, mediating reductions in the size of the latent reservoir (measured as changes in total cell associated DNA; Pace and Frater, 2018). Since blips of viremia suggest that a 'shock' is usually achieved, it is possible that the reactivated reservoir cells may either be resistant to killing (Huang et al, 2018), or fail to be eliminated after 'kill', thus warranting the need for enhancement of the immune system for control (Montaner and Riley, 2017) or are killed but belong to a much larger than previously estimated pool of inducible reservoir cells (Ho et al, 2013). Irrespective of the prevailing challenge,

more work is required in the 'kill' aspect of the agenda. Finally, and perhaps related to point two, is that most LRAs are actually drugs employed in the field of oncology with *in vitro* data suggesting that they could have some adverse effects on effector cells needed to mediate effective killing after latency has been reversed (Clutton and Jones, 2018, Walker-Sperling et al, 2016, Garrido et al, 2016). Although some data suggest this may not be the case- Pace and Frater, 2018, Sogaard et al, 2015, if this is true *in vivo*, it might present one explanation for why *in vivo* attempts at shock-and-kill have failed to reduce the size of the reservoir in candidate patients. In chapter two of this thesis, we will discuss our exploration of this third challenge with studies assessing the effect of some LRAs individually and in combinations on the activity of ES CD8+ T cell mediated immune suppression of HIV.

1.4 ENHANCING IMMUNE CELL FUNCTION FOR THE CONTROL OF HIV: FOCUS ON NK CELLS

Thus far, we have established that HIV/AIDS cannot be cured without the elimination of the latent reservoir. We also appreciate that if reservoir eliminating strategies like shock-and-kill are to work, they will depend on efficiently functioning immune responses capable of eliminating target reservoir cells after reactivation. Unfortunately, in most HIV infected individuals, such robust immune responses are absent, thus the need for ART. On one hand, this is caused by poorly targeted/functional HIV specific CD8+ T cell responses (Eller et al, 2016, Betts et al, 2006), a challenge more evident in non-controllers than in controllers (Pereyra et al, 2014). However, immune activation and subsequent exhaustion might also play an important role (Sokoya et al, 2017, Klatt et al, 2014).

As a chronic infection, the presence of sustained, uncontrolled viral replication leads to sustained levels of inflammatory cytokines, particularly interferon stimulated genes mediated by type-I interferons. These in turn result in dysregulated immune activation and subsequent immune exhaustion that renders effector cells less functional at controlling viral replication (Zhen et al, 2016, Cheng et al, 2016). Accordingly, immune activation has been determined to be the more accurate measure of disease progression than CD4+ T cell decline (Utay and Hunt, 2016). Since ART effectively inhibits viral replication, its initiation is associated with concomitant *in vivo* reductions in inflammatory cytokines and, by extension, immune activation, but never to the levels of uninfected individuals (Utay and Hunt, 2016). Indeed, unsuccessful shock-and-kill as well as vaccine trials suggest that at least one contributing factor to these failing strategies might be persistently dysfunctional immune effector cells despite continuous ART. Accordingly, efforts exist to strengthen immune responses for improved functional cure efforts, with a focus on CD8+ T cells (Perreau et al, 2017, Riley and Montaner, 2017). Of course, this focus is not unwarranted, since CD8+ T cells play an important role in the control of HIV replication. However, given their evident limitations, including mutations in viruses that lead to escape from targeted epitopes as well as down-regulation of MHC-I by viral nef proteins permitting CD8+ T cell immune evasion (Garcia et al, 2016, Wonderlich et al, 2011, Jones et al, 2004), it is imperative that the focus on immune effector potentiation amidst cure efforts be focused not only on CD8+ T cells, but also on other cells capable of complementing CD8+ T cell responses in HIV replication control. An aim of this thesis work, and the focus of chapter three, is the bid to achieve this goal with a focus on Natural Killer (NK) cells, whose potential value for control of HIV infections is well documented (Figure 4, Scully and Alter, 2016). Specifically, we demonstrate how short course treatments of

NK cells from various individuals with the protein IFN-alpha enhances multiple immune functions, while preserving, or strengthening the existing CD8+ T cell responses of study subjects.

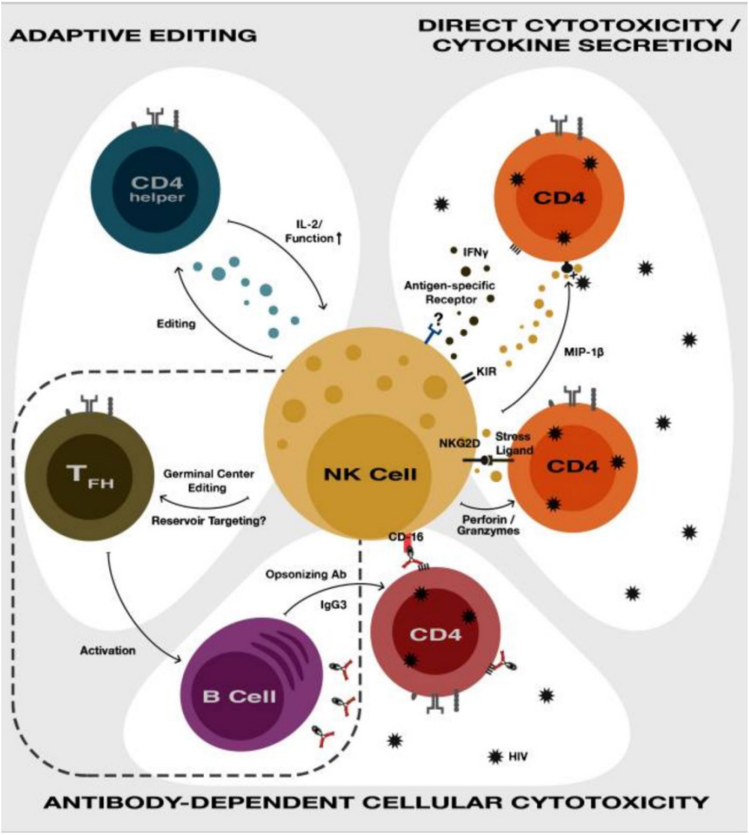

Figure 4. Varied roles of NK cells in HIV control. Different avenues by which NK cells can help mediate HIV control, including direct cytolysis, antibody dependent cytotoxicity, cytokine production, amongst others. Adapted from Scully and Alter, 2016.

2 CHAPTER 1

ELITE SUPPRESSORS HAVE LOW FREQUENCIES OF INTACT HIV-1 PROVIRAL DNA

2.1 ABSTRACT

Elite controllers or suppressors control viral replication without antiretroviral therapy. We used the intact proviral DNA assay to approximate the size of the inducible latent reservoir in elite suppressors and found that while the median frequency of both total and intact proviral DNA was markedly lower than the frequencies seen in chronic progressors on antiretroviral therapy, there was no significant difference in the ratio of intact to total proviral DNA between elite suppressors and chronic progressors.

2.2 INTRODUCTION

A cure for HIV can be achieved through complete eradication of the virus or by a functional cure where viral replication is controlled in the absence of antiretroviral therapy (ART) (Siliciano and Siliciano, 2016). Elite suppressors who maintain viral loads below the limit of detection of clinical assays are a model of a functional cure. While some studies suggest that infection with attenuated virus can lead to elite control and/or long-term nonprogression (Zaunders et al, 2011), we and others have shown that a subset of elite suppressors are infected with fully replication-competent virus (Blankson et al, 2007, Lamine et al, 2007, Bailey et al, 2008, Julg et al, 2010, Buckheit et al, 2012, Salgado et al, 2014). However, these individuals clearly have smaller inducible reservoirs compared with chronic progressors on ART as measured by the quantitative

viral outgrowth assay (QVOA). Furthermore, the QVOA has been shown to underestimate the size of the reservoir in chronic progressors (Ho et al, 2013, Bruner et al, 2016), but it is not known whether the same finding holds true in elite suppressors. A study based on more than 400 near full-length sequences of provirus from 28 chronic progressors on ART demonstrated that only 2.4% of proviruses in chronic progressors did not contain large deletions or hypermutations (Bruner et al, 2016). The intact proviral DNA assay (IPDA) is a recently developed digital droplet PCR assay that uses primers developed from near full-length proviral sequences to distinguish intact proviral DNA from most deleted and/or hypermutated proviral DNA (Bruner et al, 2019). In this study, we used the IPDA to approximate the frequency of cells with total and intact proviral DNA in a cohort of nine elite suppressors and 10 chronic progressors.

2.3 METHODS

2.3.1 Quantifying patient total and intact HIV proviral DNA

CD4+ T cells from 10 CP and 9 ES were flash frozen and sent to Accelevir Diagnostics for processing. On average, a total of $1.05e^6$ cells (median of $9.78e^5$) were delivered for processing via IPDA assay. Using a proprietary, company specific established protocol, the total DNA present in cells was quantified and from this the frequency of both total HIV DNA, as well as intact HIV DNA was quantified using unique primers in a digital droplet PCR that distinguishes intact proviruses from defective ones (Bruner et al, 2019). Specifically, the IPDA rests on a premise determined from near full-length HIV genome sequencing. Data from these show that together, amplicons from the packaging signal and rev response element (RRE) regions of the viral genome can identify more than 90% of defective viruses. As such the primers of this assay (optimized for HIV subtype B) are designed to recognize intact/wild type packaging signal and RRE. Both the

packaging signal and RRE are considered conserved regions with high chances of either small deletions or hypermutations, respectively. Consequently, the complete amplification of both (detected in specific channels by fluorescent probes) suggests the genome interrogated was intact, and the failure of one or the other or both to correctly amplify suggests some defect in the interrogated viral genome (Bruner et al, 2019). For our study, repeat samples with higher cell numbers were delivered where possible for re-testing in the event where no intact proviruses were obtained from a patient. When these repeat attempts yielded similar results, we used the Siliciano IUPMStats calculator to estimate what the highest possible frequency of intact proviral DNA might have been (Rosenbloom et al, 2015). When the intact HIV DNA was imputed in this manner, the imputed data was not employed in calculating the ratio of intact to total proviruses.

2.3.2 Statistics

All graphs and associated statistics were performed in Microsoft Excel and GraphPad Prism version 7.

2.4 RESULTS

2.4.1 Chronic progressors have significantly higher frequency of total and intact HIV DNA relative to elite suppressors

All chronic progressors studied in this project had CD4+ T cell counts of greater than 500 cells/ul (median of 758) and had undetectable viral loads on ART for at least 1 year (median of 5.5 years). The frequency of total proviral DNA was significantly higher in the chronic progressors (median of 602.52 copies per million CD4+ T cells) than the elite suppressors (median of 30.4 copies per million CD4+ T cells, $P = 0.0005$, Figure 5a). As shown in Figure 5b, the frequency of intact proviral DNA was also significantly higher in chronic progressors (median of 36.95 copies

per million CD4+ T cells) compared with elite suppressors (median of 1.61 copies per million CD4+ T cells, $P = 0.0159$).

2.4.2 There is no significant difference in the proportion of intact to total HIV DNA between elite suppressors and chronic progressors

After determining the individual frequencies of total and intact HIV DNA in both groups of patients, we next asked whether the proportion of total proviral DNA that was intact was different between the same two patient groups (Figure 5c). Interestingly, we found that, there was no significant difference in the ratio of intact to total proviral DNA in chronic progressors versus elite suppressors. While this was amongst independent patients, we also wanted to determine if this observation was true amongst transmission couples specifically discordant ones. We have previously described the transmission of virus from chronic progressor 10 to elite suppressor 9 (Bailey et al, 2008). The transmission occurred 20 years ago and elite suppressor 9 has maintained elite control since then while chronic progressor 10 has been on ART. The frequency of total proviral (Figure 5d) and intact proviral DNA (Figure 5e) was much higher in chronic progressor 10 than in elite suppressor 9, but the ratio of intact to total proviral DNA was similar in the two subjects (Figure 5f).

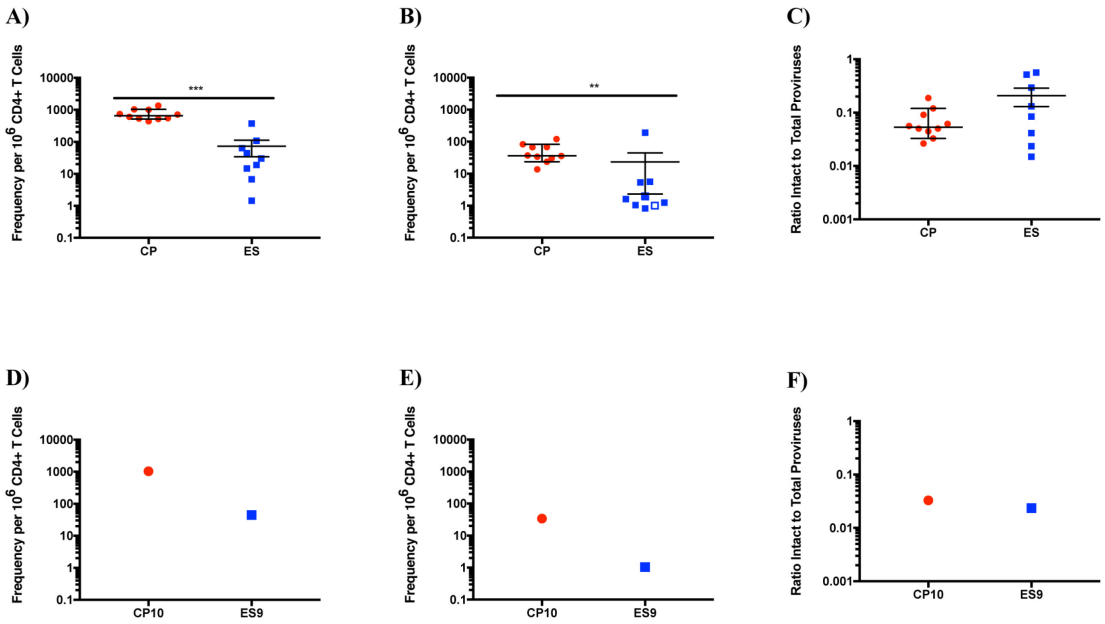

Figure 5. Total and intact provirus quantification. Frequency of total (a) and intact (b) proviruses were quantified, in 10 chronic progressors (red circles) and nine elite suppressors (blue squares) using the intact proviral DNA assay. The open symbol denotes a value that was below the limit of detection of the assay and represents the highest possible frequency of intact proviral DNA. The ratio of intact to total DNA is shown for chronic progressors and elite suppressors in (c). Bars represent the median values with 95% confidence intervals. The frequency of total (d) and intact (e) proviruses were quantified in the chronic progressor 10/elite suppressor 9 transmission pair. The ratio of intact to total DNA is shown in (f). The nonparametric, Kruskal–Wallis test was performed along with a Dunn's multiple comparisons test to compare elite suppressors and chronic progressors values.

Table 1: Clinical characteristics and reservoir measurements in ES in our study

	Gender	Years HIV positive	Years on ART	CD4 count	Protective HLA allele	Total proviral DNA per million CD4+ T cells	Intact proviral DNA per million CD4+ T cells	IUPM	Cells cultured (million)
ES3	F	26	NA	1014	HLA-B*57	6.78	< 1.00	<0.04	18
ES5	F	27	NA	918	HLA-B*57	108.1	1.61	<0.03	25
ES6	F	25	NA	916	HLA-B*57	30.41	1.25	< 0.02	29
ES9	F	21	NA	680	HLA-B*27 HLA-B*57	44.34	1.04	ND	ND
ES18	F	7	NA	970	None	19.00	5.58	ND	ND
ES24	M	8	NA	1269	HLA-B*57	372.57	191.31	4.57	5
ES31	F	11	NA	956	HLA-B*27 HLA-B*57	63.14	5.33	ND	ND
ES46	M	13	NA	667	HLA-B*57	9.85	1.95	<0.06	12
ES51	M	13	NA	1015	HLA-B*57	1.45	0.82	<0.03	20

NA: Not applicable

ND: Not done

2.5 DISCUSSION

We compared the levels of intact proviral DNA to the frequency of cells with replication-competent virus as measured by the QVOA from a separate but recent study from our group. Elite suppressor 24, who has the highest level of intact proviral DNA in our cohort (191 copies per million CD4+ T cells), also has a high frequency of cells positive for replication competent virus [4.57 infectious units per million (IUPM)] (Veenhuis et al, 2018). In contrast, five elite suppressors who had less than two copies of intact proviral DNA per million CD4+ T cells had IUPM values of less than 0.06 (Table 1), implying that low frequencies of intact proviral DNA were associated with low frequency of cells from which replication-competent virus can be cultured.

Prior studies have shown that elite suppressors have lower frequencies of total and integrated DNA (Lambotte et al, 2005, Hatano et al, 2009, Graf et al, 2011, Mendoza et al, 2012, Hatano et al, 2013, Canoui et al, 2017, Garcia et al, 2017, Avettand-Fenoel et al, 2019), lower levels of baseline and inducible cell associated RNA (Noel et al, 2016, Pohlmyer et al, 2017), and lower frequencies of cells positive for replication-competent virus (Blankson et al, 2007, Julg et al, 2010, Salgado et al, 2014, Mendoza et al, 2012, Noel et al, 2016, Chun et al, 2013). These different assays measure different aspects of the viral reservoir, and while there have been studies comparing these assays in chronic progressors (Bruner et al, 2019, Eriksson et al, 2013), this is the first time measurements of intact proviral DNA have been compared with measurements of the inducible replication-competent viral reservoir in elite suppressors. This is important as elite suppressors may represent a model of a functional cure and their small reservoir size may approximate what can be achieved with different curative interventions. We show here that while elite suppressors have much lower total and intact DNA levels than chronic progressors, the ratio of intact to total DNA is similar to the ratios present in chronic progressors. This would imply that elite suppressors do not have a higher proportion of defective viral DNA than chronic progressors. It remains unclear whether the small reservoir size is a cause or consequence of elite control, but lower peak viral loads and earlier control of viral replication has been reported in some elite suppressors (Goujard et al, 2009). It is possible that this early control of viral replication limits the seeding of the reservoir as has been described in individuals who are treated with ART during primary infection (Robb and Ananworanich, 2016). However, elite suppressor 24, had levels of intact DNA that were higher than the levels seen in our chronic progressors. This would suggest that a small reservoir is not an absolute requirement for elite control.

Our results have implications for HIV cure strategies as they further elucidate the viral reservoir in patients who may represent a model of a functional cure of HIV1 infection.

3 CHAPTER 2

THE EFFECT OF INGENOL-B ON THE SUPPRESSIVE CAPACITY OF ELITE SUPPERSSOR HIV-SPECIFIC CD8+ T CELLS

3.1 ABSTRACT

Some latency-reversing agents (LRAs) inhibit HIV-specific CD8+ T cell responses. In a prior study of protein kinase C (PKC) agonists, we found that bryostatin-1 inhibited elite controller/suppressor (ES) CD8+ T cell suppressive activity whereas prostratin had no effect. Ingenol-B is another PKC agonist with potent LRA activity both by itself and in combination with the bromodomain inhibitor JQ1; however, its effect on CD8+ T cell mediated control of HIV-1 replication is unknown. Accordingly, CD8+ T cells were isolated from ES and treated with bryostatin-1, prostratin, ingenol-B, and JQ1 as well as a combination of each PKC-agonist with JQ1. The cells were then tested in the viral suppression assay. Furthermore, to assess possible mechanisms of inhibition, CD8+ T cells were treated with the LRAs and analyzed for the expression of various immune cell markers. Our findings show that Ingenol-B had no effect on the ability of ES CD8+ T cells to suppress viral replication, however, the combination of ingenol-B and JQ1 caused a modest, but significant decrease in this suppressive capacity. The mechanism of the inhibitory effect of the JQ1 and ingenol-B combination relative to ingenol-B alone was unclear but the effect appeared to be dose dependent. In summary, ingenol-B does not inhibit HIV-specific CD8+ T cell responses in vitro. These responses are however modestly inhibited when 100nM ingenol-B is combined with JQ1. Since HIV-specific CD8+ T cell activity may be essential for the eradication of reactivated latently infected cells, the potency of latency-reversal activity of drug

combinations must be balanced against the effects of the combinations on HIV-specific CD8+ T cell responses.

3.2 INTRODUCTION

Shock-and-kill strategies have been proposed as a possible mechanism for HIV-1 eradication (Siliciano and Siliciano, 2013, Archin and Margolis, 2014). The strategies involve the use of latency-reversing agents (LRAs) such as Histone Deacetylase (HDAC) inhibitors and PKC-agonists to "shock" latently infected CD4+ T cells and myeloid cells into producing viral proteins that could then be recognized by effector cells leading to the "kill" component of the strategy. Several studies have shown that LRAs do in fact lead to an increase in HIV transcription and/or blips of viremia in clinical trials; however, this has not been accompanied by a decrease in the size of the latent reservoir (Archin et al, 2012, Elliot et al, 2014, Rasmussen et al, 2014, Sogaard et al, 2015). One possible reason for this disconnect is that the number of latently infected cells that were eradicated in these studies represent a very small proportion of the total latent reservoir. Another potential explanation is that the LRAs inhibit the responses of HIV-specific effector cells thereby leading to 'shocking' without 'killing'.

Recent studies have indeed shown that different classes of LRAs inhibit the responses of natural killer (NK) cells (Pace et al, 2016, Garrido et al, 2016), T cells (Akimova et al, 2012), and HIV-specific CD8+ T cells (Jones et al, 2014, Walker-Sperling et al, 2016, Clutton et al, 2016) and others may affect the susceptibility of CD4+ T cells to HIV-1 infection (Lucera et al, 2014). We previously showed that the PKC-agonist bryostatin-1 inhibited the suppressive capacity of primary CD8+ T cells from elite controllers/suppressors (ES) whereas the PKC-agonist prostratin had no effect on these cells (Walker-Sperling et al, 2016). Furthermore, drug combinations that

were shown to have synergistic effects on latency reversal (Laird et al, 2015) also had different effects on HIV-specific CD8+ T cells (Walker-Sperling et al, 2016). For instance, the combination of bryostatin-1 with the HDAC inhibitor romidepsin induced more inhibition of the suppressive capacity of T cells than either drug alone, whereas the combination of prostratin with the bromodomain inhibitor JQ1 did not significantly impact the said HIV-specific response (Walker-Sperling et al, 2016).

Ingenol-B is a relatively new addition to the gradually increasing list of potential candidates for latency reversal (Darcis et al, 2015, Shang et al, 2015). It is derived via a series of chemical reactions that result in a selective esterification at the carbon 3 position of ingenol obtained from ingenol esters of the Euphorbiacea tirucalli plant (Pandelo Jose et al, 2014). While ingenol itself is thought to be less efficient at enhancing viral replication (Fujiwara et al, 1998), its derivatives, such as ingenol-B and ingenol dibenzoate, are known to demonstrate potent latency reversal when used both alone (Abreu et al, 2014, Spivak et al, 2015) and in combination with JQ1 in vitro (Darcis et al, 2015, Jiang et al, 2015), and also when used with the HDAC inhibitor vorinostat *in vivo* in a recent SIV study (Gama et al, 2017). This synergy of viral reactivation exhibited when ingenol derivatives are combined with other LRAs likely stems from the different, but complimenting, mechanisms through which each drug reactivates viral LTR- with ingenol achieving this via the PKC-NFκB pathway, and JQ1 and vorinostat doing so via p-TEFb recruitment, and HDAC inhibition, respectively.

Given their efficacy at latency reversal both alone and in combination with other drugs such as JQ1, ingenol derivatives appear to be promising candidates for the shock-and-kill cure agenda. However, while much has been done to assess its potency as an LRA, no studies have assessed the effect of the drug on the ability of HIV-specific CD8+ T cells to directly inhibit viral

replication. This question is addressed in this study with a focus on the effect of the ingenol derivative ingenol-B on CD8+ T cells when used either alone or in combination with the bromodomain inhibitor JQ1. We also assessed the effects of other PKC agonists, and their combinations, on CD8+ T cell function, using CD8+ T cells form ES, since the cells of these patients have been shown to have superior, HIV-specific suppressive capacities (O'Connell et al, 2009, Migueles et al, 2002, Betts et al, 2006, Saez-Cirion et al, 2007, Migueles et al, 2008, Hersperger et al, 2010, Buckheit et al, 2013). The results have implications for HIV-1 eradication strategies.

3.3 METHODS

3.3.1 Patients

HIV-1 positive and HIV-1 negative blood samples were obtained from donors with written, informed consent and handled according to a Johns Hopkins University IRB approved protocol. Elite suppressors are patients who have maintained undetectable viral loads without antiretroviral therapy. Chronic progressors (CPs) are patients on antiretroviral therapy who have maintained undetectable viral loads for more than 1 year. Healthy donors (HDs) are HIV negative donors. The clinical characteristics of the ES individuals are summarized in Table 2.

Table 2: Clinical characteristics of patients

	CD4 count	Race/Gender/Age	First positive HIV test	HLA-A	HLA-B
ES3	1149	AA/F/65	1991	25,68	51,57
ES5	617	AA/F/65	1990	23,68	57,58
ES6	601	AA/F/60	1992	23	15,57
ES9	711	AA/F/66	1999	2,30	27,57
ES22	1141	AA/M/55	2009	30,31	15,57
ES24	1452	AA/M/63	2009	24,30	7,57
ES31	1326	AA/F/63	2008	3	27,58
ES48	978	H/F/33	2012	3,23	44,51

3.3.2 The effect of LRAs on elite suppressor CD8+ T cell suppressive capacity

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood via Ficoll Paque PLUS gradient centrifugation (GE Healthcare Life Sciences). CD8 + T cells were isolated from PBMCs of elite suppressors by positive selection with Miltenyi beads. CD4+ T cells were isolated from the CD8+ T cell negative cells by negative selection using Miltenyi beads. CD8 + T cells from elite suppressor patients were treated for six hours with either media, DMSO (negative control, 0.1%), JQ1 (1μM; Sigma Aldrich), ingenol-B (100nM; Amazoˆnia Fitomedicamentos Ltda.), bryostatin-1 at two concentrations (10nM, 1nM), prostratin (0.3μM; Sigma Aldrich), or PMA (positive control, 50ng/mL; Sigma). In some experiments, the PKC-agonists were combined with JQ1 at the same concentrations. All drug concentrations were selected based on either physiological relevance in clinical studies (bryostatin at 1nM) or previous work that demonstrated them to be optimal for latency reversal (Laird et al, 2015, Smith et al, 2011). Bulk CD4 + T cells were spinoculated at 1200 × g for two hours at 37˚C with HIV-1 NL4 − 3 Δ Env − GFP at 200ng/100,000 cells. HIV-1 NL4 − 3 Δ Env − GFP is a replication incompetent lab strain pseudotype virus with env replaced with gfp and whose expression is controlled by the HIV promoter. At the conclusion of the six-hour drug treatments, the CD8 + T cells were washed and

added at a 1:1 effector:target ratio to the spinoculated CD4 + T cells. The cells were co-cultured for three days prior to Flow cytometry (FACS) analysis (Figure 6).

3.3.3 Effects of latency reversing agents on cell death, proliferation, exhaustion, and activation immune markers

PBMCs were isolated from HDs or CPs, seeded at $1x10^6$ cells /well in a 48 well plate and treated for 6 hours at 37˚C with the individual LRAs or LRA combinations listed above. Cells were washed three times after drug treatment and re-seeded into 96 well plates at 200,000 cells/well/treatment type and assessed for immune marker expression via FACS at four time points: 6 hours (immediately after drug treatment), 24 hours, 48 hours and 72 hours.

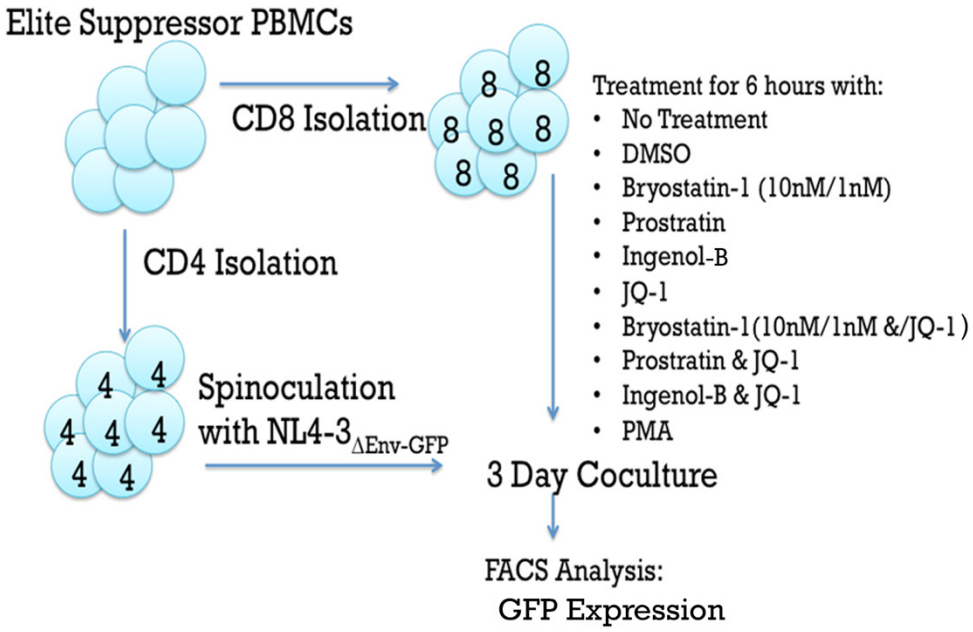

Figure 6. A representative flow diagram of the suppression assay

We also explored the potential of select LRAs/LRA combinations, to either induce proliferation, or increase cell death, in CD8+ T cells isolated from healthy donor PBMCs. In this assay, CD4+

and CD8+ T cells were isolated from donor PBMCs and combined at a 1:1 ratio. They were washed with pre-warmed PBS and exposed to 1uM CFSE (diluted in DMSO to 5uM, then warm PBS to 1uM) at a $1x10^6$ cells/ml of CFSE with gentle vortexing. Cells were then incubated for 10 minutes at 37˚C and CFSE quenched with 5 times original volume of cold media followed by a 5 minute incubation at 4˚C. Cells were washed one more time with media and treated for 6 hours with select LRAs or LRA combinations in 96 well plates at 200,000 cells/well/treatment. Drug combinations are the same as used a prior, except for the addition of ingenol-B at 10nM and PMA/Ionomycin at 50ng/ml and 1uM respectively. At the end of this incubation, cells were extensively washed, and cultured for an additional 3 days, after which cell death and proliferation were assessed by FACS. For Ki-67 staining, cells were treated with drugs for 6 hours prior to cell culture. On day 3, antibodies to surface markers were added and the cells were then fixed with 70% cold ethanol followed by intracellular staining with the Ki-67 antibody (Biolegend).

3.3.4 FACS analysis

For the suppression assay, the following antibodies were used: CD3 (Pacific Blue, clone UCHT1, BioLegend), CD8 (PE, clone RPA-T8, BD Pharmingen) and CD69 (APC, clone FN50, BioLegend). The percent infection was assessed by quantifying GFP expression using the following formula:

$$\left[1 - \frac{(\%\text{GFP+ CD4+ T cells cultured with CD8+ T cells})}{(\%\text{ GFP+ CD4+ T cells without effectors})}\right] \text{X } 100$$

The following markers were used to assess cell death, immune activation, and exhaustion: CD3 (PE, clone UCHT1, BD Pharmingen), CD8 (APC-H7, clone SK1, BD Biosciences), CD69 (APC, clone FN50, BioLegend), 7AAD (Read in PerCP/Cy5.5, BD Pharmingen), Annexin V (Read in

V450, BD Biosciences), PD1 (PerCP/Cy5.5, clone EH12.2H7, BioLegend) CD25 (FITC, clone M-A251, BD Pharmingen), HLA-DR (PE, clone L243, Biolegend) and Ki-67 (APC, clone Ki67, Biolegend). Proliferation was assessed using the CellTrace cell proliferation kit from Invitrogen and read in the FITC channel.

3.3.5 Statistics

All statistical analysis and associated graphs were generated in GraphPad Prism 7. For each parameter studied, statistical significance was assessed using a one-way ANOVA with repeated measures. The Geisser-Greenhouse Correction was also employed to account for violations of sphericity. In experiments that entailed replicates, standard deviations were used to determine variances from corresponding means. In all cases, the Dunnett's Multiple Comparison's test was used to assess variations of treatment groups from the DMSO control. Multiplicity adjusted P values were reported to assess significance with the following p-value demarcations: ns ($p>0.05$), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

3.4 RESULTS

3.4.1 Ingenol-B does not inhibit ES CD8+ T cell suppressive capacity

To determine the effect of individual drugs on the suppressive capacity of HIV-specific CD8+ T cells, we treated CD8+ T cells isolated from elite suppressors for 6 hours with three different doses of ingenol-B that have been tested in vitro, and then co-cultured them (after extensive washes) over a 3-day period with autologous CD4+ T cells spinoculated with a lab strain HIV pseudotype virus. There was no inhibition of CD8+ T cell suppressive capacity with 10nM, 100nM or 1000nM of the drug compared to the DMSO control (Figure 7). We therefore selected

the 100nM dose for comparison to other LRAs including the bromodomain inhibitor JQ1 and the other PKC agonists bryostatin-1 and prostratin.

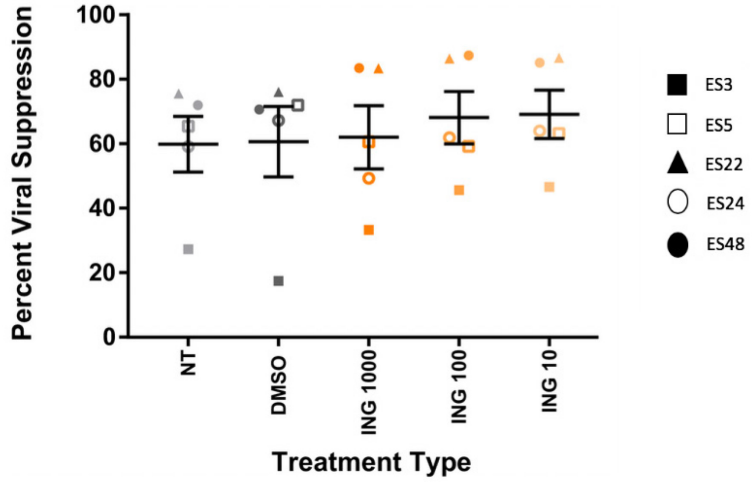

Figure 7. **Elite suppressor CD8 + T cell responses are not inhibited by ingenol-B.** CD8 + T cells from 5 ES were either not treated (NT) or pre-incubated with DMSO or ingenol-B at 3 different doses for 6 hours, washed, then added to autologous CD4 + T cells infected with a lab strain HIV-1 pseudotype virus at a 1:1 effector:target ratio. Percent suppression of viral replication was determined after 3 days. Triplicates were performed and mean values are shown for each individual.

The combination of ingenol-B and JQ1 has a modest inhibitory effect on ES CD8+ T cell suppressive capacity

As previously described (Walker-Sperling et al, 2016), bryostatin-1 caused significant inhibition of the suppressive capacity of ES CD8+ T cells at 10nM (19.22% suppression versus 59.33% for DMSO treated cells, $p = 0.002$) whereas prostratin and JQ1 individually had no effect (59.74% suppression and 63.77% suppression respectively, Figure 8). CD8+ T cells treated with 100nM ingenol-B for 6 hours suppressed viral replication as efficiently as CD8+ T cells treated

with the DMSO vehicle (65.33% suppression, p = 0.567, Figure 8). While neither100nM of ingenol-B nor JQ1 had a significant effect on ES CD8+ T cells, the combination of the 2 drugs had a modest, but significant, inhibitory effect on the suppressive capacity of the T cells (39.99% suppression compared to 59.33% suppression with DMSO, p = 0.033). A more pronounced inhibitory effect was seen with the combination of bryostatin-1 (10nM) and JQ1 (18.23% suppression), and no significant inhibition was seen with the prostratin/JQ1 combination (45.23% suppression) as previously described (Walker-Sperling et al, 2016).

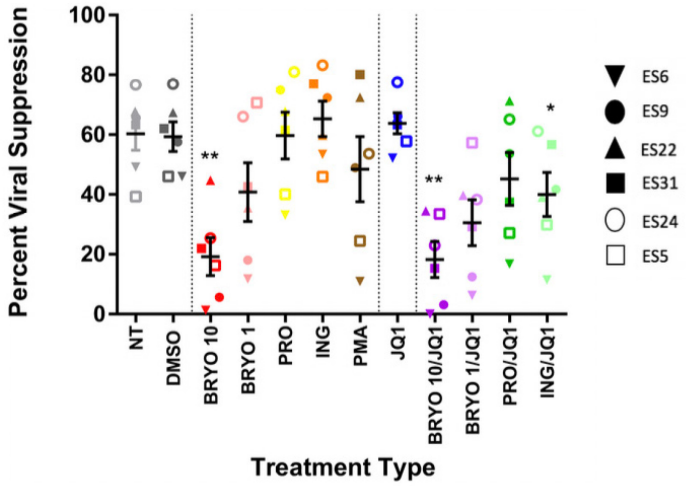

Figure 8. Elite suppressor CD8 + T cell responses are modestly inhibited by the combination of ingenol-B and JQ1. CD8 + T cells from 6 ES were pre-incubated with indicated LRAs for 6 hours, washed, then added to autologous CD4 + T cells infected with a lab strain HIV-1 pseudotype virus at a 1:1 effector:target ratio. Percent suppression of viral replication was determined after 3 days. Triplicates were performed and mean values are shown for each individual. One-way repeated measures ANOVAs were used to determine significance for each of the two sets of experiments. Symbols directly above treatments indicate differences from the DMSO control (* p< 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10 or 1nM (BRYO 10 and BRYO 1 respectively), prostratin at 0.3uM (PRO), ingenol-B at 100nM (ING), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments, the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, BRYO 1/JQ1, PRO/JQ1, ING/JQ1).

3.4.2 Ingenol-B induces high levels of CD8+ T cell activation, modest cell death and PD1 expression and no cell proliferation

In order to address the different effects of the inhibitory (bryostatin-1) and non-inhibitory (prostratin and ingenol-B) PKC-agonists, we measured the amount of immune activation they induced on CD8+ T cells from healthy donors (Figure 9). With the exception of JQ1, which slightly increased levels of CD69, all drugs induced high levels of CD69 expression after 6 hours of drug exposure. This activation was sustained for bryostatin-1 and ingenol-B, but by the 24 hour time point, CD69 expression on CD8+ T cells exposed to prostratin had begun to decline and by 2 days there was no difference in the level of expression between prostratin and DMSO treated cells. Similarly, besides prostratin and bryostatin at 1nM, cells treated with the combination of JQ1 and PKC agonists had sustained levels of activation as cells treated with the PKC-agonists alone. A similar pattern was seen with cells obtained from chronic progressors on ART (Figure 10). We also examined other activation markers and found that aside from PMA, and PMA/Iono, none of the LRAs induced significant expression of CD25 (Figure 11) or co-expression of HLA-DR and CD38 (Figure 12).

Ingenol-B treatment resulted in a modest but significant increase in annexin V expression at 2 days, but no significant increase in cell death was observed at any other time point (Figure 13 and Figure 14). The percentages of CD8+ T cells expressing PD1 after ingenol-B and ingenol-B/JQ1exposures were also slightly elevated at the 1 and 2 day time points but not enough to explain the differences in suppressive capacity of ES CD8+ T cells (Figure 15 and Figure 16). We also co-cultured purified CD4+ and CD8+ T cells to more closely approximate the experiment we performed with the ES CD8+ T cells and observed no significant increase in CD8+ T cell death on day 3 in this system, following a 6 hour exposure to ingenol-B or ingenol-B and JQ1 (Figure 17). Additionally, we asked whether non-specific proliferation of CD8+ T cells could explain the

difference in responses to ingenol-B versus ingenol-B and JQ1. We found that the LRAs did not induce any non-specific proliferation of CD8+ T cells as determined by CFSE dilution or Ki-67 expression (Figure 18).

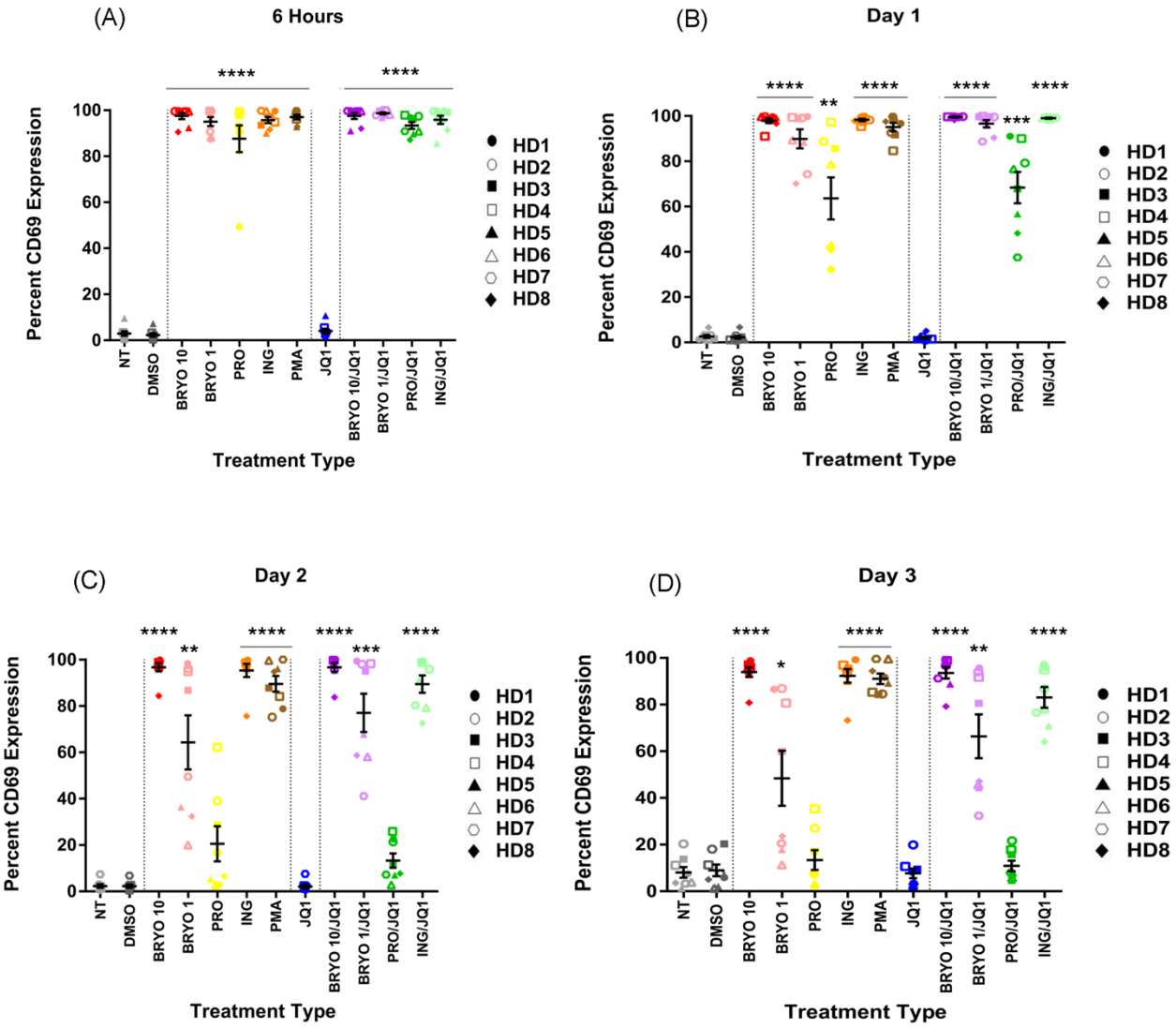

Figure 9. CD69 expression is upregulated on HD CD8+ T cells following treatment with PKC-agonists. PBMCs from 8 HDs were treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for CD69 expression on CD8 + T cells. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1, (C) day 2 and (D) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10 or 1nM (BRYO 10 and BRYO 1 respectively), prostratin at 0.3uM (PRO), ingenol-B at 100nM (ING), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, BRYO 1/JQ1, PRO/JQ1, ING/JQ1).

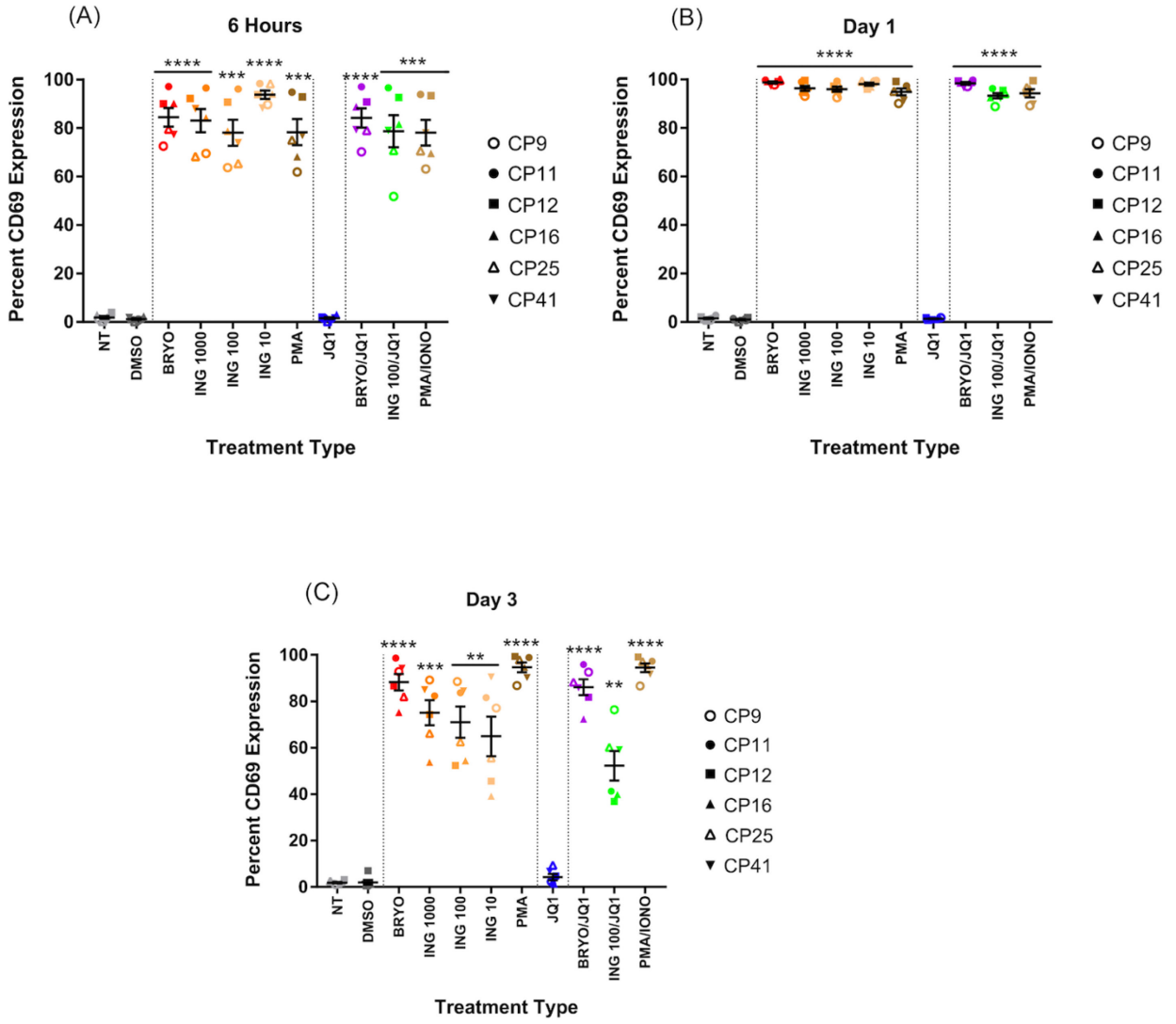

Figure 10. CD69 expression is upregulated on CP CD8+ T cells following treatment with PKC-agonists. PBMCs from 6 CPs were treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for CD69 expression on CD8 + T cells. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1 and (C) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10nM (BRYO), ingenol-B at 1000, 100 or 10nM (ING 1000, ING 100, ING 10 respectively), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, ING 100/JQ1). PMA and ionomycin (PMA/IONO, 50ng/ml and 1uM respectively) were used as a positive control.

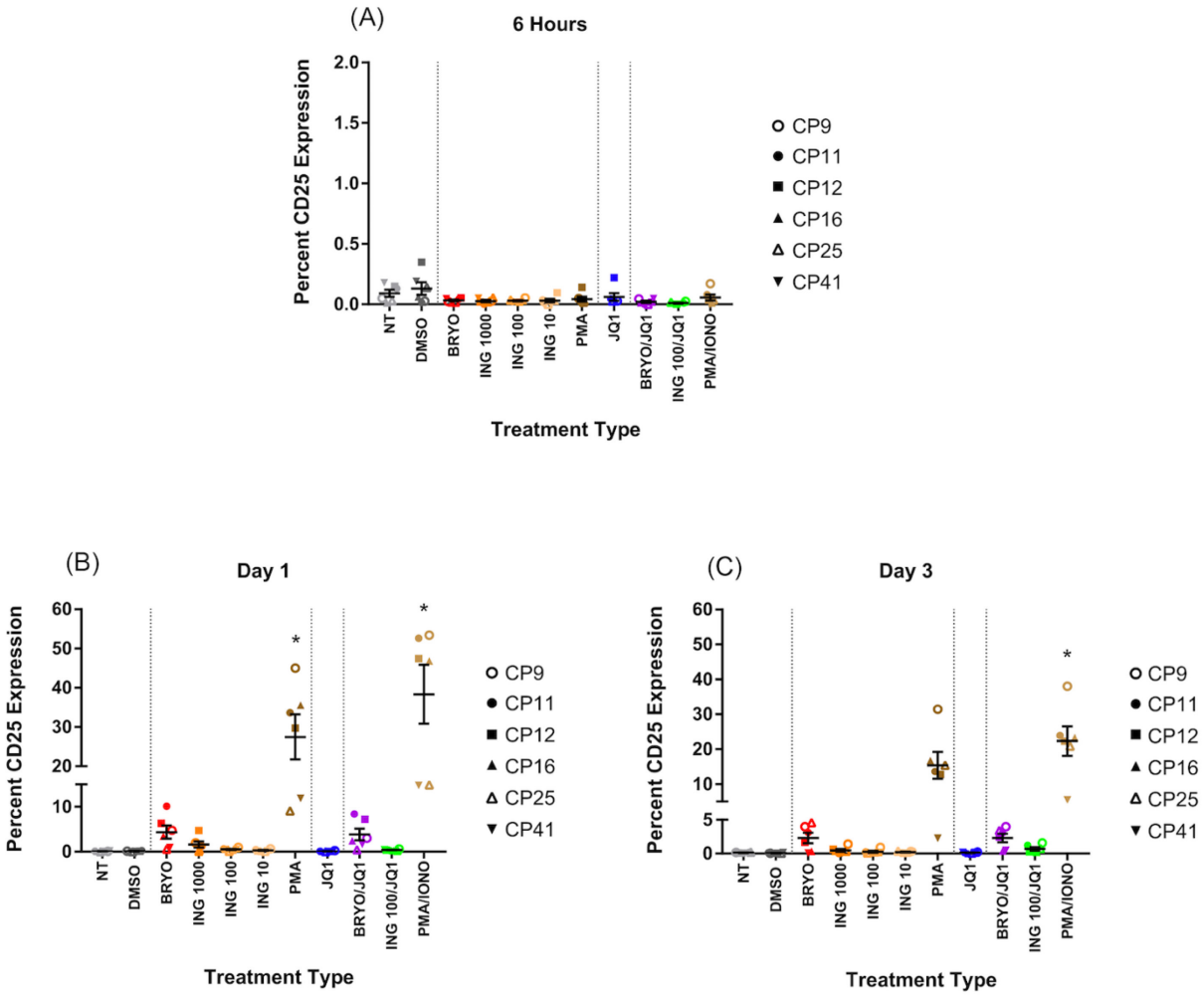

Figure 11. CD25 expression is not upregulated on CP CD8+ T cells following treatment with LRAs. PBMCs from 6 CPs were treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for CD25 expression on CD8 + T cells. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1 and (C) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p < 0.05$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10nM (BRYO), ingenol-B at 1000, 100 or 10nM (ING 1000, ING 100, ING 10 respectively), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, ING 100/JQ1). PMA and ionomycin (PMA/IONO, 50ng/ml and 1uM respectively) were used as a positive control.

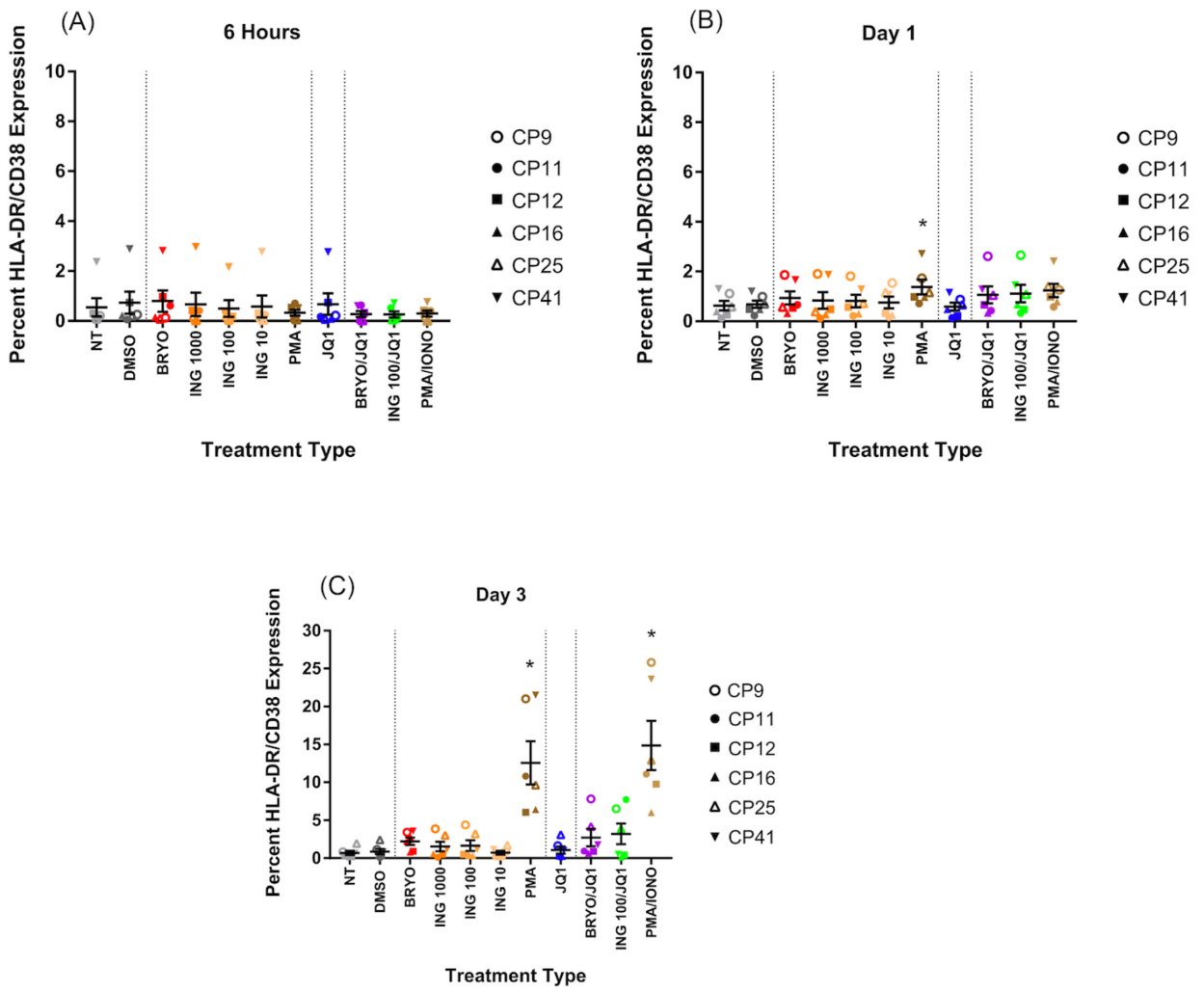

Figure 12. CD38 and HLA-DR co-expression is not upregulated on CP CD8+ T cells following treatment with LRAs. PBMCs from 6 CPs were treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for CD38 and HLA-DR co-expression on CD8 + T cells. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1 and (C) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p < 0.05$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10nM (BRYO), ingenol-B at 1000, 100 or 10nM (ING 1000, ING 100, ING 10 respectively), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, ING 100/JQ1). PMA and ionomycin (PMA/IONO, 50ng/ml and 1uM respectively) were used as a positive control.

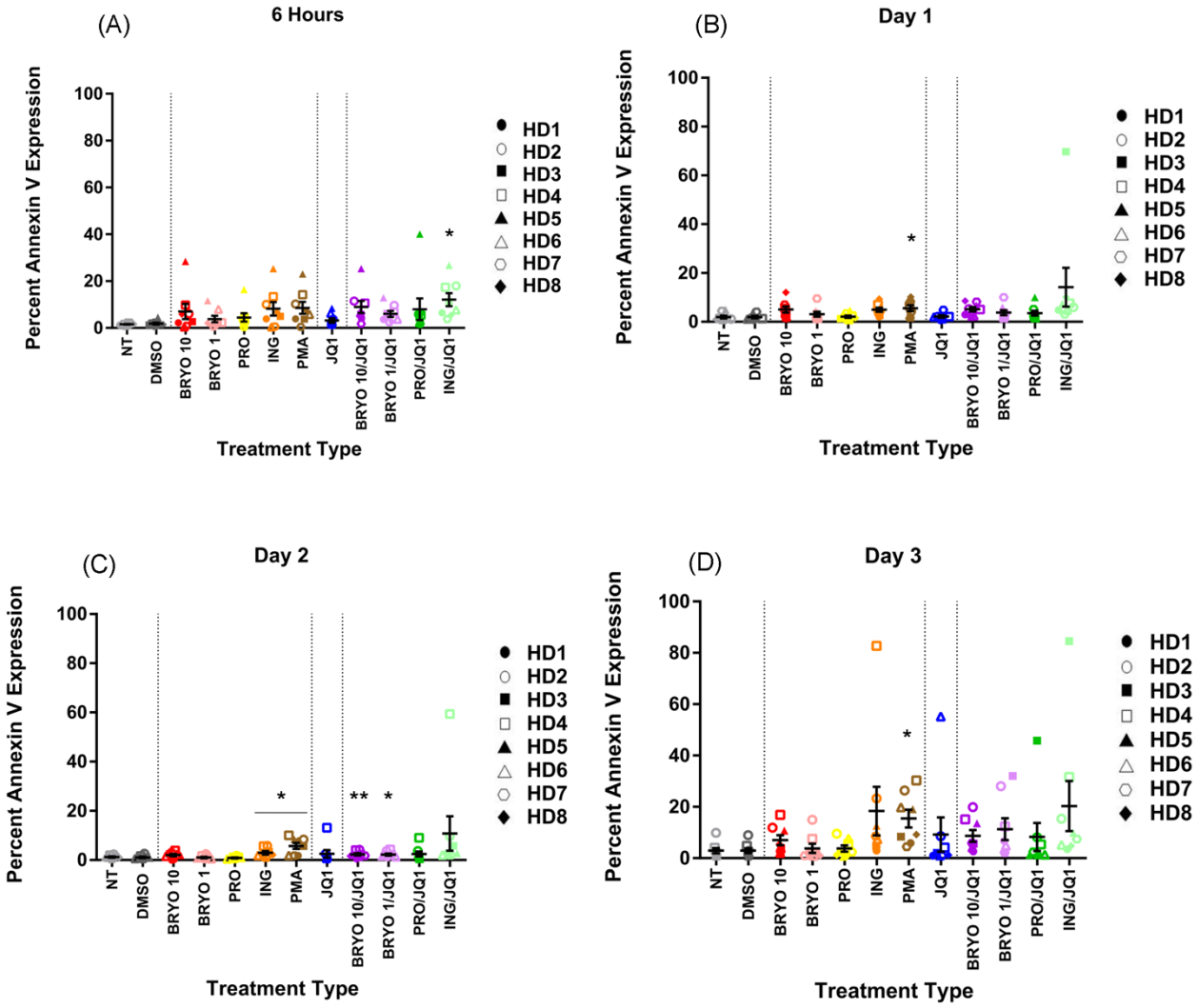

Figure 13. Ingenol-B and ingenol-B/JQ1 combination causes modest levels of cell death in HD CD8+ T cells. PBMCs from 8 HDs were isolated and treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for cell death as measured by percent annexin V expression. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1, (C) day 2 and (D) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10 or 1 nM (BRYO 10 and BRYO 1 respectively), prostratin at 0.3uM (PRO), ingenol-B at 100nM (ING), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, BRYO 1/JQ1, PRO/JQ1, ING/JQ1).

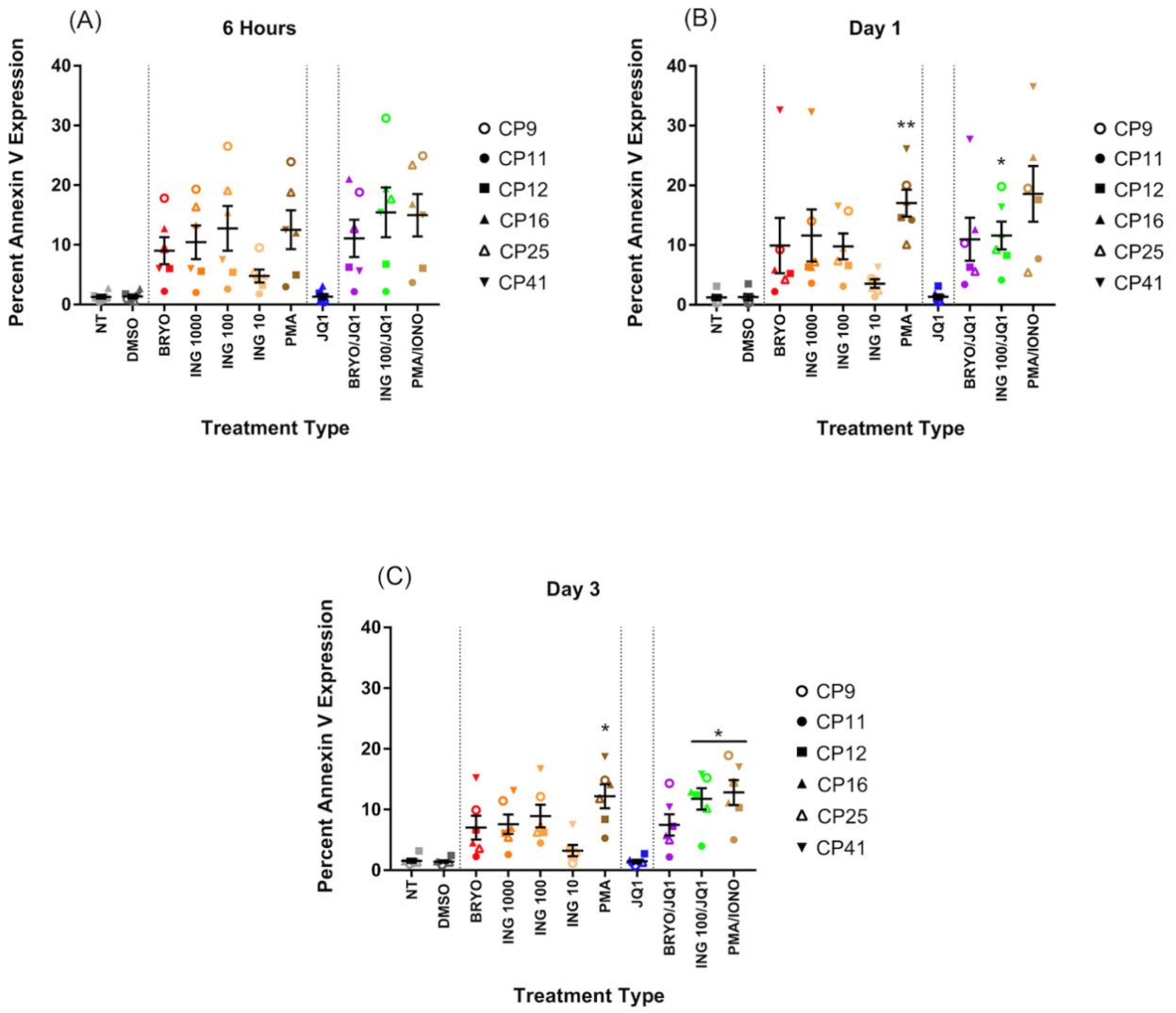

Figure 14. Ingenol-B/JQ1 combination causes modest levels of cell death in CP CD8+ T cells. PBMCs from 6 CPs were isolated and treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for cell death as measured by percent annexin V expression. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1 and (C) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p < 0.05$, ** $p < 0.01$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10nM (BRYO), ingenol-B at 1000, 100 or 10nM (ING 1000, ING 100, ING 10 respectively), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, ING 100/JQ1) or with PMA and ionomycin (PMA/IONO, 50ng/ml and 1uM respectively).

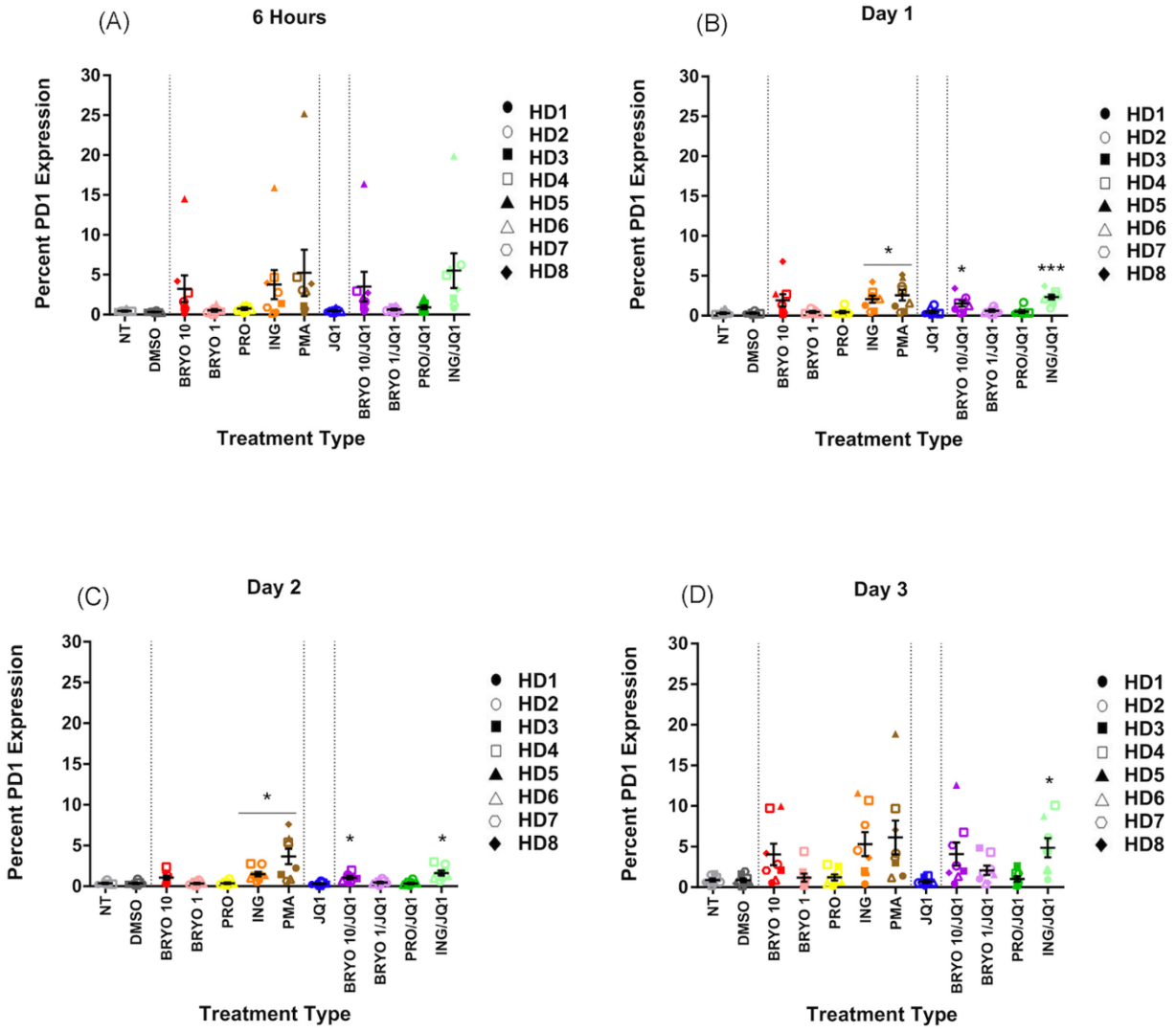

Figure 15. Ingenol-B/JQ1 combination causes transient, slightly higher levels of % PD1 expression on HD CD8+ T cells. PBMCs from 8 HDs were treated with LRAs for 6 hours before being washed and cultured for up to 3 days and examined for cell exhaustion as measured by percent PD1 expression. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1, (C) day 2 and (D) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p< 0.05$, ** $p < 0.01$, *** $p <0.001$, **** $p < 0.0001$). Cells were either non treated (NT) or treated with DMSO (1%), bryostatin-1 at 10 or 1 nM (BRYO 10 and BRYO 1 respectively), prostratin at 0.3uM (PRO), ingenol-B at 100nM (ING), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, BRYO 1/JQ1, PRO/JQ1, ING/JQ1).

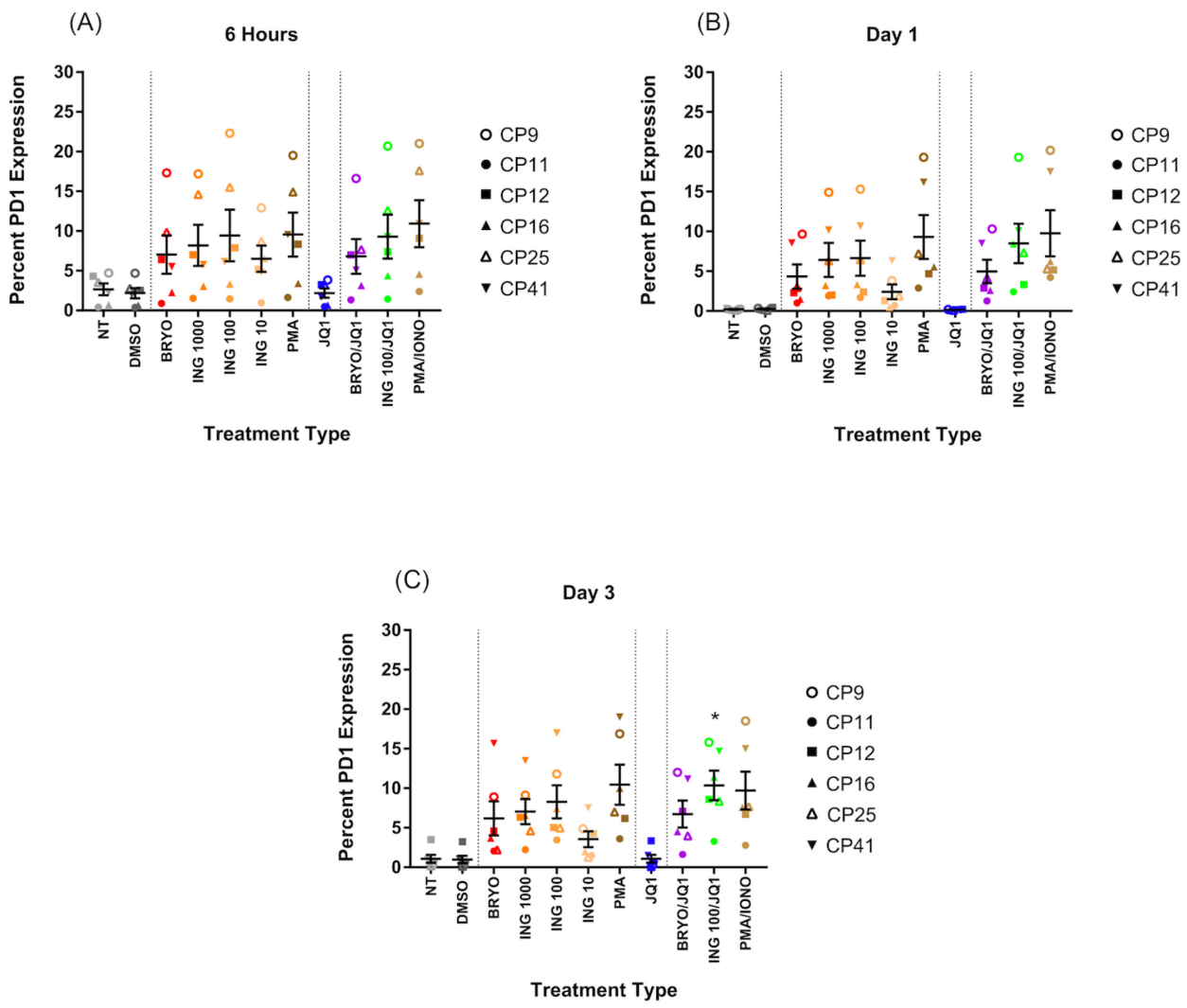

Figure 16. Ingenol-B/JQ1 combination causes slightly higher levels of % PD1 expression on CP CD8+ T cells. PBMCs from 6 CPs were isolated and treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for cell exhaustion as measured by percent PD1 expression. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1, and (C) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p < 0.05$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10nM (BRYO), ingenol-B at 1000, 100 or 10nM (ING 1000, ING 100, ING 10 respectively), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, ING 100/JQ1) or with PMA and ionomycin (PMA/IONO, 50ng/ml and 1uM respectively).

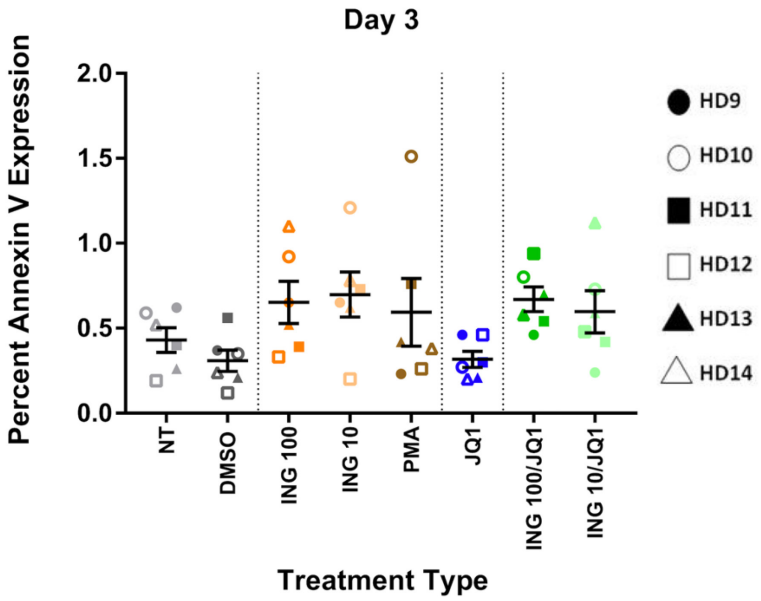

Figure 17. LRAs do not increase levels of cell death in HD CD8+ T cells following incubation with CD4+ T cells. PBMCs were isolated from 6 HDs and CD4+ and CD8+ T cells enriched. Cells were combined at a 1:1 ratio, co-cultured and treated with LRAs for 6 hours after which cells were extensively washed and incubated for an additional 3 days before examining for CD8+ T cell death by percent annexin V expression. Mean expression ± standard error is indicated for each treatment at day 3. No significant differences in cell death were observed, relative to the DMSO control. Cells were either non treated (NT) or treated with DMSO (1%), ingenol-B at 100nM or 10nM (ING 100 or ING 10), PMA at 50ng/mL (PMA), JQ1 at 1uM (JQ1) and ingenol-B/JQ1 combinations at the same concentrations (ING 100/JQ1, ING 10/JQ1).

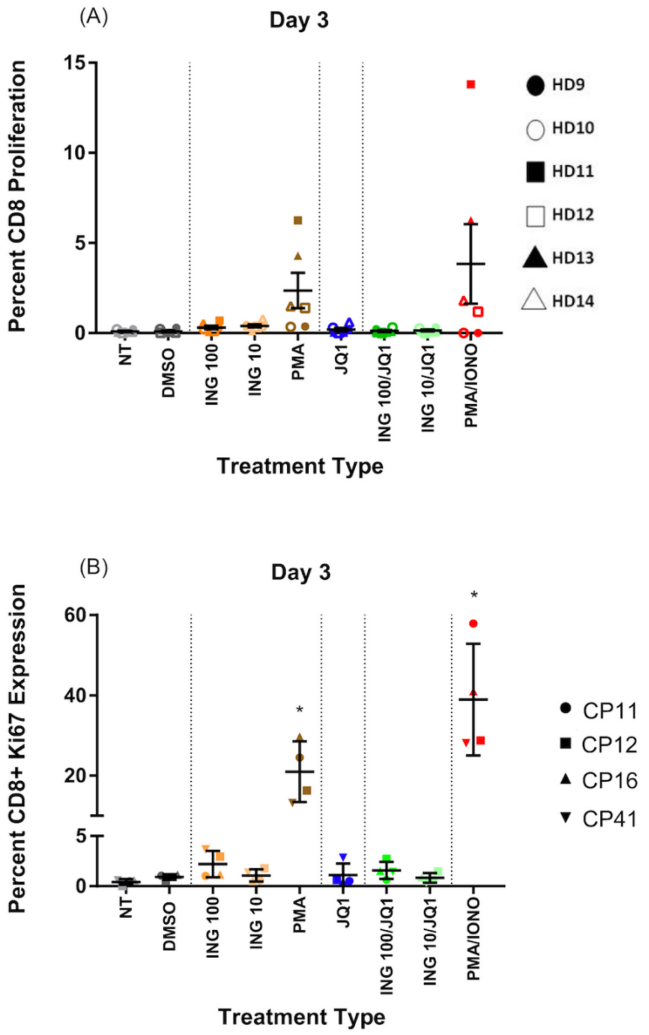

Figure 18. Ingenol-B does not induce proliferation of CD8+ T cells. (A) PBMCs were isolated from 6 HDs and CD4+ and CD8+ T cells enriched. Cells were combined at a 1:1 ratio and exposed to CellTrace CFSE. They were treated with LRAs for 6 hours incubated for an additional 3 days before examination for CD8+ T cell proliferation by percent CFSE expression. Mean expression ± standard error is indicated for each treatment at day 3. Cells were either non treated (NT) or treated with DMSO (1%), ingenol-B at 100nM or 10nM (ING 100 or ING 10), PMA at 50ng/mL (PMA), PMA/Ionomycin (PMA/IONO, 50ng/ml and 1uM respectively), JQ1 at 1uM (JQ1) and ingenol-B/JQ1 combinations at the same concentrations (ING 100/JQ1, ING 10/JQ1). (B) PBMCs were isolated from 4 CPs and treated for 6 hours with LRAs. They were then extensively washed and incubated for an additional 3 days before intracellular staining with the Ki-67 monoclonal antibody. Mean expression ± standard error is indicated for each treatment.

3.4.3 Ingenol-B and ingenol-B/JQ1 induce transient downregulation of CD3 but have no effect on surface CD8 expression

Prior studies have shown that ingenol-B causes a significant downregulation of surface CD4 expression (Abreu et al, 2014, Spivak et al 2015) and thus we asked whether a similar downregulation of CD8 might play a role in the inhibitory effect of the ingenol-B/JQ1 combination. We found that while CD8 surface levels were slightly lower in cells treated with bryostatin-1 at the day 2 time point (normalized MFI of 0.914 compared to DMSO treated cells of 0.996, $p = 0.009$), cells treated with 100nM ingenol-B and ingenol-B/JQ1 had similar levels of CD8 as cells treated with the DMSO vehicle (normalized MFIs of 1.046 and 0.943 respectively, Figure 19). In contrast, CD3 was significantly downregulated on CD8+ T cells that were treated with bryostatin-1 at 10nM and 1nM (normalized MFIs of 0.574 and 0.787 respectively), ingenol-B at 100nM (normalized MFI of 0.645), bryostatin/JQ1 (normalized MFI of 0.5113) and ingenol-B/JQ1(normalized MFI of 0.539) at the 6 hour time point (Figure 20 and Figure 21). Interestingly, this downregulation was transient for ingenol-B and ingenol-B/JQ1 treated cells, such that CD3 had normalized by the 24-hour time point. In contrast, cells treated with bryostatin-1 and bryostatin-1/JQ1 had sustained downregulation of CD3 (Figure 20).

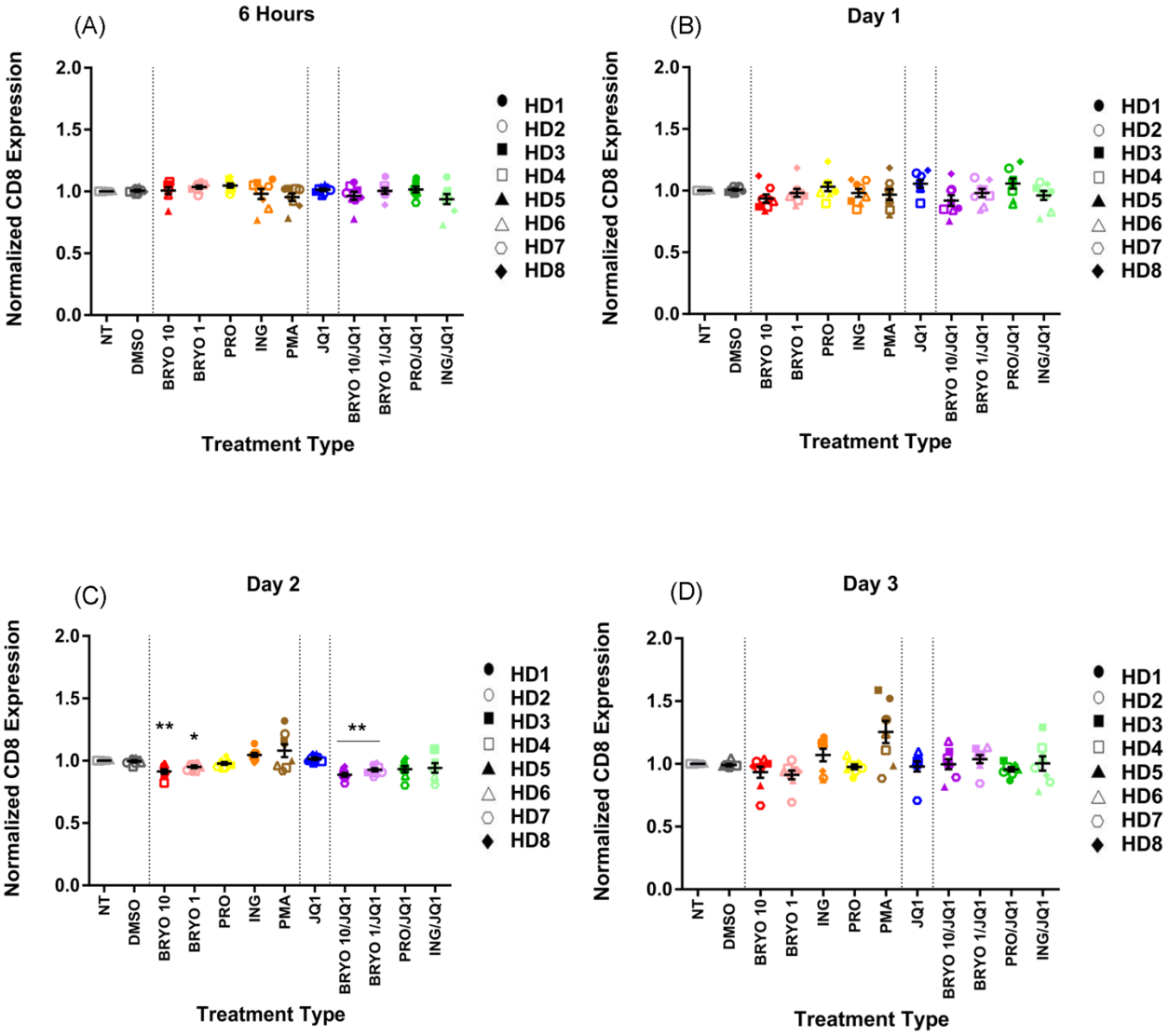

Figure 19. Surface CD8 expression in HD is modestly downregulated by some LRAs. PBMCs from 8 HDs were treated with LRAs for 6 hours before being washed and then cultured for up to 3 days and examined for CD8 expression. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1, (C) day 2 and (D) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10 or 1nM (BRYO 10 and BRYO 1 respectively), prostratin at 0.3uM (PRO), ingenol-B at 100nM (ING), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, BRYO 1/JQ1, PRO/JQ1, ING/JQ1).

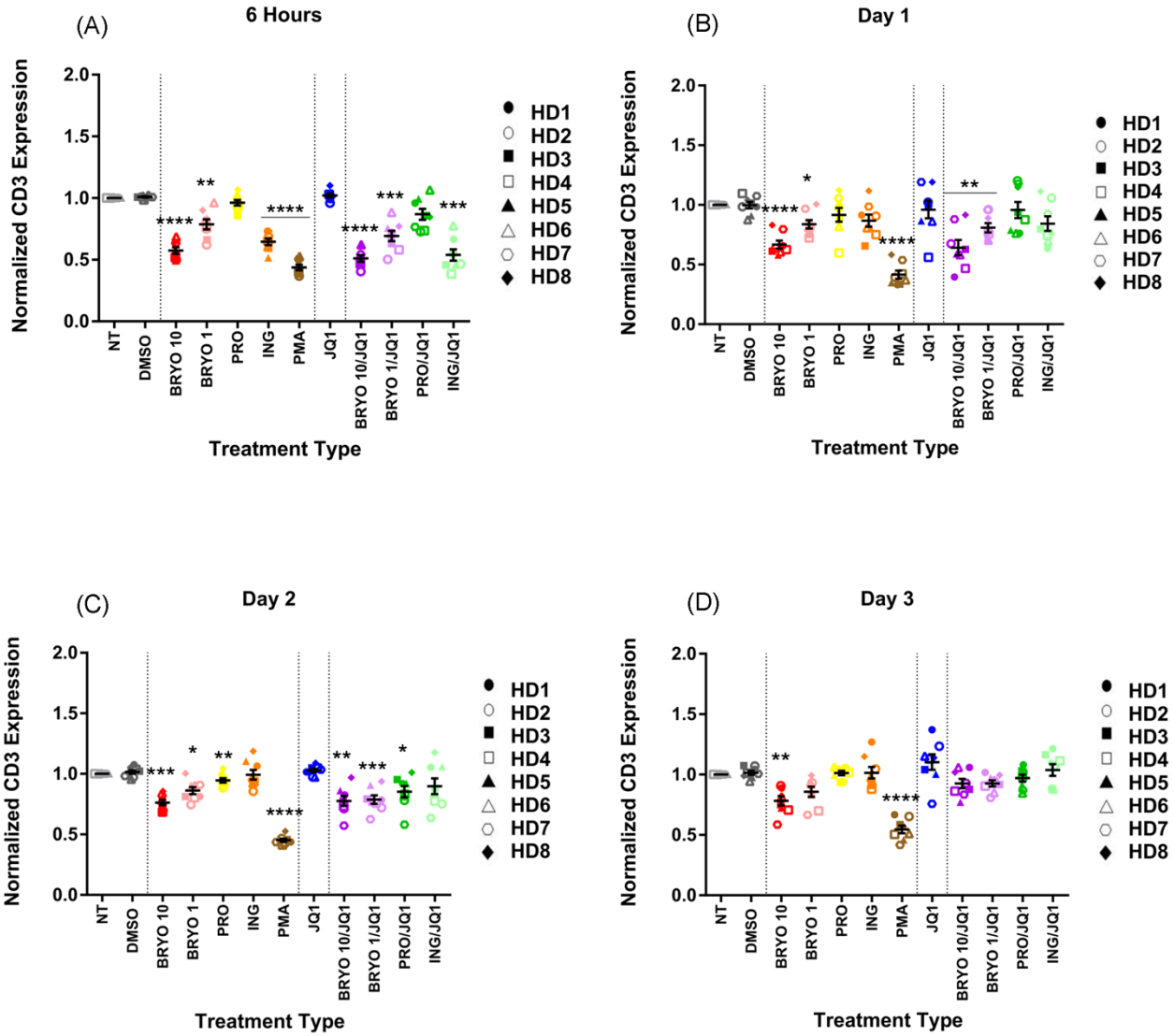

Figure 20. Surface CD3 expression in HD is downregulated following treatment with PKC-agonists. PBMCs from 8 HDs were treated with LRAs for 6 hours before being washed and then cultured for up to 3 days and examined for CD3 expression. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1, (C) day 2 and (D) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10 or 1nM (BRYO 10 and BRYO 1 respectively), prostratin at 0.3uM (PRO), ingenol-B at 100nM (ING), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, BRYO 1/JQ1, PRO/JQ1, ING/JQ1).

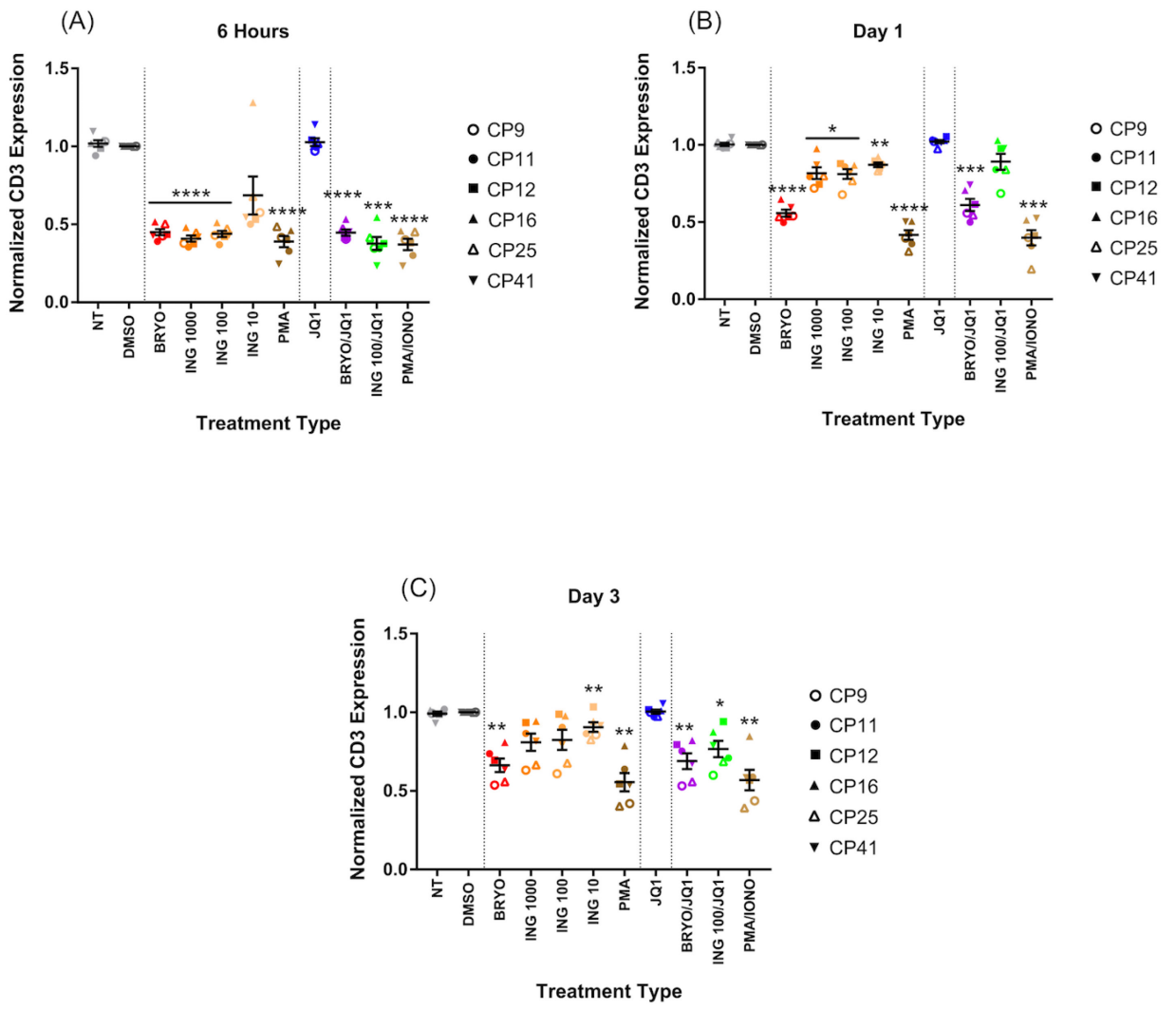

Figure 21. Surface CD3 expression in CP is downregulated following treatment with PKC-agonists. PBMCs from 6 CPs were isolated and treated with LRAs for 6 hours, washed, then cultured for up to 3 days and examined for CD3 expression. Mean expression ± standard error is indicated for each treatment at (A) 6 hours, (B) day 1, and (C) day 3. Symbols directly above treatments indicate differences from the DMSO control (* $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Cells were either non treated (NT) or treated with DMSO (0.1%), bryostatin-1 at 10nM (BRYO), ingenol-B at 1000, 100, or 10nM (ING 1000, ING 100, ING 10 respectively), PMA at 50ng/mL (PMA) and JQ1 at 1uM (JQ1). In some experiments the PKC-agonists were combined with JQ1 at the same concentrations (BRYO 10/JQ1, ING 100/JQ1) or with PMA and ionomycin (PMA/IONO, 50ng/ml and 1uM respectively).

3.4.4 The combination of a lower dose of Ingenol-B with JQ1 has no significant inhibitory effect on ES CD8+ T cell suppressive capacity

In order to determine whether the modest inhibitory effect of the combination of ingenol-B with JQ1 was dependent on the dose of ingenol-B, we performed experiments with 10nM of the drug in combination with the same dose of JQ1. As shown in Figure 22, no inhibition of the suppressive capacity of CD8+ T cells was observed with this specific combination.

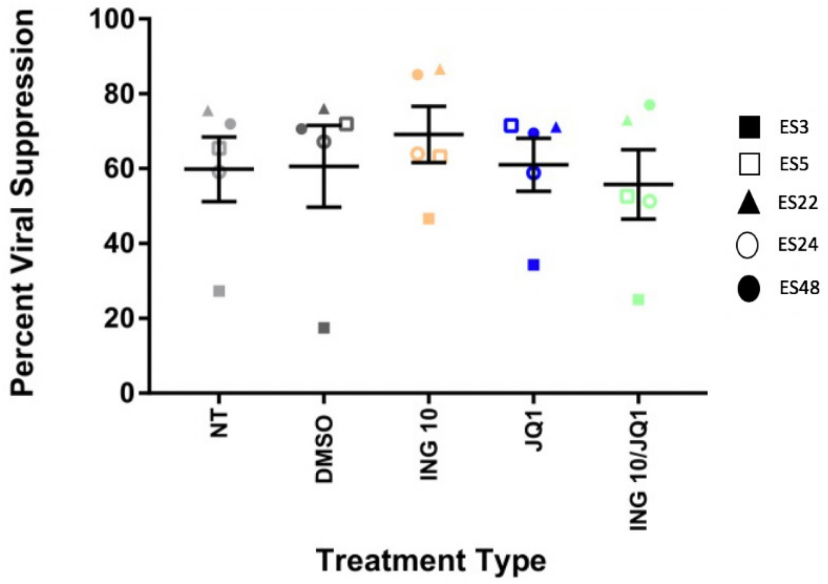

Figure 22. Elite suppressor CD8 + T cell responses are not inhibited by 10nM ingenol-B in combination with JQ1. CD8 + T cells from 5 ES were pre-incubated with 10nM ingenol-B and 1uM JQ1 for 6 hours, washed, then added to autologous CD4 + T cells infected with a lab strain HIV-1 pseudotype virus at a 1:1 effector:target ratio. Percent suppression of viral replication was determined after 3 days. Triplicates were performed and mean values are shown for each individual.

3.5 DISCUSSION

Ingenol derivatives have been shown to function efficiently both as monotherapy and in combination with other drugs such as JQ1 and vorinostat in vitro (Darcis et al, 2015, Abreu et al 2014, Spivak et al, 2015, Jiang et al, 2015) and in vivo (Gama et al 2017). While some ingenol derivatives are thought to function via alternative pathways (Fujiwara et al, 1998), it is generally established that most ingenol derivatives, including ingenol-B, function as PKC agonists, reversing latency via the PKC-NFκB pathway (Abreu et al 2014, Spivak et al, 2015, Jiang et al, 2015). Besides PKC activation, ingenol-B has also been shown to facilitate transcript elongation via the recruitment of specific P-TEFb components namely CDK9 and CyclinT1 (Pandelo Jose et al, 2014). Ingenol-B is also thought to be capable of inhibiting viral propagation by downregulating HIV receptors and co-receptors (CD4, CCR5 and CXCR4) (Abreu et al 2014, Spivak et al, 2015). This drug may therefore be a promising agent to test in clinical trials.

A recent study has shown that the reversal of latency by itself is not sufficient to eliminate latently infected CD4+ T cells, and that HIV-specific CD8+ T cells may also be needed for the eradication of these cells (Shan et al, 2012). This finding has prompted additional studies which have now produced significant data to suggest that some LRAs may inhibit NK cell and CD8+ T cell activity (Pace et al, 2016, Garrido et al, 2016, Akimova et al, 2012, Jones et al, 2014, Walker-Sperling et al, 2016, Clutton et al, 2016) and thus may not be the optimal candidates for shock-and-kill strategies. In a recent study, we showed that agents such as the HDAC inhibitors romidepsin and panobinostat, and the PKC agonist bryostatin-1, inhibited the responses of ES CD8+ T cells in viral suppression assays (Walker-Sperling et al, 2016). We also tested combinations of drugs that had been found to be synergistic and found that some combinations

like romidepsin and bryostatin-1, inhibited CD8+ T cell responses to a greater degree than either drug alone.

In this study, we screened ingenol-B to determine whether it inhibited HIV-specific CD8+ T cell responses. We used CD8+ T cells from ES since these patients have potent HIV-specific CD8+ T cell responses that are probably responsible for their control of HIV-1 infection (O'Connell et al, 2009, Migueles et al, 2002, Betts et al, 2006, Saez-Cirion, 2007, Migueles et al, 2008, Hersperger et al, 2010, Buckheit et al, 2013). While HIV-1 specific CD8+ T cells in these subjects are not representative of the CD8+ T cell responses seen in the vast majority of HIV-infected subjects, it is possible that successful therapeutic vaccination of these subjects will enhance CP HIV-specific CD8+ T cell responses to a similar level. We found that neither ingenol-B at 100nM nor JQ1 individually inhibited the suppressive capacity of ES CD8+ T cells, but the combination of the two drugs had a modest inhibitory effect. While a difference in CD3 downregulation kinetics could potentially, partially, explain the difference in the effects of bryostatin-1 and ingenol-B on HIV-specific CD8+ T cells, it does not explain the difference observed between ingenol-B and ingenol-B/JQ1 treated cells.

Besides CD3 modulation, we also looked at the expression levels of other cell surface markers such as CD8, annexin V and PD1, to determine if these could explain the difference in suppressive capacities of CD8+ T cells treated with either ingenol-B or its combination with JQ1. We found that ingenol-B alone and in combination with JQ1 does not significantly modulate CD8 or PD1 expression on T cells. We also found that treatment with ingenol-B or ingenol-B/JQ1 resulted in minimal cell death which confirms studies by Abreu et al (Abreu et al, 2014).

Our data are also consistent with a recent study that showed that treatment of peripheral blood mononuclear cells from healthy donors and HIV-infected patients with ingenol-B increased

the number of antigen specific CD8+ T cells that expressed perforin and CD107a (Clutton et al, 2016). However, while the drug had no effect on the total number of CD8+ T cells that proliferated in response to antigen over a 5 day period, it inhibited the number of times antigen-specific CD8+ T cells proliferated (Clutton et al, 2016). Taken together, it is possible that while this drug may not have an effect on the short-term ability of HIV-specific CD8+ T cells to control infected CD4+ T cells, it could have a negative impact on the ability of HIV-specific CD8+ T cells to expand in vivo. Clinical trials will be needed to assess the effects of this drug alone, and in combination with JQ1, on HIV-specific CD8+ T cells in vivo. The effects of the drugs will likely be dose dependent and while we used concentrations of LRAs that have reversed latency in vitro, it is not clear that these are concentrations of ingenol-B and JQ1 that can be safely achieved in vivo.

In summary, our data suggest that drug combinations should be tested before being used in clinical trials because even LRAs that do not inhibit HIV-specific CD8+ T cell responses by themselves may have significant effects when they are used in combination with each other. Furthermore, while certain combinations of LRAs may be more potent at inducing viral transcription than individual LRAs, the advantages of this enhanced potency may be neutralized by the inhibitory effects of the drug combinations on HIV-specific CD8+ T cell responses

4 CHAPTER 3

INTERFERON ALPHA ENHANCES NK CELL FUNCTION AND THE SUPPRESSIVE CAPACITY OF HIV-SPECIFIC CD8+ T CELLS

4.1 ABSTRACT

Current shock-and-kill strategies for the eradication of the HIV-1 reservoir have resulted in blips of viremia but not in a decrease in the size of the latent reservoir in patients on suppressive antiretroviral therapy (ART). This discrepancy could potentially be explained by an inability of the immune system to kill HIV-1-infected cells following the reversal of latency. Furthermore, some studies have suggested that certain latency-reversing agents (LRAs) may inhibit CD8+T cell and natural killer (NK) cell responses. In this study, we tested the hypothesis that alpha interferon (IFN-α) could improve the function of NK cells from chronic progressors (CP) on ART. We show here that IFN-α treatment enhanced cytokine secretion, polyfunctionality, degranulation, and the cytotoxic potential of NK cells from healthy donors (HD) and CP. We also show that this cytokine enhanced the viral suppressive capacity of NK cells from HD and elite controllers or suppressors. Furthermore, IFN-α enhanced global CP CD8+ T cell cytokine responses and the suppressive capacity of ES CD8+ T cells. Our data suggest that IFN-α treatment may potentially be used as an immunomodulatory agent in HIV-1 cure strategies.

4.2 INTRODUCTION

Attempts to cure HIV-1 have been unsuccessful due to the presence of latent HIV reservoirs (Dahabieh et al, 2015, Siliciano and Greene, 2011). Several approaches have thus far

been proposed to meet this challenge, including the "shock-and-kill" strategy, where latency-reversing agents (LRAs) are used to pharmacologically reverse latency in order to render formerly quiescent reservoirs susceptible to both antiretroviral therapy (ART) and the immune system (Cary and Peterlin, 2016, Marsden and Zack, 2015, Martin and Siliciano, 2016). However, while treatments with certain LRAs have resulted in viral blips, no trial has demonstrated changes in the size of the latent reservoir (Sogaard et al, 2015, Archin et al, 2012, Archin et al, 2014, Spivak et al, 2014, Rasmussen et al, 2014, Elliott et al, 2014, Elliott et al, 2015), owing perhaps to the dysfunctional state of potential effector cells. This awareness has drawn attention to the need to potentiate immune responses in order to efficiently eliminate reactivated reservoirs (Perreau et al, 2017, Riley and Montaner, 2017).

As part of these efforts, there has been a lot of attention focused on CD8+ T cells. However, while these cells play a critical role in the control of HIV-1 replication, they also possess limitations. They are slower to respond to viral infections due to the timing of the adaptive immune response, they are susceptible to viral escape strategies such as HLA downregulation (Collins et al, 1998), and they are typically excluded from B cell follicles in lymph nodes (Folkvord et al, 2005, Connick et al, 2007, Fukazawa et al, 2015), which can then serve as sanctuaries for sustained productive infection (Dahabieh et al, 2015). For these reasons, it is important to also analyze other immune effector cells capable of complementing CD8+ T cell effector function. Natural killer (NK) cells are ideal candidates for this role. They respond to viral infection without a need for clonal expansion, are thought to home to and control viral replication in lymph node sanctuaries (Huot et al, 2017), and are equipped to kill infected cells that evade CD8+ T cell elimination by HLA downregulation (Cohen et al, 1999).

NK cell function can be augmented by various cytokines, including interleukin-15 (IL-15), IL-18, and IL-21, as well as type 1 interferons (IFNs) (Zwirner and Domaica, 2010). IL-15 in particular has recently been shown to enhance NK cell-mediated antiviral activity in humanized mice (Seay et al, 2015) and in vitro following latency reversal in CD4+ T cells from patients on ART (Garrido et al, 2018). In this study, we focused on alpha interferon (IFN-α) since it has been used clinically for the treatment of hepatitis C (Maughan and Ogbuagu, 2018). As a member of the type 1 IFN family, IFN-α helps create antiviral immune states that in turn help control the spread of viral infections (Odorizzi and Wherry, 2013). The efficiency of this IFN-mediated control, however, is principally dependent on the timing and concentration levels of IFN-α (Odorizzi and Wherry, 2013, Telenti, 2014). Specifically, in the SIV model of HIV-1 infection, type 1 IFNs ramp up the immune system and tip the scales in favor of an antiviral state that helps control infection spread in primary infection (Sandler et al, 2014). However, several studies have suggested that IFN-α also plays a role in the immune dysfunction seen in the chronic stages of HIV-1 infection (Hardy et al, 2013, Sedaghat et al, 2008). Therefore, to maximize the benefits of type 1 IFN (IFN-α) for NK cells, a fine line must be drawn in choosing a concentration and regimen that facilitates an antiviral response without causing excessive immune activation that triggers a transition away from that antiviral state.

While several studies have shown that IFN-α enhances NK cell cytokine secretion and cytotoxic responses in healthy donors (Ellis et al, 1989, Jewett et al, 1995, Tomescu et al, 2007, Tomescu et al, 2015) and, in a subset of HIV controllers (Tomescu et al, 2012), similar studies have not been performed with chronic progressor (CP) NK cells. We therefore sought to determine via in vitro assays, whether treatment of CP NK cells over short periods of time (termed a “pulse”) with IFN-α would enhance their cytokine-producing capacities, as well as their cytotoxic

responses, without inhibiting general CD8+ T cell function. We also sought to determine whether similar treatments would enhance NK cell-mediated HIV-1 replication suppression in elite suppressor (ES) NK cells, again without inhibiting the HIV-specific suppressive capacities of CD8+ T cells. Our results suggest that IFN-α pulse therapy could be employed for enhancing NK cell effector function in HIV-1 cure strategies.

4.3 METHODS

4.3.1 Patients

Blood samples from HIV-negative and HIV-positive donors were obtained with written informed consent and subsequently handled in accordance with protocols approved by the Johns Hopkins University IRB. An elite suppressor (ES) refers to a patient who has maintained undetectable viral loads in the absence of ART (Blankson, 2010), while a chronic progressor (CP) refers to a patient who has maintained undetectable viral loads for more than a year on ART. Viremic progressors (VP) were patients who were not on ART and were viremic. VP1 had a CD4 count of 192 cells/μl and a viral load of 7,250 copies/ml and VP2 had a CD4 count of 296 cells/μl and a viral load of 17,100 copies/ml. Healthy donors (HD) refer to HIV-negative individuals.

4.3.2 NK cell cytokine secretion and degranulation assay

PBMCs were collected from donor whole blood after Ficoll-Paque Plus gradient centrifugation (GE Healthcare Life Sciences, Baltimore, MD). Next, NK cells were isolated from HD and CP PBMCs with NK cell-specific Miltenyi beads (Miltenyi Biotec, Gaithersburg, MD). The NK cell purity was generally in the range of 90%. Sample purity levels can be found in Figure 23. Purified NK cells were treated for 6 hours (termed a pulse) with either medium (RPMI

supplemented with 10% fetal bovine serum [FBS], 1% Pen-Strep, and 10 U/ml IL-2) or various concentrations of IFN-α (2a) diluted in medium (1 U/ml to 200 U/ml; PBL Assay Science, Piscataway, NJ). At the end of IFN-α pulses, cells were washed three times and cocultured for 4 hours at a 1:1 effector/target ratio with K562 cells to assess the relative effect of IFN-α on NK degranulation and cytokine production. NK cells treated with IFN-α were also washed and cultured alone to determine any nonspecific effects of IFN-α on NK cell function in the absence of target cell stimulation. Untreated NK cells were also cultured alone as a negative control. All cultures were performed in medium supplemented with protein transport inhibitors (Golgi Plug, 1 μg/ml; Golgi Stop, 0.7 μg/ml; CD49d, 1 μg/ml [BD Bioscience, San Diego, CA]), as well as an antibody to CD107a (FITC; BD Bioscience, clone H4A3). K562 cells were used because their lack of MHC-I surface expression makes them targets for NK cells. At the end of the 4 hours, the cells were washed three times and stained for surface markers: CD3 (PB; BD Bioscience, clone UCHT1), CD16 (PerCPcy5.5; BD Bioscience, clone 3G8), and CD56 (APCH7 [BioLegend, San Diego, CA], clone HCD56). Cells were also probed for the production of cytokines via intracellular staining with a focus on IFN-γ (PECy7, BioLegend, clone B27) and MIP-1β (PE, BD Bioscience, clone D21-1351).

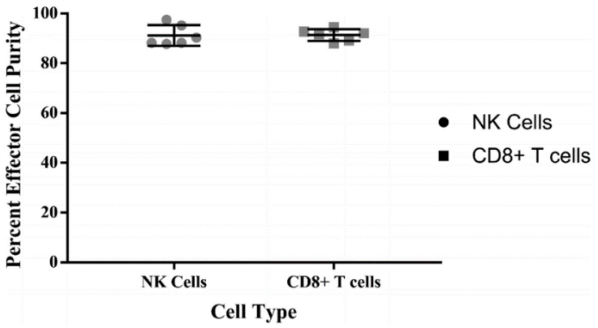

Figure 23. Representative plot of percent purity of effector cells prior to each experimental assay run. NK cells and CD8+ T cells employed as effectors in each experiment were obtained by cell specific Miltenyi isolation kits, with an average 90% cell purity.

4.3.3 NK cell cytotoxicity assay

The robust production of cytokines by an effector cell may not always correlate with cytotoxicity. Therefore, to assess NK cytotoxicity, we used the following assay adapted from Derby et al. (2001). Briefly, effector NK cells from CP PBMCs were isolated as previously described. CD4+ T cells were also isolated from a portion of patients PBMCs. Next, NK cells were pulsed as described above with media for a baseline control or media with 100 or 200 U of IFN-α. CD4+ T cells, however, were pulsed with medium only. To distinguish between effector and target cells, K562 cells were labeled with 2.5 μM of CFSE (carboxyfluorescein succinimidyl ester) at 10^6 cells/ml of CFSE with gentle vortexing. CFSE was then quenched with lukewarm FBS. The K562 cells were then washed three times and cocultured for 4 hours with pretreated, washed, NK cells at a 5:1 effector/target ratio (targets at 100,000 cells per well in 96-well flat bottom plates). Killing was determined by flow cytometry as CFSE-positive cells that co-expressed 7AAD and annexin V. To affirm that any cytotoxicity observed was NK cell specific, CFSE-labeled K562s were also cultured with CD4+ T cells at the same effector/target ratios. Any background death observed in CD4/K562 wells was subtracted from NK:K562 cultures at each effector/target ratio.

4.3.4 NK cell and CD8+ T cell suppression assay

PBMCs were isolated from HD and ES blood as described above. In ES these PBMCs were divided into two sets: from one set CD8+ T cells were isolated (by positive selection) and from the other NK cells were isolated by negative selection. CD4+ T cells were then isolated, by negative selection, from the flow-through of the CD8+ T cell isolation. In HD, PBMCs were split into two sets with one set used for NK cell isolation and the other for CD4+ T cell isolation. All cells were isolated with cell type-specific Miltenyi beads. As with NK cells, CD8+ T cell purity levels were generally in the 90% range (Figure 23). Effector cells (CD8+ T cells or NK cells) were then pulsed with either medium or various concentrations of IFN-α as described above. Next, target cells (bulk CD4+ T cells) were infected by spinoculation at 30 ng of HIV-1 p24/100,000 cells with a pseudotype virus (HIV-1-NL4-3ΔEnv–GFP) for 2 hours at 1,200 × *g* and 37°C. HIV-1-NL4-3ΔEnv–GFP is a lab strain of HIV-1 that has *env* replaced with *gfp*. At the end of the cytokine pulse, effector cells were washed three times and co-cultured over 72 hours with infected CD4+ T cells at an effector/target ratio of 1:1. The percent GFP expression, as measured by flow cytometry, was subsequently used to assess the percent viral suppression according to the following formula: [1 – (%GFP + $CD4^+$ T cells cultured with effectors)/(%GFP + $CD4^+$ T cells without effectors)] × 100.

The median percentage of GFP infection in CD4+ T cells that were cultured in medium alone was 6% for HD and 13% for ES. In VP, an average of 6% GFP in infected CD4+ T cells was seen in the two subjects studied. For transwell experiments, 1 million infected CD4+ T cells were cultured at the bottom of 12-well transwell plates, while 1 million CD8+ T cell effectors were cultured in the transwell inserts. Infected CD4+ T cells were cultured alone to determine the maximum percent infection, and infected cells were cocultured in the same well with CD8+ T cells

to determine the effect of direct contact of the effector and target cells. Cultures were maintained for 3 days, and viral replication suppression was measured as a percentage of GFP expression via flow cytometry.

4.3.5 CD8+ T cell cytokine secretion and degranulation assay

To ensure that the concentrations of IFN-α used in this assay to enhance NK cell function did not impair the cytokine-producing abilities of patient CD8+ T cells, general CD8+ T cell cytokine production was also assessed in CP. Specifically, CD8+ T cells were isolated from CP PBMCs (Miltenyi Biotec) and pulsed with either medium or various concentrations of IFN-α diluted in medium as before. At the end of this pulse, cells were washed three times and stimulated for 4 hours with either medium alone (baseline control) or anti-CD3 MAb at 1 μg/ml (BD Bioscience), all in the presence of protein transport inhibitors, as well as an antibody to CD107a. The cells were then washed and stained for surface markers CD3 (PB, BD Bioscience, clone UCHT1) and CD8 (APCH7, BD Biosciences, clone SK1), as well as cytokine production via intracellular staining for IFN-γ (PECy7, BioLegend, clone B27) and MIP-1β (PE, BD Bioscience, clone D21-1351).

4.3.6 CD8+ T cell in IFN-γ ELISpot assays

Beyond general CD8+ T cell function, we also sought to determine whether IFN-α treatment of CP CD8+ T cells inhibited their ability to release the type II interferon (IFN-γ) in an HIV-specific manner. We performed IFN-γ-specific ELISpot assays as previously described (Pohlmeyer et al, 2018), following pretreatment of patient cells with the candidate type I IFN (IFN-α 2a). Specifically, PBMCs were isolated from CP whole blood, seeded at 250,000 cells per well, and treated for 6 hours with either medium as a baseline control or 100 U of IFN-α. Cells were then washed three times, transferred into 96-well ELISpot plates precoated with IFN-γ-specific

antibodies (Mabtech, Human IFN-γ ELISpot Plus), and stimulated for 18 hours with either medium alone or 10 μg/ml of overlapping consensus B Gag and Nef peptides (NIH AIDS Reagent Program). The plate was then processed according to the manufacturer's protocol and read, blinded, by an independent investigator using an automated reading system.

4.3.7 Statistics

Statistics were generated by GraphPad Prism 7. For each set of experiments, the one-way repeated-measures analysis of variance (ANOVA) was used to assess statistical significance, and levels of significance were parsed out with the ensuing P value demarcations as follows: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$. To account for any violations in sphericity, the Geisser-Greenhouse correction was used during data analysis. Finally, in all cases, a Dunnett's multiple-comparison test was used to assess variations of treatment groups from no-treatment baseline controls. Standard deviations were used to determine the variations from the mean within treatment groups.

4.4 RESULTS

4.4.1 IFN-α enhances degranulation and cytokine production in CP NK cells

The goal of this study was to determine whether short treatments of IFN-α could be used to enhance CP NK cell effector function without incapacitating the effector functions of CD8+ T cells. To answer this question, assays measuring NK cell degranulation and cytokine production were used. The presence of residual intracellular drugs prevents effective superinfection of CP CD4+ T cells. We therefore assessed the effects of IFN-α on CP NK cell function by measuring the response of these cells to K562 cells. Briefly, isolated CP NK cells were pulsed with media alone (baseline control) or various concentrations of IFN-α and subsequently cocultured with K562

targets at a 1:1 effector/target ratio in the presence of a CD107a antibody and protein transport inhibitors. NK cells from healthy donors (HD) were treated in a similar fashion as a means of comparison to results from CP-treated NK cells. The percent expression of various cytokines was then assessed via flow cytometry. Relative to untreated NK cells, there was little to no nonspecific production of cytokines from NK cells treated with IFN-α and cultured in the absence of target cells (data not shown). However, in the presence of targets, IFN-α treatment significantly increased the cytokine-producing capacity of HD NK cells. As shown in Figure 24A, IFN-α-treated HD NK cells, relative to baseline, showed a marked increase in CD107a degranulation ($P < 0.0008$). IFN-α treatment also significantly increased the production of MIP-1β (Figure 24B, $P < 0.0006$) and IFN-γ (Figure 24C, $P < 0.004$). CP NK cells also yielded significant amounts of CD107a (Figure 24D, $P < 0.001$), MIP-1β (Figure 24E, $P < 0.0001$), and IFN-γ (Figure 24F, $P < 0.002$), with stimulation of K562 cells following IFN-α pulses.

More importantly, not only were individual cytokine secretion and degranulation profiles of IFN-α-treated NK cells enhanced, but their polyfunctional profiles were enhanced as well. As shown in Figure 25, pretreatment of CP NK cells with IFN-α significantly increased the co-expression of CD107a and MIP-1β (Figure 25A, $P < 0.002$), CD107a and IFN-γ (Figure 25B, $P < 0.005$), and MIP-1β and IFN-γ (Figure 25C, $P < 0.004$). In addition, there was also enhanced co-expression of all three proteins in CP (Figure 25D, $P < 0.0007$) and HD (Figure 25E, $P < 0.0008$). There was no significant difference in the percentage of polyfunctional NK cell responses between HD and CP. We also assessed whether longer treatments with IFN-α would further increase CP NK cell polyfunctionality and found that while 6 hours of treatment resulted in a more potent response than one hour, no added benefit was seen with 18 or 24 hours of pre-incubation with the cytokine (Figure 26).

4.4.2 IFN-α significantly enhances CP NK cytotoxicity

In assessing whether IFN-α treatment actually enhanced CP NK cell cytotoxic responses against target cells, we labeled K562 cells with CFSE and used that as a means of detecting cell death in the target cell population within an effector-target co-culture. As described in Materials and Methods, death was assessed via flow cytometry as CFSE+ cells that were double positive for 7-aminoactinomycin D (7AAD) and annexin V (Figure 27). Of the six CP studied at the 5:1 effector/target ratio, brief IFN-α treatments significantly enhanced CP NK cell cytotoxicity with an approximate net difference in death of 20% in IFN-α-treated NK cells relative to untreated baseline NK cells (Figure 28, P = 0.0004 for 100 U and 0.0001 for 200 U). To ensure that cytotoxic responses in these experiments were NK cell specific, co-culture of K562 cells with CD4+ T cells (as effectors) was performed simultaneously with co-cultures of K562 cells with NK cells. Death observed in these wells was subtracted from that observed in the NK/K562 cocultures.

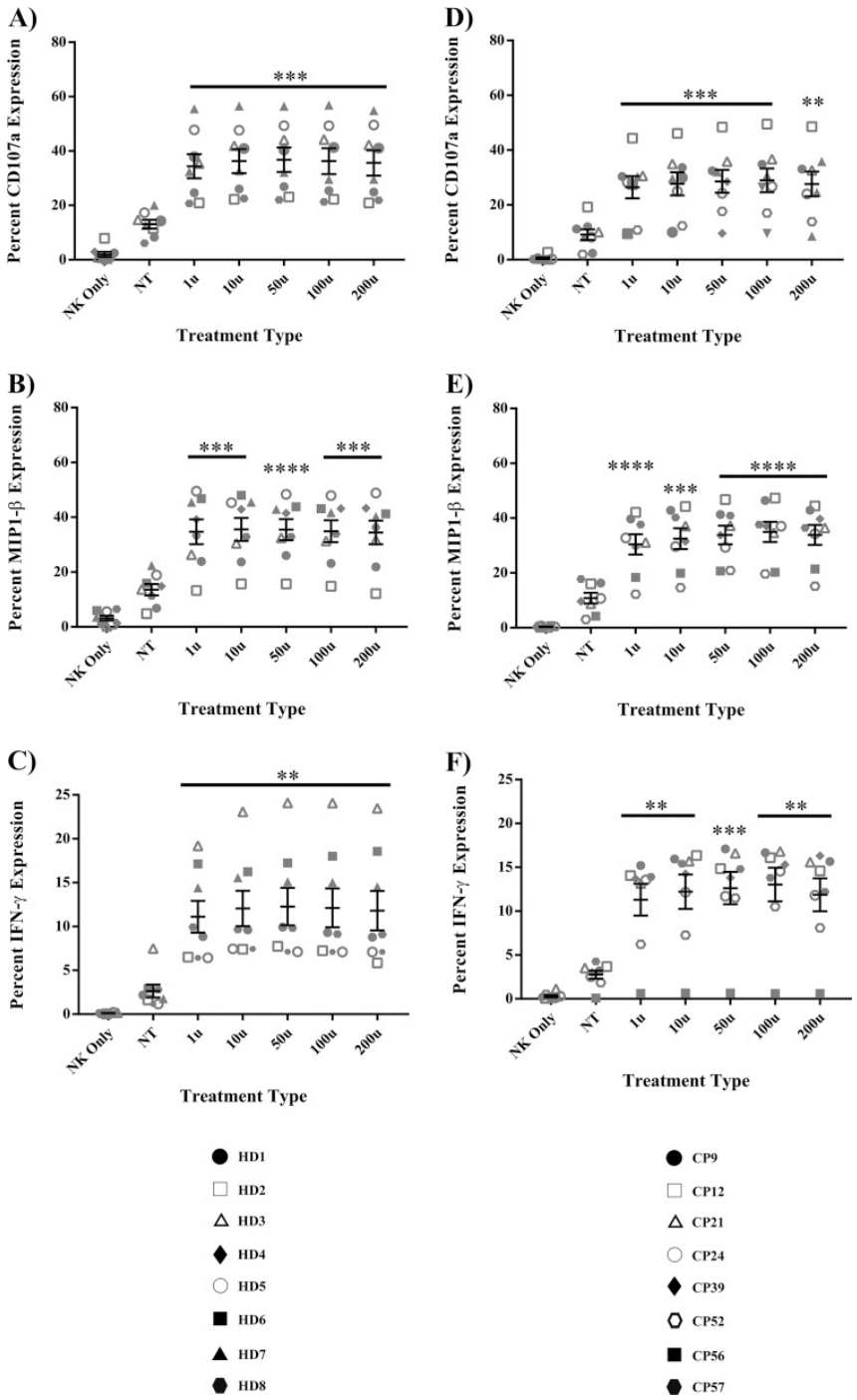

Figure 24. IFN-α enhances degranulation and cytokine production in HD and CP NK cells. NK cells from 8 HD and 8 CP were treated with either medium alone (NT baseline) or various concentrations of IFN-α for 6 h, washed, and then cocultured for 4 h with target K562 cells or medium alone (NK only) in the presence of a CD107a antibody and protein transport inhibitors. Enhancement of degranulation and cytokine production was subsequently studied by flow cytometry following surface and intracellular staining. (A) CD107a degranulation in HD; (B) MIP-1β in HD; (C) IFN-γ in HD; (D) CD107a degranulation in CP;

(E) MIP-1β in CP; (F) IFN-γ in CP. Triplicates were performed for each patient with error bars representing standard deviation from mean within treatment groups. One-way repeated-measures ANOVA was used to determine difference between baseline and specific treatments. Asterisks indicate differences from a no-treatment baseline control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

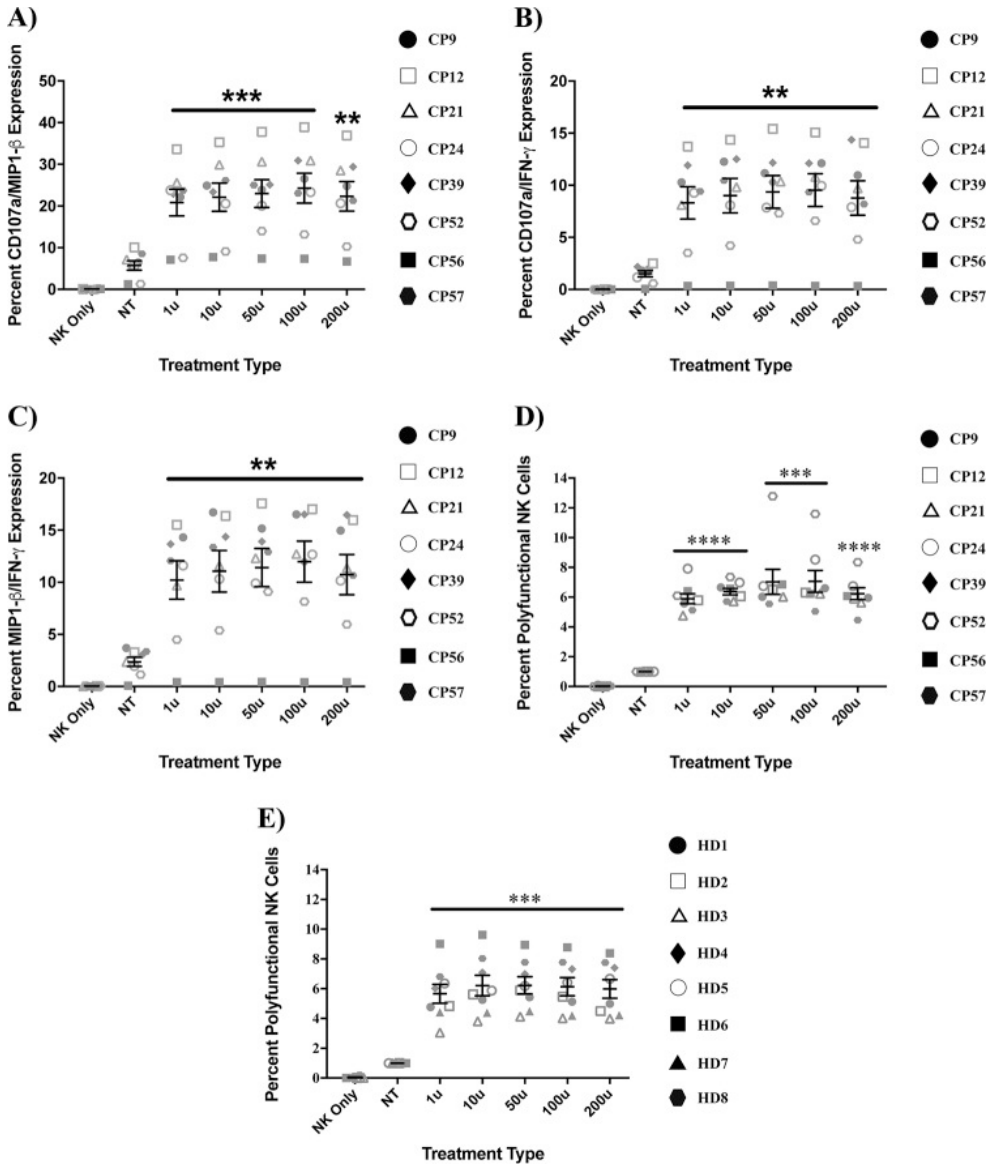

Figure 25. IFN-α enhances HD and CP NK cell polyfunctionality. NK cells from 8 HD and 5 to 8 CP were pulsed with either medium (NT baseline) or various concentrations of IFN-α for 6 h, washed, and then cultured alone (NK only) or for 4 h with target K562 cells in the presence of antibodies to CD107a and protein transport inhibitors. The assessment of enhancement of degranulation, bi- and trifunctional protein production was subsequently studied via flow cytometry following surface and intracellular staining. In our analysis, bulk CP NK cells were probed for the simultaneous production of either CD107a and MIP-1β (A), CD107a and IFN-γ (B), and MIP-1β and IFN-γ (C). Bulk CP (D) and HD (E) NK cells were then also probed for the simultaneous production of CD107a, MIP-1β, and IFN-γ. In all experiments, triplicates were performed for each subject, with error bars representing standard deviations from mean within treatment groups. One-way repeated-measures ANOVA was used to determine the differences between baseline and specific treatments. Asterisks indicate differences from a no-treatment baseline control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

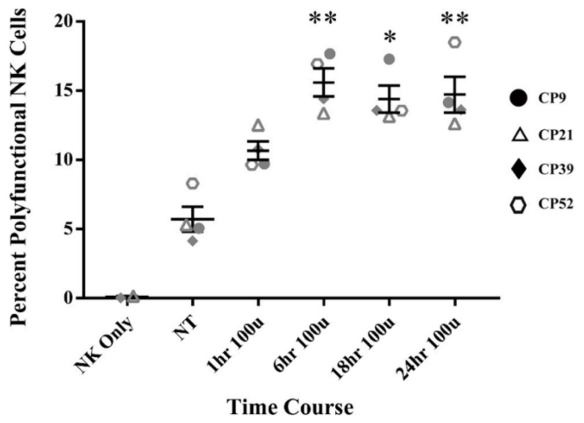

Figure 26. Extended incubation beyond 6 hours of NK cells with IFN-α does not further enhance their polyfunctional responses. NK cells were isolated from 4 CPs and treated with either media alone (NT baseline), or a single concentration of IFN-α (100u) over either 1, 6 18 or 24 hours. At the end of the incubation, cells were washed, then cultured alone (NK only) or for 4 hours with target K562 cells in the presence of antibodies to CD107a and protein transport inhibitors. The assessment of polyfunctional protein production was subsequently studied via flow cytometry following surface and intracellular staining. In analysis, bulk NK cells were probed for the simultaneous production of CD107a, MIP1-β and IFN-γ. In all experiments, triplicates were performed for each subject with error bars representing standard deviation from mean within treatment groups. One way repeated measures ANOVA was used to determine difference between baseline and specific treatments. Stars above show differences from no treatment baseline control with * p<0.0001.

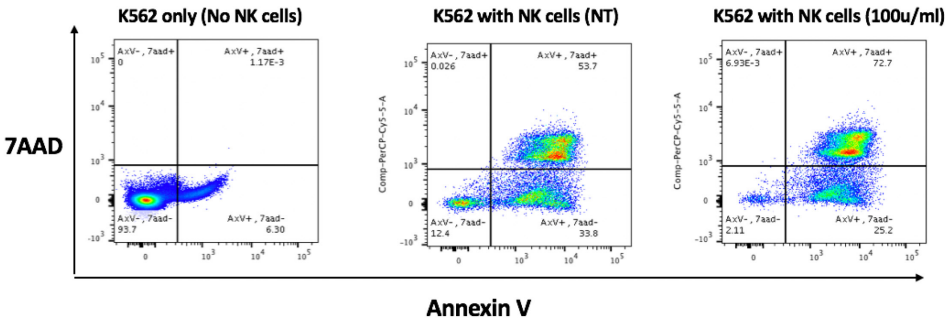

Figure 27. Representative flow plot of NK:K562 cytotoxicity assay. NK cells (or CD4+ T cells) were isolated from 6 CP and treated with either media alone (NT baseline), or varying concentrations of IFN-α for 6 hours, washed, then co-cultured for 4 hours at a 5:1 effector:target ratio with CFSE labelled target K562 cells. Cell death was then assessed by the co-expression of 7AAD and Annexin V on K562 cells. From the left, cell death assessment in: CFSE positive K562 cells without NK effectors, CFSE positive K562 cells co-cultured with untreated NK cell effectors and CFSE positive K562 cells co-cultured with effector NK cells treated a prior, for 6 hours, with 100u of IFN-α.

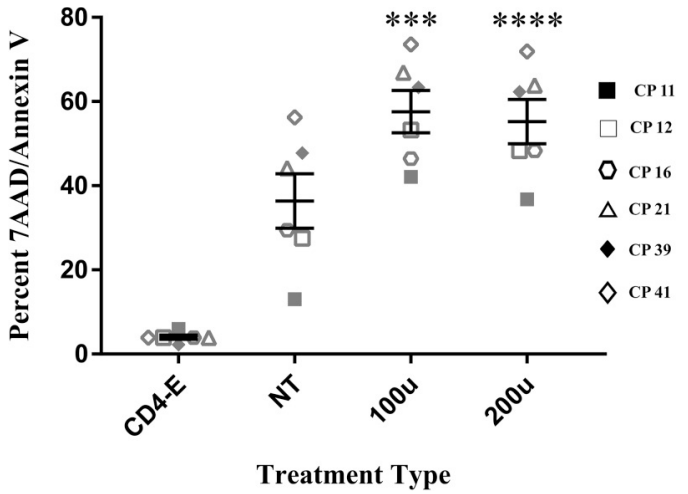

Figure 28. IFN-α enhances CP NK cell cytotoxicity. NK cells and CD4+ T cells were isolated from 6 CP and treated with either medium alone (NT baseline) or various concentrations of IFN-α for 6 hours, washed, and then co-cultured for 4 hours with CFSE-labeled target K562 cells. Cell death was assessed based on the co-expression of 7AAD and annexin V on K562 cells. Experiments were conducted at a 5:1 effector/target ratio. In each experiment, CD4+ T cell effectors (CD4-E) were used at the same effector/target ratio as a control to affirm NK-specific cytotoxicity. Asterisks indicate differences from a no-treatment baseline control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Duplicates were performed for each patient with error bars representing standard deviations from the mean within treatment groups. One-way repeated-measures ANOVA was used to determine the difference between baseline and specific treatments

4.4.3 IFN-α enhances the suppressive capacity of HD and ES NK cells

To determine whether IFN-α treatment of NK cells could impact their control of HIV-1 replication in vitro, we assessed the suppressive capacities of HD and ES NK cells with or without IFN-α treatment. CP were not used for these experiments, since the presence of residual antiretroviral drugs in CD4+ T cells from these patients prevents efficient superinfection of their cells in vitro.

NK cells from 8 HD and 10 ES were treated for 6 hours with either medium alone (baseline control) or various concentrations of IFN-α. The cytokine was then washed off, and the NK cells were co-cultured with autologous CD4+ T cells infected with a lab strain of HIV-1 as described in

Materials and Methods. Suppression, defined as a reduction in the percentage of target cells expressing GFP, was calculated following flow cytometry, with infected targets cultured without effectors being used to normalize the percent infection. As expected, not all HD NK cells were able to suppress viremia at baseline. However, some concentrations of IFN-α, particularly 50 U and higher, significantly improved NK cell suppression capacity in these subjects (Figure 29A, $P < 0.04$). A higher baseline level of suppression was seen with ES NK cells, and this was further enhanced after treatment with 10 U or higher of IFN-α (Figure 29B, $P < 0.04$).

4.4.4 IFN-α treatment enhances degranulation and the cytokine producing capacity of CP CD8+ T cells

Having shown that IFN-α significantly enhanced NK cell polyfunctionality and HIV-1 suppressive capacity, we next sought to determine whether this cytokine had inhibitory effects on $CD8^+$ T cell responses, as has been previously shown in untreated HIV-1 infection. To answer this question, assays measuring CD8+ T cell cytokine production and degranulation after treatment with anti-CD3 monoclonal antibody (MAb) were employed. Figure 30 summarizes results for CD8+ T cells. Specifically, IFN-α treatment increased degranulation in CD8+ T cells following stimulation, as measured by CD107a (Figure 30A, $P < 0.0008$). MIP-1β production was also similarly increased after IFN-α treatment relative to untreated, stimulated, CD8+ T cells (Figure 30B, $P < 0.04$). In addition, CD8+ T cells from CP treated with IFN-α also showed significant increases in IFN-γ production (Figure 30C, $P < 0.02$). Stimulation of $CD8^+$ T cells with IFN-α alone resulted in minimal MIP-1β and IFN-γ production (data not shown).

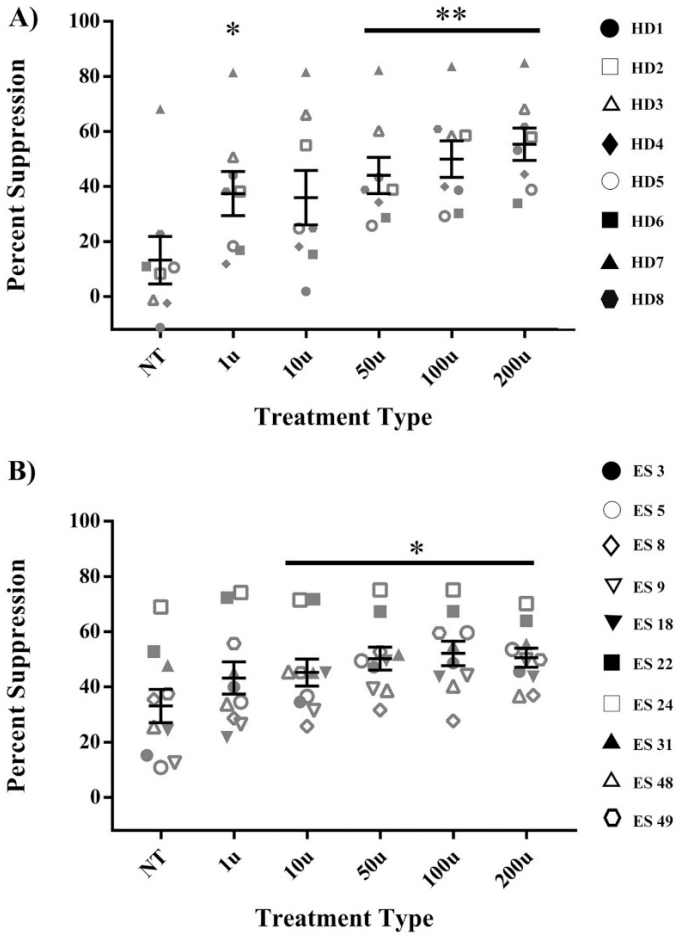

Figure 29. IFN-α enhances the suppressive capacity of HD and ES NK cells. NK cells from 8 HD (A) and 10 ES (B) were treated with either medium alone (NT) or various concentrations of IFN-α for 6 hours, washed, and then co-cultured over 3 days at a 1:1 ratio with autologous CD4+ T cells infected with a GFP-tagged pseudotype lab strain of HIV-1. Viral replication suppression was measured as a percentage of GFP expression via flow cytometry. Triplicates were performed for each patient, with error bars representing the standard deviation, from means within treatment groups. One-way repeated-measures ANOVA was used to determine the difference between baseline and specific treatment groups. Asterisks indicate the differences from a no-treatment baseline control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

4.4.5 IFN-α treatment does not inhibit CP HIV specific CD8+ T cell IFN-γ responses

Having demonstrated that IFN-α treatment enhanced global CP CD8+ T cell responses, we next analyzed its effect on CP HIV-specific CD8+ T cells responses. To do this, we performed enzyme-linked immunospot (ELISpot) studies using peripheral blood mononuclear cells (PBMCs) from CP pulsed with a 100U dose of IFN-α for 6 hours prior to stimulation with overlapping Gag and Nef peptides. As shown in Figure 31A, there was minimal nonspecific production of IFN-γ with IFN-α treatment. Furthermore, pretreatment of PBMCs with IFN-α had no effect on the number of IFN-γ-producing cells following stimulation with Gag and Nef peptides.

4.4.6 IFN-α enhances the suppressive capacity of ES CD8+ T cells

We next analyzed the effect of IFN-α on the suppressive capacity of ES CD8+ T cells. Similar to the suppression experiments performed with ES NK cells, ES CD8+ T cells were isolated from eight ES and pulsed with either medium alone (baseline control) or various concentrations of IFN-α for 6 hours. The cells were then washed and co-cultured at a 1:1 effector/target ratio with autologous CD4+ T cells infected with a green fluorescent protein (GFP)-tagged pseudotype lab strain of HIV-1. The culture was maintained over 3 days, and suppression, defined as a reduction in the percentage of target cells expressing GFP, was measured by flow cytometry. Similar to the suppression observed with NK cells, each IFN-α treatment significantly improved the suppressive capacities of CD8+ T cells (Figure 31B, $P < 0.03$). In ES22 we demonstrated that suppression was contact dependent since separating the $CD8^+$ T cells from the target cells with a transwell abrogated the inhibitory response (Figure 32). In summary, IFN-α did not inhibit, but rather enhanced suppressive capacities of ES CD8+ T cells.

4.4.7 IFN-α enhances the suppressive capacity of VP CD8+ T cells

Because we were unable to determine the effect of IFN-α on the suppressive capacity of CP CD8+ T cells due to the presence of residual ART, we looked at the effect on two viremic subjects. In both cases, we saw enhanced suppressive capacity when CD8+ T cells were preincubated for 6 hours with IFN-α (Figure 31C).

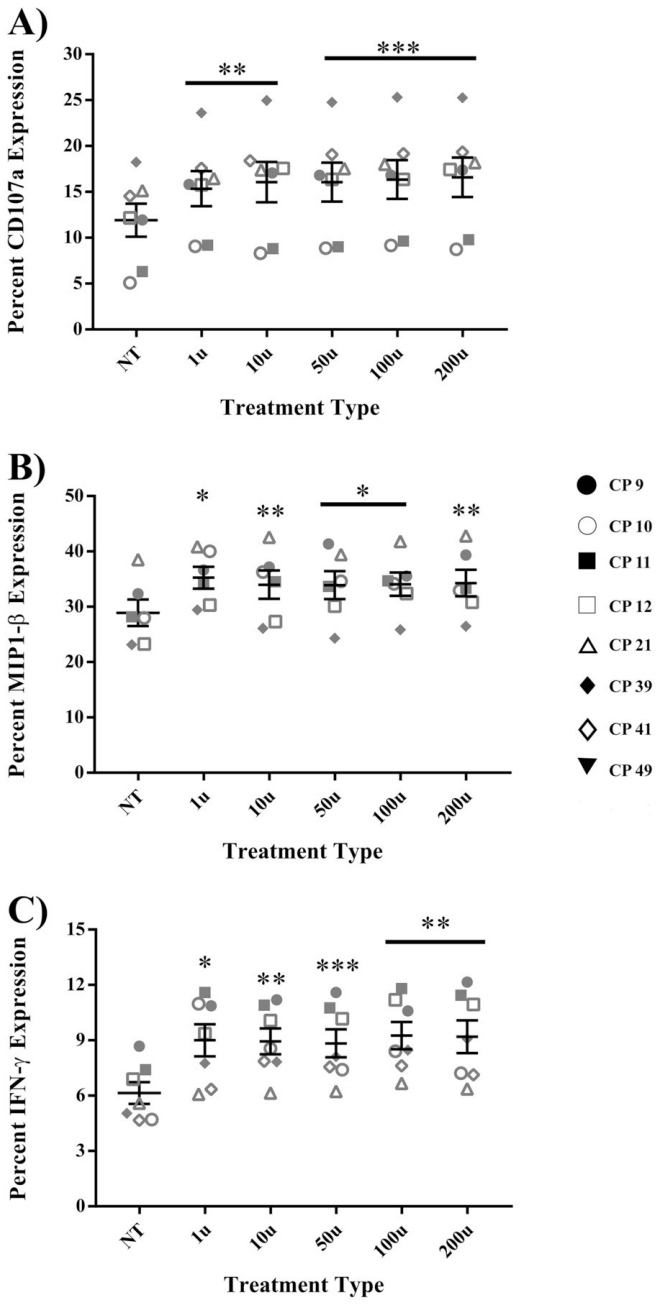

Figure *30*. IFN-α treatment enhances CP CD8+ T cell degranulation and cytokine production. CD8+ T cells from 6 to 8 CP were pulsed with either medium alone (baseline) or various concentrations of IFN-α for 6 hours, washed, and then cultured for 4 hours in the presence of a CD107a antibody and protein transport inhibitors. Enhancement of degranulation and cytokine production was subsequently studied via flow cytometry following surface and intracellular staining. Specifically, the cells were either not treated but stimulated (NT) or treated with various units of IFN-α and stimulated with anti-CD3 MAb. (A) CD107a degranulation; (B) MIP-1β; (C) IFN-γ. Triplicates were performed for each patient, with error bars representing the standard deviations from the mean within treatment groups. One-way repeated-measures ANOVA was used to determine the differences between baseline and

specific treatments. Asterisks indicate the differences from a no-treatment baseline control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

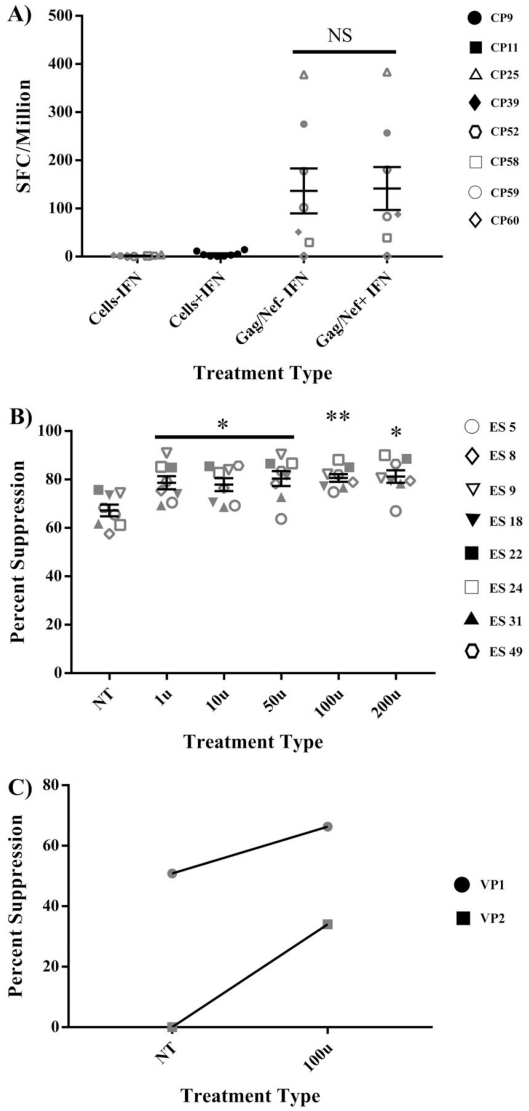

Figure 31. IFN-α enhances the suppressive capacity of VP and ES CD8+ T cells without inhibiting HIV-specific IFN-γ responses of CP CD8+ T cells. (A) PBMCs from 8 CP were pulsed with either medium alone (baseline) or 100 U/ml of IFN-α for 6 h, washed, and then cultured for 18 h with or without CMV, Gag, or Nef peptides. An IFN-γ ELISpot assay was then used to assess the effect of IFN-α treatment on the production of IFN-γ by CP CD8+ T cells. Duplicates were performed for each patient, with error bars representing the standard deviations from the mean within treatment groups; saturated responses (CMV) were not included in data. One-way repeated-measures ANOVA was used to determine the difference between baseline and 100-U treatments. (B) CD8+ T cells from 8 ES were pulsed with either medium alone (NT) or various concentrations of IFN-α for 6 h,

washed, and then cocultured over 3 days at a 1:1 ratio with autologous CD4+ T cells infected with a GFP-tagged pseudotyped lab strain of HIV-1. Viral replication suppression was measured as a percentage of GFP expression via flow cytometry. Triplicates were performed for each patient, with error bars representing the standard deviations from the mean within treatment groups. One-way repeated-measures ANOVA was used to determine the difference between baseline and specific treatments. Asterisks indicate the differences from a no-treatment baseline control *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (C) CD8+ T cells from 2 VP were pulsed with either medium alone (NT) or 100 U/ml of IFN-α for 6 h, washed, and then cocultured over 3 days at a 1:1 ratio with autologous CD4+ T cells infected with a GFP-tagged pseudotyped lab strain of HIV-1. Viral replication suppression was measured as a percentage of GFP expression via flow cytometry. Triplicates were performed for each patient.

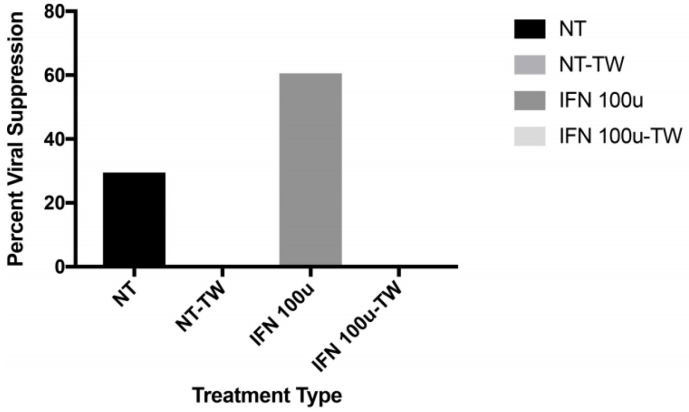

Figure 32. Viral suppression in ES CD8+ T cells is contact dependent. CD8+ T cells from an ES (ES22) were pulsed with either media alone (NT) or 100u of IFN-α for 6 hours. Cells were then washed, and co-cultured over 3 days at a 1:1 effector:target ratio (at 1 million cells for either effectors or targets) with autologous CD4+ T cells infected with a GFP tagged pseudotyped lab strain of HIV-1. Effector target cultures were either done together, or in transwells (TW) with target cells in the bottom of the plate and effectors in transwell inserts. Infected CD4+ T cells were also cultured alone for the purpose of normalization, and viral replication suppression was measured as a percentage of GFP expression via flow cytometry.

4.5 DISCUSSION

Several clinical trials with LRAs have not resulted in reductions in the size of the latent reservoir (Sogaard et al, 2015, Archin et al, 2012, Archin et al, 2014, Spivak et al, 2014, Rasmussen et al, 2014, Elliott et al, 2014, Elliott et al, 2015). One possible explanation for this might be that effector immune cells critical to the "killing" aspect of the strategy are inefficient. Several studies have also shown that some LRAs inhibit NK cell (Garrido et al, 2016, Pace et al, 2016) and CD8+ T cell (Jones et al, 2014, Walker-Sperling et al, 2016, Kwaa et al, 2017) function. To address these concerns, efforts are now being directed at enhancing immune effector function for the control of HIV (Perreau et al, 2017, Riley and Montaner, 2017). While many of these are directed toward CD8+ T cells, NK cells would be important effector cells to consider for the purpose of complementing CD8+ T cell function in HIV control (Scully and Alter, 2016, Carrington and Alter, 2012). In the present study, we confirm prior studies that showed that pre-treatment of NK cells with IFN-α enhances cytokine secretion, viral suppression, and the cytotoxic potential of HD (Ellis et al, 1989, Jewett and Bonavida, 1995, Tomescu et al, 2007, Tomescu et al, 2015) and ES NK (Tomescu et al, 2012) cells against target K562 cells. While Portales et al. (2003) showed an enhancement in NK cell perforin and granzyme A expression in patients who were treated with pegylated IFN-α2b, this is to our knowledge the first demonstration of IFN-α directly enhancing cytokine secretion and the killing capacity of CP NK cells. IFN-α pulses also enhanced the ability of HD and ES NK cells to suppress HIV-1 replication in autologous CD4+ T cells. While we were not able to study this particular function in CP NK cells, the fact that CP and HD NK cells had similar levels of degranulation and cytokine production in response to IFN-α following stimulation with K562 cells strongly suggests that CP NK cells would also have enhanced antiviral activity.

Furthermore, in addition to studies showing improvement in direct NK cell cytotoxic activity, Tomescu et al. showed that IFN-α enhances NK cell-mediated antibody-dependent cell-mediated cytotoxicity (Tomescu et al, 2017). Thus, it appears that this cytokine enhances multiple facets of NK cell function.

Betts et al. demonstrated that the polyfunctionality of CD8+ T cells was a major correlate of HIV-specific immunity (Betts et al, 2006). These results have since been corroborated not only in CD8+ T cell studies (Migueles and Connors, 2015) but also in NK cell studies. Specifically, in two separate reports, Boulet et al. (2010) and Kamya et al. (2011) demonstrate that HIV^+ patients with the HLA-Bw4 ligand and its cognate NK receptor, KIR3DL1, had more polyfunctional NK cells, which accordingly contributed to slower disease progression. We show here for the first time that IFN-α enhances polyfunctionality in CP NK cells to a level that is similar to that seen in HD NK cells. This enhanced NK cell polyfunctionality may lead to better control of HIV-1 replication in shock-and-kill strategies.

We minimized our IFN-α pre-incubation exposure for two reasons. The first was to determine the feasibility of efficiently enhancing NK cell effector function within the narrow time frame that effector cells have for the elimination of infected CD4+ T cells prior to the release of virions after latency reversal (Walker-Sperling et al, 2015). We show here that 6 hours of pre-incubation with IFN-α was as effective as 24 hours. Our finding that this short window of exposure was sufficient to efficiently and significantly enhance the suppressive, cytotoxic, and polyfunctional responses of NK cells in CP is exciting and suggests that it may be possible to optimize NK cell activity while avoiding IFN-α-mediated immune exhaustion in both NK cells and CD8+ T cells (Odorizzi and Wherry, 2013, Telenti, 2014).

Several studies have suggested that type I IFNs can lead to apoptosis of memory T cells in mice, thereby compromising the adaptive immune response (Welsh et al, 2012, McNally et al, 2001, Bahl et al, 2006). Other studies have shown that IFN-α induces immune activation of CD8+ T cells from HIV-positive subjects in vitro (Rodriguez et al, 2006, Hua et al, 2014) and in vivo (Manion et al, 2012). Furthermore, studies in humanized mice have shown that blocking the IFN-α/β receptor enhances HIV-specific immune responses (Cheng et al, 2017, Zhen et al, 2016). In contrast to these studies, we show here for the first time that pre-treatment with IFN-α for a limited period of time did not inhibit HIV-specific CD8+ T cell IFN-γ responses and resulted in a moderate enhancement of global CD8+ T cell responses in CP. Furthermore, there was also a modest enhancement in HIV-suppressive CD8+ T cell responses in ES and in two VP. These responses are not due to non-specific activation of CD8+ T cells by IFN-α; instead, we show here that the suppression was contact dependent, which is consistent with our prior studies (Shan et al, 2012, Walker-Sperling et al, 2014, Veenhuis et al, 2018). However, further studies will be needed to definitively determine whether this is due to direct cytotoxic activity, as has been previously demonstrated for IFN-α-treated NK cells (Tomescu et al, 2015). If this enhanced HIV-specific CD8+ T cell response is confirmed with in vivo studies, it would suggest that pulse therapy with IFN-α could enhance both innate and adaptive antiviral immune responses in CP. A clinical trial has already shown that treatment with IFN-α prolongs the time to viral rebound following the cessation of ART (Azzoni et al, 2013). In another study, NK cell activation was associated with a decline in cell-associated HIV-1 DNA in HIV-1/HCV-co-infected subjects treated with pegylated IFN-α and ribavirin (Hua et al, 2017). While a recent study in SIV-infected monkeys on ART suggested that pegylated IFN-α2a treatment did not significantly affect the size of the viral reservoir (Palesch et al, 2018), this could potentially be because there was no LRA given in

conjunction with this immunomodulatory agent. An exciting new direction, given these data, would be the coupling of short course IFN-α therapy with LRAs in shock-and-kill trials with the goal of eliminating reactivated reservoirs without exacerbating pre-existing immune exhaustion profiles of CP effector cells.

5 CONCLUSIONS AND FUTURE DIRECTIONS

It has now been decades since its discovery, yet viral latency continues to stand as a barrier towards persistent efforts towards a cure to HIV/AIDS. The goal of this thesis work, therefore, was to gather novel data that would contribute to the growing body of knowledge on the nature of the latent reservoir and how we might combat it.

In our first chapter, we look to define the size of the inducible latent reservoir in ES relative to CP as a (possible) guide to what a goal of inducible reservoir frequency could be in order to attain a functional cure. Specifically, we employ the newly described IPDA and make two important observations. First, our findings from a cohort of ES and CP demonstrates that the frequency of both total and intact proviruses in ES is significantly smaller than in CP, but that the proportion of intact to total proviruses does not significantly differ between the two patient groups. If this ratio had been significantly smaller in ES, it might have suggested that ES achieve elite control possibly due to a disproportionate infection with defective viruses (Calugi et al, 2006, Kirchoff et al, 1995, Miura et al, 2010). That there was no difference, however, reinforces data from many studies (Blankson et al, 2007, Lamine et al, 2007, Bailey et al, 2008, Julg et al, 2010, Buckheit et al, 2012, Salgado et al, 2014) that show that while the afore mentioned concern may be true in some ES, it is not true for all. The second important finding we make is that although most ES do have smaller inducible reservoirs (Salgado et al, 2014, Mendoza et al, 2012, Noel et al, 2016) a small latent reservoir may not be required for either elite control or a functional cure. We conclude this from our observation of one patient, ES24, who had the largest frequency of intact proviruses relative to all other patients studied, yet had at the time of study, maintained control in the absence of ART. Overall therefore, even though this study is limited by a small sample size, it sheds important light not only on our current understanding of elite control as it relates to the size of the inducible versus non-inducible reservoir, but also on the need to

completely decipher the phenomenon of elite control in order to improve our chances of obtaining a functional cure to HIV/AIDS, for all.

After quantifying the reservoir via IPDA in ES, we move on to chapter 2 to assess how drugs targeted at reactivating the viral reservoir might impact immune effector cell function. This is an important topic to focus on given that shock-and-kill remains the most widely clinically tested attempt at eliminating the latent reservoir despite data suggesting that drug combinations that successfully and efficiently reverse latency *in vitro* might simultaneously adversely impact immune effector cell function (Pace et al, 2016, Garrido et al, 2016, Akimova et al, 2012, Jones et al, 2014, Walker-Sperling et al, 2016, Clutton et al, 2016). Using *in vitro* studies, we specifically focused on ingenol-B and its combination with other LRAs to determine its effect on the suppressive capacity of ES CD8+ T cells. After studying an array of individuals (HD, ES and CP), we determined that this drug did not adversely affect HIV suppressive function of ES CD8+ T cells except when combined, at higher doses, with the bromodomain inhibitor JQ1. Thus far, a clinical trial using a derivative of ingenol (ingenol mebutate) has demonstrated that this active ingredient as part of a topical treatment for actinic keratosis, disrupted latent HIV in the skin tissue microenvironment, and achieved this without excessive immune activation or harm to immune cells (Jiang et al, 2019). While this corroborates our *in vitro* findings, it is a single trial with a single drug, locally administered and the concern from this work rests in combining ingenol with other LRAs. As such, as we strive to reverse latency and potentially eliminate inducible reservoirs in an attempt at a cure, the need for caution in balancing drug dosing and drug combination choices with the potential for adverse effects on immune effector cell function remains.

Currently, the advent of effective ART currently enables HIV infected individuals to live relatively normal lives. Yet the fact remains that given a choice, a cure is preferred to taking a pill a day, for life. Furthermore, not all patients can adhere to the medication regimen (due to health or other socio-economic reasons) and taking a pill every day for life can also have adverse side effects on individuals. For this reason, a cure (even a functional one) is necessary (Deeks et al, 2016), and yet data suggest that untreated HIV+ individuals unable to control infection fail to do so due to several factors including impaired cytokine production and/cytotoxic effector cell function. As such, the success of cure agendas including that of shock-and-kill will probably depend on enhancing patient effector cell function (Perreau et al, 2017, Riley and Montaner, 2017).

In the final chapter of this work, we demonstrate the ability of short-course alpha interferon (IFN-α) treatments to effectively enhance such effector functions in chronic progressor NK cells without inhibiting general CD8+ T cell function. These results point to the possibility of exploring such short-course IFN-α treatments for the enhancement of effector cell function in HIV+ patients in future cure strategies. While much of this work involved the use of an X4-tropic virus, data suggest that the majority of patients are first infected with an R5-tropic virus that then evolves into an X4-tropic infection (Weinberger and Perelson, 2011). Furthermore, the viruses employed were mainly single round pseudotyped reporter viruses with the absence of envelope precluding opportunities for studies on other aspects of NK cell function namely antibody dependent cellular cytotoxicity or ADCC. In one trial using IFN-α as an immunotherapy to treat CP (Azzoni et al, 2013), findings from ex-vivo studies showed that genes associated with ADCC were identified in IFN-α responders and absent in non-responders (Papasavvas et al, 2019). Given this data, an important future direction would be to repeat these studies (with a possible cocktail of cytokines) using replication competent R5-tropic viruses with a focus on ADCC function via both broadly

neutralizing and non-neutralizing antibodies. Since broadly neutralizing antibodies and NK cells are gradually gaining renewed traction in the HIV cure agenda arena, the findings from these studies could have implications for cure strategies.

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7 CURRICULUM VITAE

EDUCATION

Johns Hopkins University — Doctor of Philosophy in Infectious Diseases: Expected May 2020
Cumulative GPA: N/A

Mount Holyoke College — Bachelor of Arts: May 2012
Cumulative GPA: 3.68/4.00 — Major: Biology Minor: English

RELEVANT COURSE WORK/EXPERIENCE

Immunology Genetics and Molecular Biology Cellular Biology Biostatistics Retail Investing

RELEVANT LABORATORY EXPERIENCE

Graduate Student, Department of Infectious Diseases- Johns Hopkins University School of Medicine Apr. 2016- May 2020
- Assessing, and enhancing, the role of Natural Killer Cells in HIV/AIDS suppression and control

Research Intern, Immunology Department- Noguchi Memorial Institute for Medical Research Ghana Apr. 2015- Jun. 2015
- Studied the role of Endothelial Progenitor Cells in the pathogenesis of Cerebral Malaria

Student Researcher, Immunology Laboratory- Mount Holyoke College Sept. 2011- May 2012
- Investigated the role of the immunosuppressant, IL-10, in MAIDS susceptibility.

Research Intern, Undergraduate Student Scholars Program- University of Pennsylvania Jun. 2011- Aug. 2011
- Studied the role of autophagy in the development of Barrett's esophagus.

Research Intern, Undergraduate Student Scholars Program- University of Pennsylvania Jun. 2010- Aug. 2010
- Studied, and helped discover, the role of Cdx2, an intestine specific transcription factor, in murine intestinal fat transport.

WORK EXPERIENCE

Research Technician, Department of Genetic Medicine- Weill Cornell Medical College Jun. 2012- Oct. 2014
- Assessing therapeutics of AAV mediated Gene Therapy in murine model of Hurler's Syndrome.
- Optimizing nicotine and cocaine vaccines via Molecular Biology.

Technology Consultant, Library, Information & Technology Services- Mount Holyoke College Jun. 2009- May 2012
- Taught weekly classes to approximately 7 students in Adobe Photoshop, Adobe Dreamweaver and Microsoft Excel.
- Provided solutions to students and faculty with questions pertaining to campus networking as well as Adobe and Microsoft Office applications via one-on-one communication.

Regional Consultant, Mount Holyoke College Office of Admissions Sept. 2009- Sept. 2011
- Maintained correspondence with, and responded to inquiries of, approximately 150 prospective international students.
- Evaluated application essays, reviewed files and gave admission recommendations for prospective students especially from Africa.

SKILLS

- **Computer:** Microsoft Word, Excel, PowerPoint and Publisher; Adobe Photoshop and Dreamweaver; STATA.
- **Operation:** Virus preparation, Western Blotting, Transfections, Protein purification, DNA isolation and Gel Electrophoresis, Sequence analysis, Cell culturing, Expression cloning, ELISA, Immunofluorescence, PCR/RT-PCR, Immunohistochemistry, Murine husbandry and organ isolations, Flow Cytometry, Chromatography.
- **Languages Spoken:** English, French, Twi,

AWARDS

New Investigator Scholarship, Conference on Retroviral and Opportunistic Infections Mar. 2020
New Investigator Scholarship, Conference on Retroviral and Opportunistic Infections Mar. 2019
New Investigator Scholarship, Conference on Retroviral and Opportunistic Infections Mar. 2018
Department of Biological Sciences High Honors Thesis May 2012
Mary Lyon Scholar May 2012
Assoc. Member, Sigma XI Mount Holyoke Chapter May 2012
Jackson Memorial Scholarship Feb. 2012
Abbey Howe Turner Award for Excellence in Biological Sciences May 2011
Bernice Maclean Award for Excellence in Biological Sciences May 2011

7 CURRICULUM VITAE

EXTRACRRICULAR ACTIVITIES

Head of Family, THREAD: Dec. 2017- Dec. 2018

Lead in a team of 3 mentoring underperforming high school student with the goal of assisting them succeed and transition from high school to college.

Retail Trading/Investing: Jul. 2017- present

Bloomberg Market Concepts certified, enthusiastic investor with a focus on health care securities.

CONFERENCE ABSTRACTS

- **Kwaa AK**, Caroline Garliss, Kristen D. Ritter, Gregory M. Laird, and Joel N. Blankson. Mar. 2020
  HIV controllers have low frequencies of intact proviral DNA (*CROI)*
- **Kwaa AK**, Eileen P. Scully, Joel N. Blankson Mar. 2019
  Rapid decline of immune activation with ART in HIV controllers with low CD4 counts (*CROI)*
- **Abena K. Kwaa,** Eileen P. Scully, Joel Blankson Mar. 2018
  Rapid decline of immune activation with ART in HIV controllers with low CD4 counts
  Conference on Retroviral and Opportunistic Infections
- **Abena K. Kwaa,** Joel Blankson. Mar. 2018
  IFN-alpha enhances NK cell and CD8+ T cell mediated suppression of HIV replication
  Conference on Retroviral and Opportunistic Infections
- **Abena K. Kwaa,** Kennedy Goldsborough, Lucio Gama, Joel Blankson. May 2017
  Ingenol-B as a potential latency reversing agent and its effect on CD8 viral suppression efficiency in vitro
  The Journal of Immunology

  Benjamin G. Van de Graaf, Marlene Wang, **Abena Kwaa**, Neil Hackett, Ronald G. Crystal, Dolan Sondhi May 2014
  AAV-Mediated phenotypic correction of the CNS manifestations of a mouse model of Mucopolysaccharidosis Type I
  The American Society of Gene & Cell Therapy
- Maria Ayala Ramirez, Yemstratch Akalu, **Abena Kwaa**, and Sharon Stranford. May 2013
  Evaluating early differences in regulatory T cell levels in the MAIDS model of AIDS
  The Journal of Immunology
- Yemstratch Akalu, **Abena Kwaa**, Lindsey Sceats, and Sharon Stranford May 2012
  Analysis of the differential immunosuppressive patterns in the MAIDS model
  The Journal of Immunology

SELECTED PUBLICATIONS

- **AK Kwaa**, CC Garliss, KD Ritter, GM Laird, JN Blankson
  Elite suppressors have low frequencies of intact HIV-1 proviral DNA
  AIDS 2020
- Veenhuis RT, **Kwaa AK**, Garliss CC, Latanich R, Salgado M, Pohlmeyer C, Nobles CL, Gregg J, Scully EP, Bailey JR, Bushman FD, Blankson JN.
  Long-term remission despite clonal expansion of replication-competent HIV-1 isolates
  JCI Insight, 2018
- **Kwaa AK,** Talana CAG, Blankson JN.
  Interferon-α enhances NK cell function and the suppressive capacity of HIV-specific CD8+ T cells
  J. Virol., 2018
- **Kwaa AK**, Goldsborough K, Walker-Sperling VE, Pianowski LF, Gama L, Blankson JN.
  The effect of Ingenol-B on the suppressive capacity of elite suppressor HIV-specific CD8+ T cells.
  PLoS ONE, 2017
- May ME, **Kwaa AK**, Blankson JN.
  HIV-1 reservoirs in elite controllers: clues for developing a functional cure?
  Future Microbiol., 2017