



The HIV Latency Reversal Agent HODHBt Enhances NK Cell Effector and Memory-Like Functions by Increasing Interleukin-15-Mediated STAT Activation

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ABSTRACT Elimination of human immunodeficiency virus (HIV) reservoirs is a critical endpoint to eradicate HIV. One therapeutic intervention against latent HIV is “shock and kill.” This strategy is based on the transcriptional activation of latent HIV with a latency-reversing agent (LRA) with the consequent killing of the reactivated cell by either the cytopathic effect of HIV or the immune system. We have previously found that the small molecule 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HODHBt) acts as an LRA by increasing signal transducer and activator of transcription (STAT) factor activation mediated by interleukin-15 (IL-15) in cells isolated from aviremic participants. The IL-15 superagonist N-803 is currently under clinical investigation to eliminate latent reservoirs. IL-15 and N-803 share similar mechanisms of action by promoting the activation of STATs and have shown some promise in preclinical models directed toward HIV eradication. In this work, we evaluated the ability of HODHBt to enhance IL-15 signaling in natural killer (NK) cells and the biological consequences associated with increased STAT activation in NK cell effector and memory-like functions. We showed that HODHBt increased IL-15-mediated STAT phosphorylation in NK cells, resulting in increases in the secretion of CXCL-10 and interferon gamma (IFN- γ) and the expression of cytotoxic proteins, including granzyme B, granzyme A, perforin, granzyme lysin, FASL, and TRAIL. This increased cytotoxic profile results in increased cytotoxicity against HIV-infected cells and different tumor cell lines. HODHBt also improved the generation of cytokine-induced memory-like NK cells. Overall, our data demonstrate that enhancing the magnitude of IL-15 signaling with HODHBt favors NK cell cytotoxicity and memory-like generation, and thus, targeting this pathway could be further explored for HIV cure interventions.

IMPORTANCE Several clinical trials targeting the HIV latent reservoir with LRAs have been completed. In spite of a lack of clinical benefit, they have been crucial to elucidate hurdles that “shock and kill” strategies have to overcome to promote an effective reduction of the latent reservoir to lead to a cure. These hurdles include low reactivation potential mediated by LRAs, the negative influence of some LRAs on the activity of natural killer and effector CD8 T cells, an increased resistance to apoptosis of latently infected cells, and an exhausted immune system due to chronic inflammation. To that end, finding therapeutic strategies that can overcome some of these challenges could improve the outcome of shock and kill strategies aimed at HIV

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eradication. Here, we show that the LRA HODHBt also improves IL-15-mediated NK cell effector and memory-like functions. As such, pharmacological enhancement of IL-15-mediated STAT activation can open new therapeutic avenues toward an HIV cure.

KEYWORDS HIV, HODHBt, LRA, NK cells, STAT signaling, memory-like NK cells

Human immunodeficiency virus (HIV) has caused more than 35 million deaths worldwide. Management of the disease requires the daily administration of a combination of antiretroviral (ART) drugs for the life of the infected individual. This is due to the presence of an intact and inducible latent reservoir of HIV that rebounds after discontinuation of ART therapy (1–3). Elimination of this latent reservoir is a critical endpoint to eradicate HIV. Therapeutic interventions against latent HIV have been mainly focused on “shock and kill” strategies (4–14). These strategies are based on the transcriptional activation of latent HIV with a latency-reversing agent (LRA) with the consequent killing of the reactivated cell by either the cytopathic effect of HIV or the immune system. Several clinical trials targeting the latent reservoir with LRAs have been completed (9, 15). In spite of a lack of clinical benefit of these initial trials, they have been crucial to elucidate hurdles that shock and kill strategies have to overcome to promote an effective reduction of the latent reservoir to lead to a cure. Among others, these hurdles include (i) low reactivation potential mediated by LRAs through promoting only transcription with low translation of HIV proteins (16), (ii) the negative influence of some LRAs on the activity of natural killer (NK) and effector CD8 T cells (17–19), (iii) an increased resistance to apoptosis of latently infected cells (20–23), and (iv) an exhausted immune system due to chronic inflammation (24, 25).

Interleukin-15 (IL-15) is a common gamma chain (γ c) cytokine that promotes its biological effects through activation of the transcription factors signal transducer and activator of transcription 1 (STAT1), STAT3, and STAT5 (26). The IL-15 superagonist N-803 is a clinical candidate because it has enhanced biologic activity *in vivo* due to a longer serum half-life than recombinant IL-15, but they have the same mechanism of action (27, 28). Currently, there are five clinical trials involving the IL-15 superagonist N-803 in ART-suppressed people living with HIV (PLWH) (ClinicalTrials registration numbers NCT04808908, NCT04340596, NCT04505501, NCT05245292, and NCT02191098). The clinical trial with registration number NCT02191098 was a recently completed phase I study of N-803 that demonstrated N-803 to be safe to administer in PLWH (29). IL-15 and N-803 have been shown to (i) reactivate latent HIV both *ex vivo* and *in vivo* (18, 30, 31), (ii) enhance NK cell activity against HIV (32, 33), (iii) improve HIV-specific CD8 T cell responses (34), and (iv) promote the migration of NK and CD8 T cells to B cell follicles, a major compartment harboring latently infected cells (35, 36). However, the clinical benefit of IL-15 or N-803 can be hindered by the transient nature of cytokine signaling. Upon the binding of IL-15 to its receptor and activation of the Janus kinase (JAK)/STAT pathway, a series of negative feedback loops, including suppressor of cytokine signaling (SOCS) proteins, dephosphorylation by phosphatases, SUMOylation, and ubiquitination, reduce the transcriptional activity of STATs (37–42). We have recently published results showing that 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HODHBt) enhanced γ c cytokine signaling in CD4 T cells by increasing the phosphorylation and transcriptional activity of STATs upon cytokine stimulation (43, 44). Our previous studies showed that HODHBt increased STAT5 phosphorylation (pSTAT5) after cytokine signaling concomitant with a reduction in SUMOylation, leading to increased STAT5 nuclear presence and transcriptional activity and binding of STAT5 to the HIV long terminal repeat (LTR) in primary CD4 T cells (43). HODHBt also enhanced the LRA activity of IL-15 in cells isolated from PLWH (44). As such, targeting this pathway may enhance the efficacy of using IL-15 or N-803 for cure approaches (35).

IL-15 is also critical for NK cell development, maturation, survival, proliferation, and cytotoxic function (45). In this work, we evaluated whether enhancing IL-15-mediated STAT activation with HODHBt could also improve NK cell effector and memory-like functions. We demonstrated that HODHBt enhanced IL-15-mediated NK cell activation,

as demonstrated by increased expression of activation markers CD25 and CD69, as well as components of cytotoxic cell granules like granzyme B (GZM B), granzyme A (GZM A), perforin, and granulysin and death receptor ligands APO2L/TRAIL and CD95L/FASL. Furthermore, HODHBt enhanced IL-15-mediated secretion of interferon gamma (IFN- γ) and CXCL-10 by NK cells. These phenotypical changes were also associated with enhanced IL-15-mediated cytotoxicity against different tumor cell lines and HIV-infected CD4 T cells. Finally, IL-15, in combination with IL-12 and IL-18, has been shown to confer memory-like properties to NK cells. These properties include a quantitative increase in IFN- γ secretion upon restimulation (46). We found that the addition of HODHBt during the generation of memory-like NK cells led to enhanced IFN- γ production upon IL-12 and IL-15 recall while maintaining the ability to kill HIV-infected cells.

In conclusion, our results indicate that enhancing cytokine-induced STAT activation with HODHBt, or other small molecules targeting this pathway, may be a suitable pharmacological strategy to both reactivate latent HIV (43, 44) and enhance NK cell effector and memory-like functions and improve HIV cure strategies.

RESULTS

HODHBt enhances IL-15-mediated STAT phosphorylation and transcriptional activity in NK cells. First, we confirmed whether HODHBt enhanced STAT phosphorylation upon cytokine stimulation on NK cells, as we have shown before for CD4 T cells (43, 44). For that, NK cells were isolated from peripheral blood mononuclear cells (PBMCs) and treated overnight with dimethyl sulfoxide (DMSO), recombinant human IL-15 (rhIL-15), HODHBt, or a combination of IL-15 plus HODHBt. After incubation, the levels of STAT5, STAT1, and STAT3 phosphorylation were analyzed by flow cytometry. Treatment with HODHBt alone did not substantially increase the levels of phosphorylation of any of the STATs analyzed (Fig. 1A). This agrees with our previous studies demonstrating that the activity of HODHBt is dependent upon a γ c cytokine (43). rhIL-15 induced phosphorylation of STAT5 compared to its phosphorylation in the DMSO control. The combination of rhIL-15 plus HODHBt induced higher phosphorylation levels of the three STATs than did rhIL-15 alone (Fig. 1A). Next, we evaluated the transcriptional changes associated with increasing STAT phosphorylation with HODHBt. NK cells were treated overnight with DMSO, rhIL-15, HODHBt, or a combination of rhIL-15 plus HODHBt. Upon incubation, RNA was isolated and subjected to RNA sequencing (RNA-Seq) (Table S1 in the supplemental material). rhIL-15 and the combination of rhIL-15 plus HODHBt changed the transcription of 2,775 and 4,212 genes, respectively, compared to DMSO control (Fig. 1B and Table S2). Interestingly, HODHBt alone did not significantly change the transcription of any gene, in agreement with our previously published data showing minimal activity in the absence of a γ c cytokine (Fig. 1B) (43). When comparing the differentially expressed (DE) genes induced by rhIL-15 and rhIL-15 plus HODHBt versus DMSO, 89.1% of the genes that were differentially expressed with rhIL-15 were also differentially expressed with rhIL-15 plus HODHBt. rhIL-15 plus HODHBt induced an additional 1,738 DE genes compared to rhIL-15 alone (Fig. 1C). We then compared the DE genes between rhIL-15 and rhIL-15 plus HODHBt. Only 202 were statistically significant, several of which are involved in the effector function of NK cells, including GZM B, GZM A, IFN- γ , and CXCL-10, and are well known downstream targets of cytokine signaling (Fig. 1D and Table S3) (47). Moreover, reactome pathway analysis indicated that the DE genes identified were involved in cytokine signaling pathways, confirming the specific role of HODHBt in enhancing cytokine signaling (Fig. 1E and Table S4).

We then confirmed the induction at the protein level of these four and other genes involved in NK cell effector function in cells isolated from additional 7 to 14 HIV-negative donor participants (Fig. 2). We first evaluated the secretion of the chemokine CXCL-10 and the cytokine IFN- γ by NK cells. The combination of rhIL-15 plus HODHBt enhanced the secretion of both compared to their secretion by rhIL-15 alone (Fig. 2A and B). Importantly, the presence of HODHBt was not associated with cell toxicity either alone or in combination with rhIL-15 (Fig. 2C). Flow cytometric analysis also

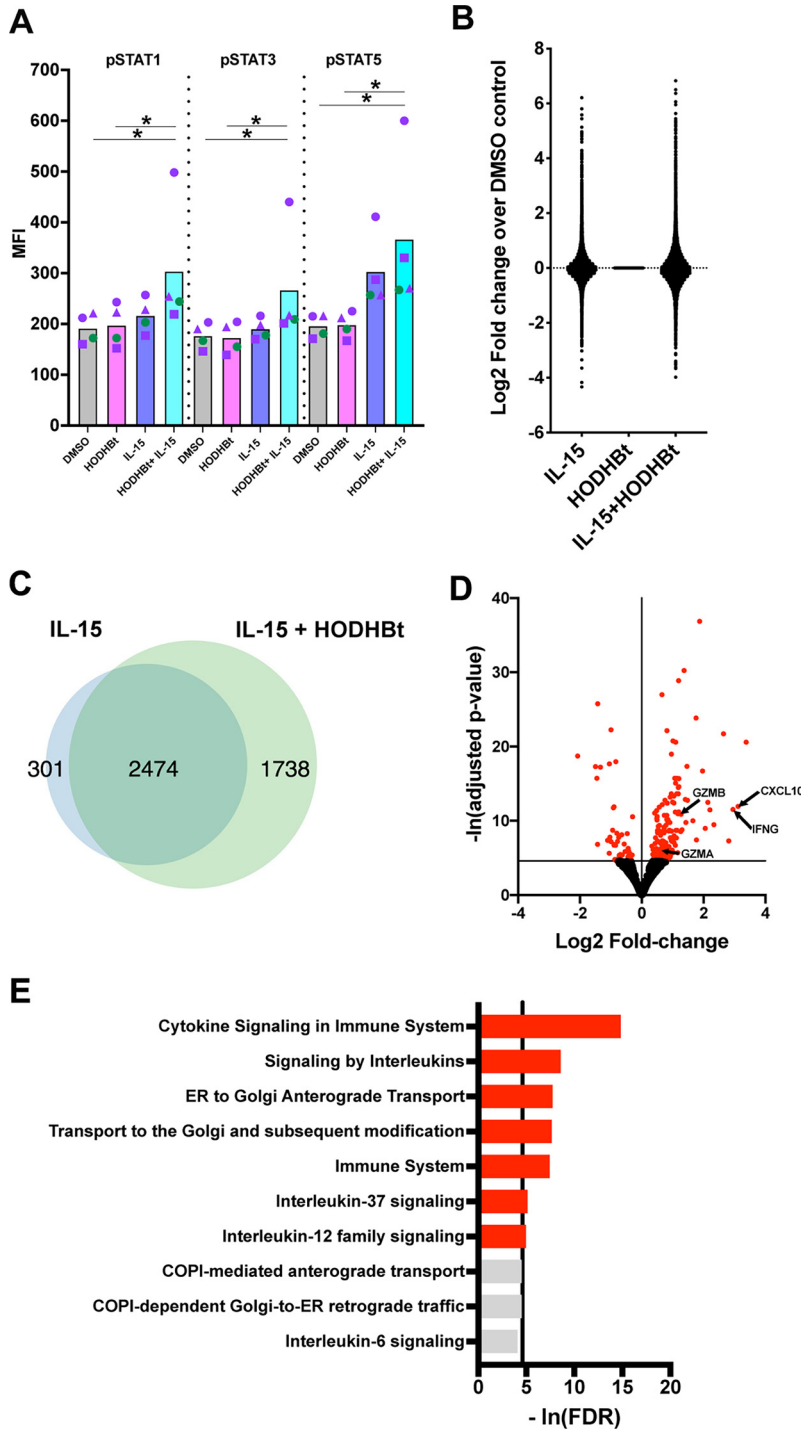


FIG 1 Biological effects of HODHBt in NK cells. (A) Levels of phosphorylated STAT1 (pSTAT1), pSTAT3, and pSTAT5 in NK cells from 4 donors. NK cells were treated with DMSO, IL-15 (100 ng/mL), HODHBt (100 μ M), or a combination of both for 24 h. MFI, mean fluorescence intensity. *, $P < 0.05$, by Dunn's multiple-comparison test. (B) Log₂ fold changes over the expression in the DMSO control of genes regulated by IL-15, DMSO, or a combination of IL-15 and HODHBt as determined by RNA-Seq. (C) Venn diagram of genes differentially expressed between IL-15 and IL-15 plus HODHBt treatment compared to their expression in the DMSO control. (D) Volcano plot of genes differentially expressed between IL-15 and IL-15 plus HODHBt treatment. Genes with a change in expression having an adjusted P value of < 0.01 are indicated in red. IFNG, IFN- γ . (E) Reactome pathway analysis of genes differentially expressed between IL-15 and IL-15 plus HODHBt treatment. Pathways with a false discovery rate (FDR) of < 0.01 are indicated in red.

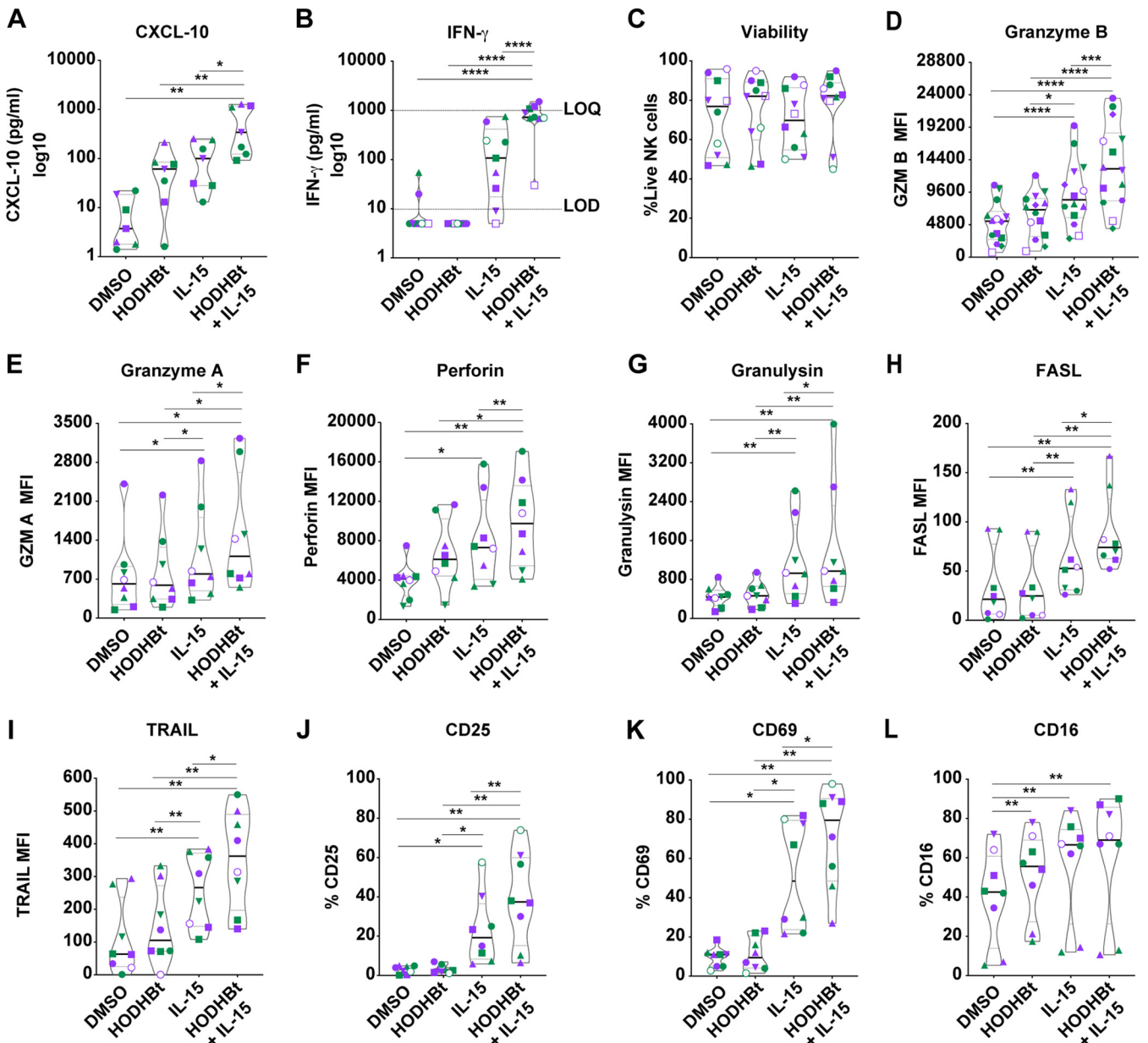


FIG 2 HODHBt enhances the cytotoxic profile of NK cells. (A, B) Levels of CXCL-10 (A) and IFN- γ (B) were quantified in cell culture supernatants after incubation of NK cells with the indicated treatments for 48 h. (C to L) Flow cytometric analysis of markers of viability (C), cytotoxicity (D to I), and activation (J to L) after incubation of NK cells with the indicated treatments for 48 h. Nonparametric Wilcoxon matched-pairs signed-rank test was used to calculate *P* values. Each symbol corresponds to a different donor. Purple symbols represent male and green female participants. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001, by analysis of variance (ANOVA) with Bonferroni correction.

confirmed enhanced protein expression of GZM B and GZM A under the rhIL-15 plus HODHBt condition compared to their expression with rhIL-15 alone (Fig. 2D and E). Next, we evaluated the expression of other proteins involved in NK cell cytotoxicity and activation. We observed increased protein expression of perforin, granulysin, FASL, and TRAIL, as well as the activation markers CD25 and CD69, but not CD16, under the rhIL-15 plus HODHBt condition compared to rhIL-15 alone (Fig. 2F to L). Interestingly, HODHBt alone was sufficient to increase the protein expression of CD16 compared to its expression in the DMSO control (Fig. 2L). However, this increase was not associated with increased gene transcription, based on our RNA-Seq analysis (Table S1).

Next, we wanted to address whether the increase in protein expression mediated by HODHBt was associated with JAK/STAT activation or with a potential off-target effect of HODHBt. To that end, NK cells were treated overnight with DMSO, rhIL-15,

HODHBt, or a combination of rhIL-15 plus HODHBt in the presence or absence of the JAK inhibitor tofacitinib. Tofacitinib completely abrogated GZM B induction by both rhIL-15 and rhIL-15 plus HODHBt (Fig. 3A). Finally, we observed a significant amount of variability in the response of NK cells to either rhIL-15 or the combination of rhIL-15 plus HODHBt. We decided to evaluate whether the biological sex or age of the donors could be a contributor in the variability observed in the induction of GZM B expression. Interestingly, the induction of GZM B (calculated as fold induction over that in the DMSO control) was significantly associated with age for rhIL-15 plus HODHBt ($P = 0.0007$, Spearman $r = 0.81$) (Fig. 3B). On the other hand, we did not observe any difference between cells from female and male donors regarding the ability of rhIL-15 or the combination of rhIL-15 plus HODHBt to induce the expression of GZM B (Fig. 3C). Overall, we confirmed that HODHBt enhanced rhIL-15-induced STAT phosphorylation and transcriptional activity on isolated NK cells, leading to the increased expression of several cytotoxic molecules important for NK cell function.

HODHBt improves the ability of IL-15-activated NK cells to kill tumor cells and HIV-infected cells. Based on the previous results demonstrating that enhancing STAT activation with HODHBt increased the expression of several proteins involved in NK cell cytotoxicity, we were interested to test whether HODHBt also increased the ability of IL-15-activated NK cells to kill different cancer cell lines, including both hematologic and solid tumors, as well as HIV-infected cells. We first tested whether HODHBt enhanced the ability of IL-15-activated NK cells to kill the erythroleukemia K562 cell line, which lacks major histocompatibility complex (MHC) class I, making these cells a target for NK cells (48). NK cells pretreated with either DMSO or HODHBt had low capacity to kill K562 cells at a 1:1 effector-to-target (E/T) ratio (Fig. 4A and B). Pretreatment of NK cells with rhIL-15 alone induced higher target killing than DMSO or HODHBt alone, while NK cells treated with the combination of rhIL-15 plus HODHBt had the highest killing ability of the four groups (Fig. 4A and B). We then used the Bliss independence model to evaluate whether the enhanced killing observed with the treatment combination was synergistic (49). The Bliss model is based on probability theory and assumes that when two drugs act through independent mechanisms, the expected ($f_{a_{xy,e}}$) combinatorial effect should be the sum of the two fractional responses (f_a) minus their product [$(f_a + f_b) - (f_a \times f_b)$]. The interaction of each combination is described by the difference between the observed (o) and the expected (e) response ($\Delta f_{a_{xy}} = f_{a_{xy,o}} - f_{a_{xy,e}}$). Bliss independence analysis yields synergistic ($\Delta f_{a_{xy}} > 0$), independent ($\Delta f_{a_{xy}} = 0$), or antagonistic ($\Delta f_{a_{xy}} < 0$) combinatorial interactions (49). In this case, the fraction of cell death induced by either HODHBt-treated or rhIL-15-treated NK cells alone was used to calculate $f_{a_{xy,e}}$ and compared with the fraction of cell death induced by NK cells treated with a combination of both ($f_{a_{xy,o}}$). This analysis demonstrated that the combination of rhIL-15 with HODHBt was synergistic ($\Delta f_{a_{xy}} = 0.05$). The increased NK cell killing capacity mediated by the combination of rhIL-15 with HODHBt was also concomitant with an increase in NK cell degranulation and cytokine release, as measured by CD107a expression (Fig. 4C) and tumor necrosis factor alpha (TNF- α) production (Fig. 4D), respectively. Next, we were interested in testing whether HODHBt would increase NK cell killing of HIV-infected CD4 T cells. As HODHBt has been shown to reactivate latent HIV (43, 44), it will be important to address whether targeting this pathway can also enhance the ability of NK cells to kill HIV-infected cells. Furthermore, it is known that HIV-infected CD4 T cells are naturally resistant to NK cell killing (50–52). We observed that coculturing IL-15-activated NK cells with HIV_{NL43}-infected autologous CD4 T cells induced higher killing of HIV-infected CD4 T cells than coculturing them with DMSO-treated NK cells at 1:1 and 2:1 E/T ratios, similar to results previously published by others (Fig. 4E and F) (32). The addition of HODHBt to rhIL-15 enhanced the killing of infected cells over that of HODHBt-treated NK cells at all E/T ratios tested and over that of rhIL-15 alone at a 1:8 E/T ratio (Fig. 4F). A similar increase was observed using the transmitted/founder (TF) virus CH058 (Fig. 4G).

Next, we extended our studies to four other transformed cell lines, including the human ovarian carcinoma cell line A2780, the glioblastoma cell line U87, the germinal

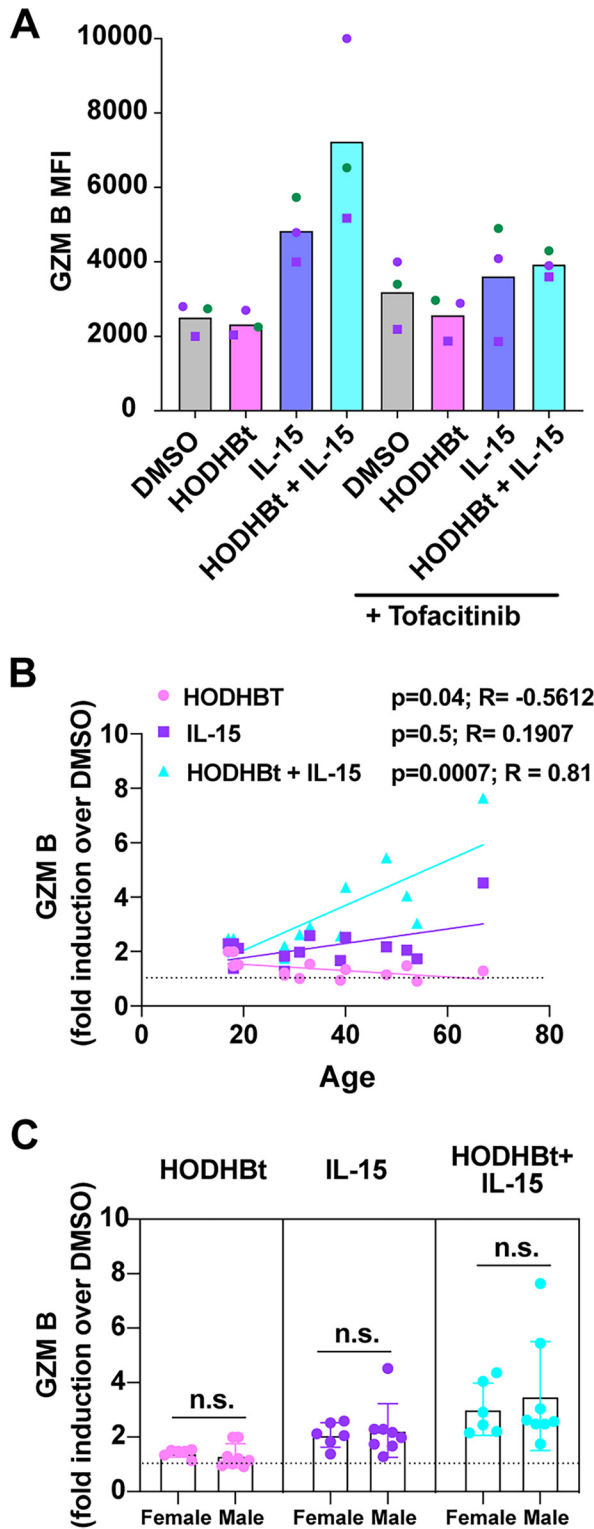


FIG 3 Influence of age and sex on granzyme B expression upon stimulation. (A) Levels of granzyme B after NK cells from 3 donors were incubated with the indicated treatments for 48 h in the presence of the JAK inhibitor tofacitinib. (B) Correlation between the induction of granzyme B expression (calculated as fold induction compared to that in the DMSO control) and age of the donors. (C) Comparison between the levels of expression of granzyme B by NK cells from male and female donors. Correlations were determined using the two-tailed nonparametric Spearman correlation coefficient. Mann-Whitney U test was used for comparisons between NK cells from female and male donors. n.s., not significant.

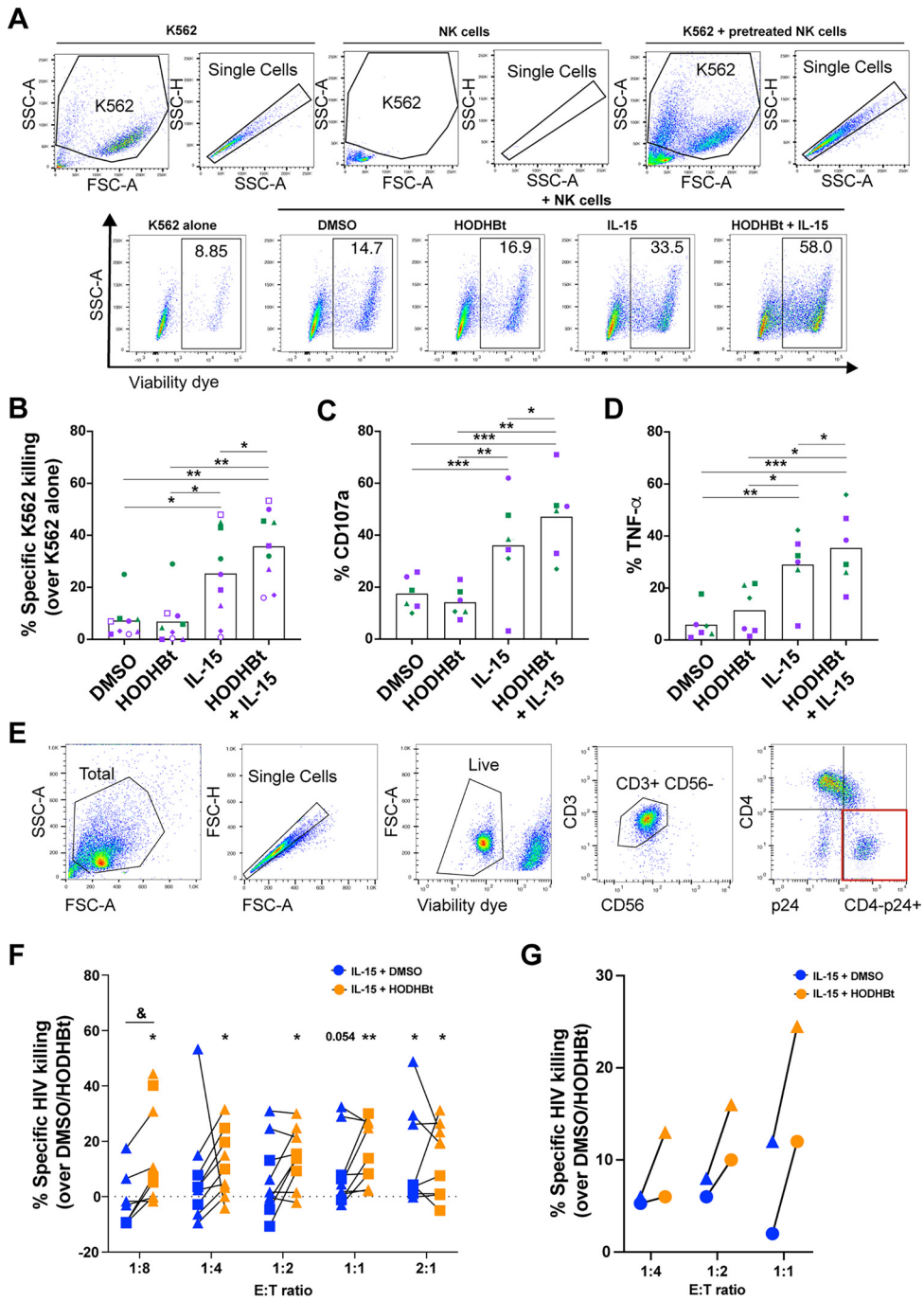


FIG 4 HODHBt enhances IL-15-mediated cytotoxicity of NK cells against HIV-infected cells. (A) Representative flow cytometry gating strategy from one experiment, indicating selection of cells to measure cell death of K562 cells cultured alone or in the presence of NK cells preincubated with the indicated treatments for 24 h at a 1:1 effector-to-target (E/T) ratio. SSC, side scatter; FSC, forward scatter. (B) K562 cell death mediated by pretreated NK cells from 6 male (purple) and 3 female (green) participants. (C, D) Surface expression of CD107a (C) and intracellular TNF- α (D) in NK cells cocultured with K562 cells. Purple symbols represent male and green female participants. (E) Representative flow cytometry gating strategy from one experiment indicating selection of CD3-positive cells to measure the reduction of p24⁺ CD4 T cells in the absence or presence of pretreated NK cells. (F) Percentages of specific HIV-infected-cell killing compared to the results for the DMSO or HODHBt control in overnight cocultures of NK cells pretreated with rIL-15 plus DMSO or HODHBt and HIV-infected CD4 T cells at different E/T ratios. Square symbols represent male and triangles female participants. (G) Specific killing of CH058-infected CD4 T cells by pretreated NK cells at different E/T ratios. &, $P < 0.05$, by two-tailed Wilcoxon matched-pairs signed-rank test, comparing IL-15 plus DMSO with IL-15 plus HODHBt treatment; *, $P < 0.05$; **, $P < 0.01$, by Wilcoxon signed-rank test, to evaluate killing compared to the results for the DMSO or HODHBt control condition.

center cell line OCILy1, and the B cell lymphoma cell line OCILy10. Treatment with rhIL-15 enhanced the NK cell killing of A2780 and U87 cells at different E/T ratios (Fig. 5A). As for K562 cells, HODHBt alone did not increase NK cells' cytotoxicity, but it did enhance the ability of IL-15-treated NK cells to kill both tumor cell lines (Fig. 5A). However, the ability of rhIL-15 or the combination of rhIL15 plus HODHBt to increase the killing capacity of NK cells was not observed in all cancer types studied. Neither rhIL-15 nor the combination substantially increased the ability of NK cells to kill the germinal center OCILy1 or B cell lymphoma OCILy10 cell lines in comparison to the killing ability of untreated NK cells (Fig. 5B). In conclusion, we demonstrated that, upon rhIL-15 stimulation, HODHBt could enhance the killing capacity of NK cells on HIV-infected CD4 T cells and different cancer cell models.

Long-term exposure to HODHBt does not cause exhaustion of NK cells. Higher activation of NK cells with HODHBt could lead to anergy and alteration of NK cell killing ability. To evaluate whether long-term exposure to HODHBt and chronic STAT activation could be detrimental for NK cell function, we used a commercial medium for NK cell expansion that requires the addition of the γ c cytokine IL-2. IL-2, like IL-15, activates STAT5, -3, and -1, and we have shown that HODHBt enhances the activation of STATs mediated by IL-2 (43). We followed NK cell expansion with this protocol for 14 days. The presence of HODHBt through the 14-day period did not alter the expansion

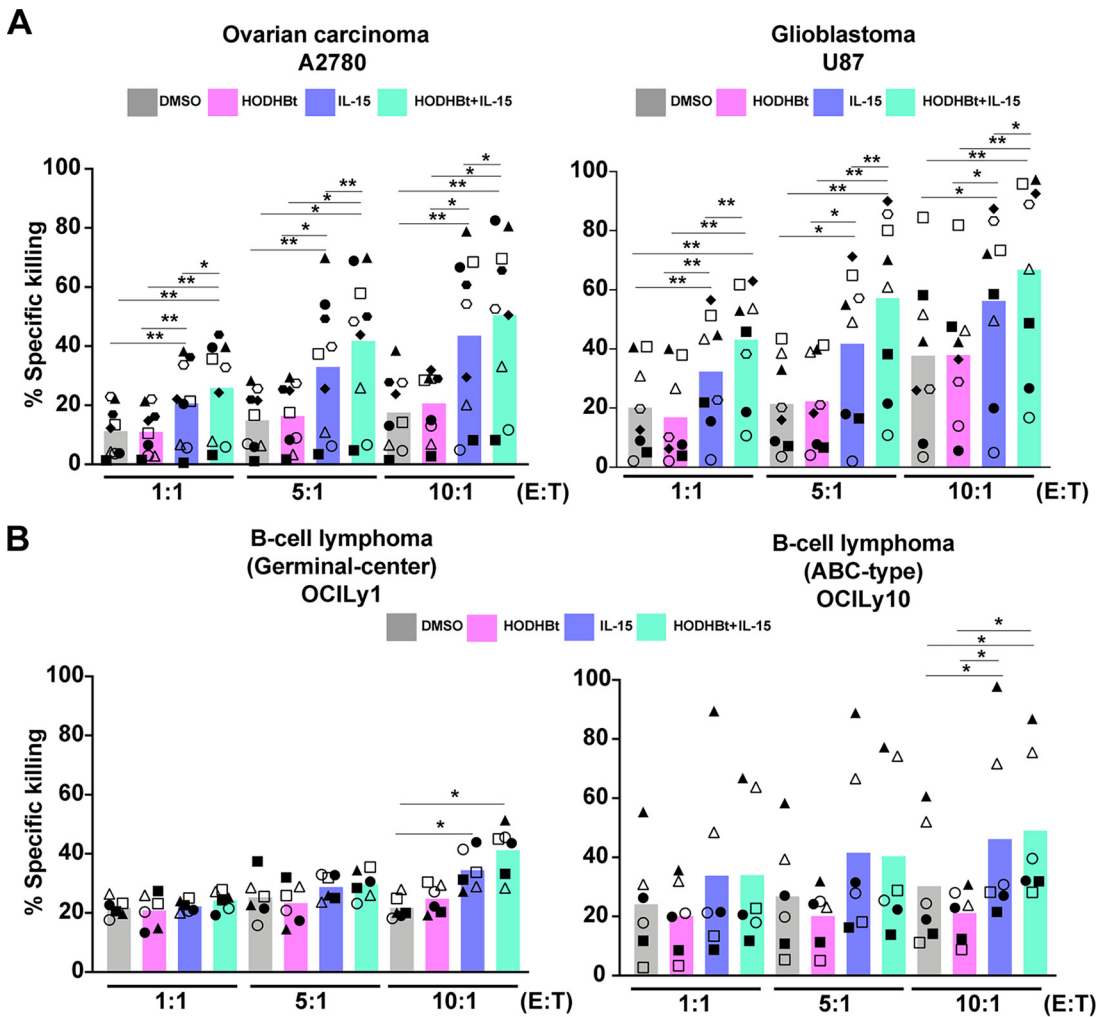


FIG 5 HODHBt enhances IL-15-mediated cytotoxicity of NK cells against cancer cell lines. Percentages of A2780 and U877 (A) and OCILy1 and OCILy10 (B) target cell lysis after incubation with pretreated NK cells at different E/T ratios. Black symbols represent male and white female participants. *, $P < 0.05$; **, $P < 0.01$, by two-tailed Wilcoxon matched-pairs signed-rank test.

sion of NK cells or their phenotype based on CD56 and CD16 expression (Fig. 6A and B). Finally, we evaluated whether long-term exposure to HODHBt would alter the killing capacity of NK cells, using the erythroblastoma cell line K562 as the target. We did observe that, in general, expansion in the presence of HODHBt increased the killing capacity of NK cells, albeit it was only statistically significant at the lowest E/T ratio tested (0.06:1), possibly due to the high variability between participants (Fig. 6C). These results suggested that long-term exposure of NK cells to HODHBt and continued STAT activation did not induce anergy and might have increased the overall killing capacity of NK cells.

HODHBt increases the generation of CIML NK cells. NK cells have been shown to have “adaptive” or “memory-like” properties (53–55). These properties include a quantitative and qualitative increase in effector response upon restimulation, characterized by enhanced IFN- γ production (56). In humans, memory-like NK cell differentiation can be induced by cytokine stimulation with a combination of rhIL-12, rhIL-18, and rhIL-15, and those cells are called cytokine-induced memory-like (CIML) natural killer cells (56, 57). CIML NK cells are characterized by an epigenetic remodeling of the IFN- γ locus, including reduced DNA methylation and enhanced IFN- γ upon restimulation in response to rhIL-12 and rhIL-15, and enhanced survival *in vivo* (56, 57). Since IL-12 mediates STAT4 activation and IL-15 activates STAT5 (53, 58), we decided to investigate whether HODHBt could increase the generation of CIML NK cells by enhancing STAT activation. First, to evaluate whether HODHBt enhanced CIML NK cell generation, we preactivated NK cells using rhIL-15 (1 ng/mL) (control) or rhIL-12 (10 ng/mL), rhIL-18 (50 ng/mL), and rhIL-15 (1 ng/mL) (CIML) in the presence of DMSO or HODHBt. On the next day, cells were washed and cultured in low concentrations of rhIL-15 (1 ng/mL) without additional DMSO or HODHBt. After 7 days, NK cells were restimulated with 100 ng/mL of rhIL-12 and rhIL-15, and intracellular production of IFN- γ was measured by flow cytometry (Fig. 7A). In general, NK cells preactivated with IL-15 alone (control) produced less IFN- γ upon cytokine recall than CIML NK cells regardless of whether cells were preactivated in the presence of DMSO or HODHBt (Fig. 7B). Interestingly, HODHBt increased the percentage of cells producing IFN- γ upon cytokine recall for both control and CIML NK cells (Fig. 7B). Compared with the IL-15 control, CIML NK cell stimulation was associated with an increase in the CD56^{bright} population (Fig. 7C) and with an increase in the levels of GZM B (Fig. 7D), but the presence of HODHBt did not alter the overall phenotype or GZM B expression. Increased cytokine production by NK cells has been associated with a reduction in their cytotoxic potential (59, 60). The generation of CIML NK cells in the presence or absence of HODHBt did not interfere with the killing of autologous HIV_{AD8}-infected CD4 T cells compared with their killing by the IL-15 con-

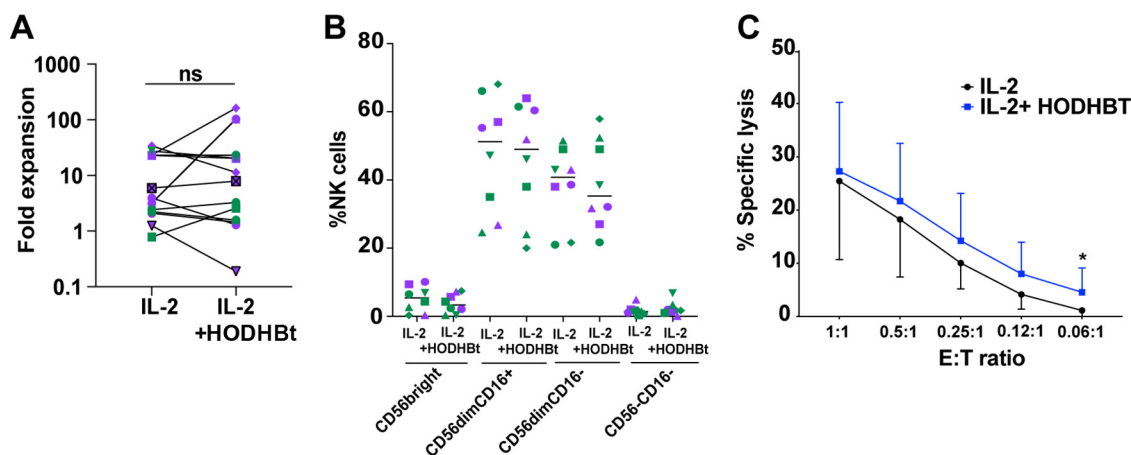


FIG 6 Long-term exposure to HODHBt does not affect NK cell proliferation, phenotype, or cytotoxicity. (A) Two-week fold expansion of NK cells with either rhIL-2 or rhIL-2 plus HODHBt. (B) Percentages of 4 different NK cell subsets based on CD56 and CD16 expression after 14 days of culture. Purple symbols represent male and green female participants. (C) Killing of K562 cells by expanded NK cells at different E/T ratios. *, $P < 0.05$, by 2-tailed Wilcoxon matched-pairs signed-rank test, to compare stimuli.

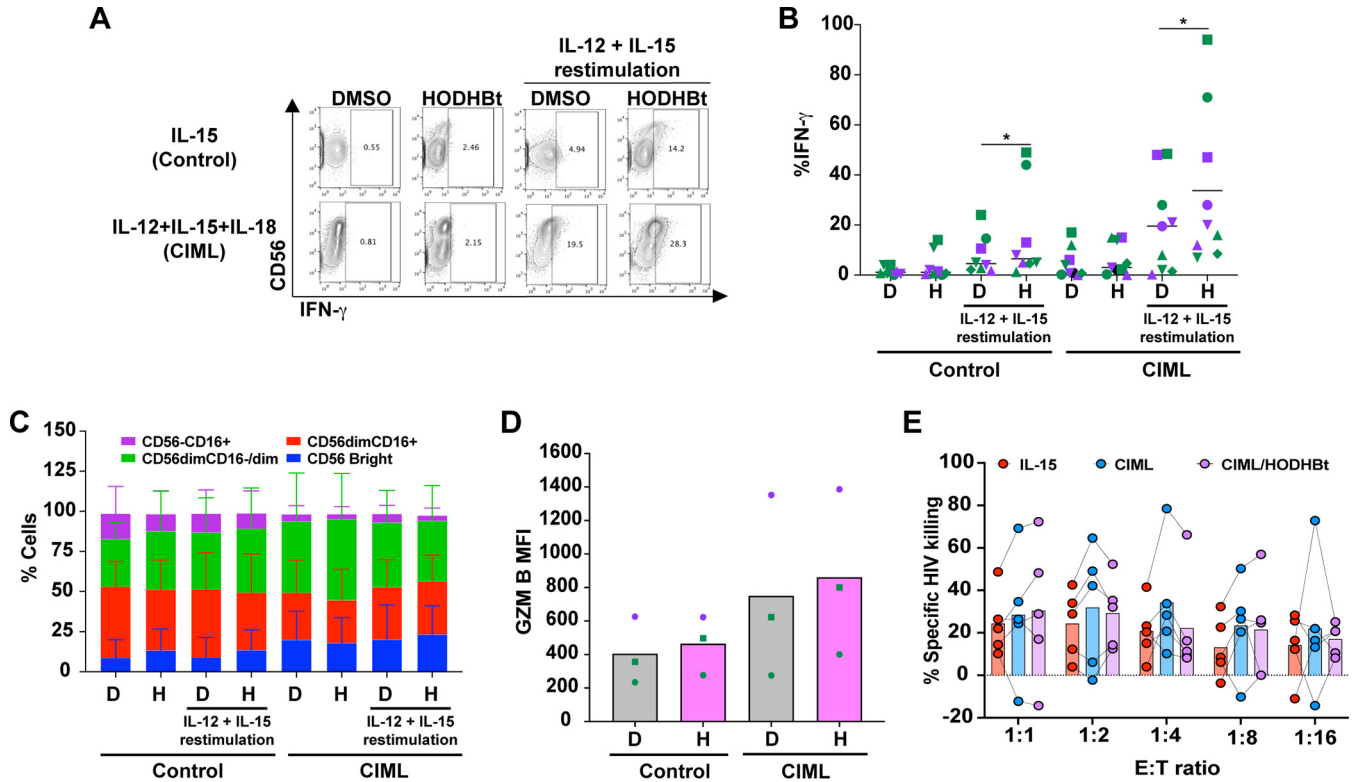


FIG 7 *In vitro* generation of human CIML NK cells with HODHBt results in enhanced memory response upon recall. (A) Representative flow cytometry plots denoting NK cells preactivated with either rhIL-15 (Control) or a combination of rhIL-12, rhIL-15, and rhIL-18 (CIML) in the presence of DMSO or HODHBt expressing IFN- γ with and without recall with rhIL-12 and rhIL-15. (B) Intracellular IFN- γ after recall in NK cells from 4 male (purple) and 5 female (green) participants. (C) Comparisons of NK cell subsets based on CD56 and CD16 expression in cells from 3 male and 4 female participants. (D) Levels of GZM B in control or CIML NK cells from 1 male and 2 female participants. (E) Percentages of specific killing of HIV-infected CD4 T cells in overnight cocultures of control or CIML NK cells generated with either DMSO or HODHBt and infected CD4 T cells at different E/T ratios. D, DMSO; H, HODHBt. *, $P < 0.05$, by 2-tailed Wilcoxon matched-pairs signed-rank test.

trol (Fig. 7E). In conclusion, increasing STAT activation with HODHBt enhanced the generation of CIML NK cells *in vitro*, indicating that the magnitude of the STAT activation could be a factor contributing to the generation of memory-like responses in NK cells, albeit the CIML phenotype did not seem to contribute to an enhanced ability to kill HIV-infected cells.

DISCUSSION

In this study, we tested whether enhancing STAT activation with the LRA HODHBt could enhance cytokine-mediated NK cell cytotoxicity and memory-like generation *in vitro*. We observed that HODHBt increased the phosphorylation of STAT5, STAT3, and STAT1 in NK cells treated with IL-15. This increase in phosphorylation by HODHBt was associated with higher STAT transcriptional activation, leading to an increased cytotoxic profile phenotype. This was demonstrated by increased expression of activation markers (CD25 and CD69), cytotoxic proteases (GZM A, GZM B, perforin, and granulysin), death receptor ligands (TRAIL and FASL), and cytokine production (IFN- γ and CXCL-10). Moreover, HODHBt enhanced the ability of IL-15-activated NK cells to kill HIV-infected CD4 T cells and different tumor cell lines, including chronic myelogenous leukemia, ovarian carcinoma, and glioblastoma cell lines. Finally, HODHBt also enhanced the generation of CIML NK cells.

NK cells are part of the innate immune system and play an important role in controlling HIV infection. NK cells are important in the control of simian immunodeficiency virus (SIV) replication in the B cell follicles of African green monkeys (AGMs) (61). Furthermore, a recent vaccination study using a pentavalent HIV vaccine that builds upon the RV144 study revealed a decrease in acquisition of simian-human

immunodeficiency virus (SHIV) due to an increase in the activity of NK cells (62, 63). Finally, Saez-Cirion et al. showed stronger NK cell responses in posttreatment controllers than in noncontrollers from the VISCONTI study (64). IL-15, through the activation of STATs, is critical for NK cell development, maturation, survival, proliferation, and cytotoxic function (45, 65). In fact, STAT5 has been proposed as the master transcriptional regulator and plays a role in NK cell maturation, survival, and cytotoxicity (66–68). Using an *in vitro* primary model, we observed that IL-15 plus HODHBt enhanced the killing of HIV-infected cells compared to the killing with IL-15 alone. As such, our results suggested that pharmacologic enhancement of IL-15-mediated STAT activation could improve NK cell activity against HIV. For example, robust NK cell effector functions could lead to better control of HIV replication (62–64). Chronic HIV-1 infection also leads to pathological changes in NK cells, including defective functionality, and control of viremia with ART has been reported to restore some but not all NK cell activity in PLWH (69–72). Those impairments include lower NK cell cytotoxicity and IFN- γ production, especially in ART patients with incomplete recovery of CD4 counts (73). Moreover, HIV infection even in the context of ART has been associated with higher risks of developing certain types of cancer (74, 75). As such, identifying pathways that can enhance NK cell effector function may benefit PLWH not only to control HIV infection but also to reduce the risk for cancer development. Furthermore, there are five clinical trials involving the use of the IL-15 superagonist N-803 in ART-suppressed PLWH to promote the elimination of latent HIV. Although HODHBt is not currently a clinical candidate, our studies suggest that enhancing IL-15-mediated STAT activation could be a potential mechanism to synergize with N-803 to reactivate latent HIV and rescue NK cell effector activity. Further efforts will be needed to develop this class of molecules into a clinical candidate or to identify novel compounds that target the same pathway.

Modulation of STAT activation with HODHBt could have an immediate application. The clinical evaluation of NK cells for use in adoptive cell immunotherapies has increased in the last decade (76–81). NK cells are an alternative to T cell immunotherapies because they preferentially target transformed cells, without the need for prior sensitization or known antigens. Also, NK cells are not thought to elicit graft-versus-host disease, and “universal donor” off-the-shelf NK cells are being developed. Despite their promise, chronic exposure to cancer cells leads to impairment of NK cell function. For this reason, multiple strategies have been developed to boost the antitumor effect of NK cells and abolish tumor resistance. Some examples include adoptive transfer of NK cells after *ex vivo* activation and expansion, restoration of NK cell function using immune checkpoint inhibitors and monoclonal antibodies, and cytokine treatment. In this work, we have performed a proof-of-concept study to demonstrate that enhancing IL-15-mediated STAT activation using the small molecule HODHBt can improve NK cell responses. We have observed that HODHBt improved NK cell-mediated killing of several cancer cell lines representative of erythroblastoma, ovarian cancer, and glioblastoma compared to treatment with IL-15 alone. However, we have observed that B cell lines are less sensitive to enhanced NK cell killing by IL-15 alone or in combination with HODHBt. These data indicate that modulating the STAT pathway may not be sufficient to enhance NK cell killing of some cancer types due to intrinsic cell resistance and that additional strategies will be needed. It will be interesting to test whether combination therapy of HODHBt with other current strategies against B cell lymphomas, such as NK cells expressing chimeric antigen receptors (CARs) against myeloid antigens, could overcome this resistance (82).

Adoptive NK cell therapy exhibits promise for both cancer and HIV therapy, and the development of additional robust methods to expand large numbers of highly effective NK cells is an important area of research. In this study, we tested the effect of HODHBt supplementation in a commercially available NK cell expansion medium with IL-2. The supplementation did not affect the proliferation rate nor the phenotype of the NK cells but increased the ability of NK cells to kill K562. It would be interesting to test whether HODHBt would enhance expansion using different proliferation protocols and other cytokines (83–85).

Although NK cells have traditionally been classified as cells of the innate immune system, they have been demonstrated to have memory features, mounting responses upon recall (55, 86, 87). In the context of HIV, preexisting memory-like NK cells can control viremia in primary infection (88, 89). Furthermore, SIV-infected macaques but not uninfected macaques have memory-like NK cells able to kill in an antigen-specific manner dendritic cells pulsed with either Gag or Env peptides (90). A recent study by Wang et al. has identified that ongoing viral replication in HIV-infected individuals increases the proportion of memory-like NK cells (91). In addition, it has been demonstrated that NK cells preactivated *in vitro* with rhIL-12, rhIL-15, and rhIL-18 (so called CIML NK cells) produced higher levels of IFN- γ upon cytokine restimulation (56, 57, 92). In this work, we were able to demonstrate that HODHBt also enhanced the generation of CIML NK cells, based on a higher IFN- γ recall response, without detrimental effects on their killing capacity for HIV-infected cells. This suggests that the magnitude of STAT activation could be a contributing factor molding the development of a memory-like phenotype in NK cells. Further studies are warranted to delineate the specific mechanisms involved in this process.

Our studies are not without caveats. We have observed a large variation among donors in the response to IL-15, as well as to IL-15 plus HODHBt. Multiple subsets of NK cells have been identified, and how IL-15 or HODHBt affects all these subsets is largely unknown (93). We have restricted our studies to identified changes in the main NK cell subsets that are characterized by the expression of CD56 and CD16. We did not observe a significant change in the proportion of any of these subsets upon HODHBt treatment (Fig. 6 and 7). Further studies using single-cell RNA-Seq will be warranted to fully understand the phenotypic changes associated with HODHBt treatment and enhancement of STAT activity. Besides natural cytotoxicity, NK cells can exert their cytotoxicity by antibody-dependent cell cytotoxicity (ADCC). ADCC requires the expression of CD16 in NK cells (94). HODHBt did not alter the expression of CD16 in combination with IL-15 (Fig. 2). However, further studies will be warranted to evaluate whether HODHBt can influence ADCC.

In conclusion, our data show that enhancing cytokine-induced STAT activation with HODHBt significantly increases the NK cell cytotoxicity phenotype and function and the generation of CIML NK cells. HODHBt could be further exploited for cell adoptive immunotherapeutic approaches using NK cells against cancer and HIV. Furthermore, the development of clinically relevant compounds targeting this pathway could be an attractive area of research to enhance *in vivo* the effector function of NK cells for HIV cure approaches or against different malignancies or other infectious diseases.

MATERIALS AND METHODS

Cell lines and reagents. rhIL-2 and rhIL-15 were provided by the BRB/NCI Preclinical Repository. rhIL-12 was obtained from PeproTech and rhIL-18 from R&D systems. HODHBt was purchased from AK Scientific. K562-green fluorescent protein (GFP) (ATCC CCL-242-GFP) cell culture was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine, and penicillin-streptomycin. A2780 cell culture was maintained in RPMI 1640 medium supplemented with 10% FBS, glutamine, and penicillin-streptomycin. U87 cell culture was maintained in DMEM supplemented with 10% heat-inactivated FBS, glutamine, OCILy1 and OCILy10 cell cultures were maintained in Iscove's modified Dulbecco's medium (IMDM) medium supplemented with 10% human serum, glutamine, and penicillin-streptomycin. Nelfinavir and raltegravir were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, and HIV-1_{NL4-3} from Malcolm Martin.

Primary cell culture. Primary NK cells and naive CD4 T cells were isolated from PBMCs by negative selection with cell-type-specific EasySep (Stem Cell Technologies) according to the manufacturer's protocol. The purity of isolated NK cells (CD3⁻ CD56⁺ CD16^{-/+}) was verified by flow cytometric analysis. NK cells were stimulated with DMSO, 100 ng/mL rhIL-15, 100 μ M HODHBt, or rhIL-15 plus HODHBt overnight or at the times indicated in the figure legends. For experiments using the JAK inhibitor tofacitinib, NK cells were pretreated with 30 ng/mL of tofacitinib (Cayman Chemical) for 1 h prior to stimulation. For expansion experiments, enriched NK cells were cultured in MACS medium (order no. 130-114-429) from Miltenyi Biotec for 14 days (with 500 IU/ML of rhIL-2 in the absence or presence of 100 μ M HODHBt) following the manufacture's protocol. For the CIML NK cell experiments, NK cells were plated at 2 to 5×10^6 cells/mL and preactivated for 16 h using rhIL-12 (10 ng/mL), rhIL-18 (50 ng/mL), and rhIL-15 (1 ng/mL) in the presence of HODHBt or of DMSO as a control and cultured in complete RPMI 1640 medium containing 10% human AB serum (Sigma-Aldrich). As a control, NK cells with only rhIL-15 were plated in medium in the presence of DMSO or HODHBt. On the next day, cells were washed, counted,

and replated with rhIL-15 (1 ng/mL) to support survival, with 50% of the medium being replaced every 2 or 3 days along with fresh cytokine. After 7 days, cells were harvested, washed, and restimulated with IL-12 (10 ng/mL) plus IL-15 (100 ng/mL) for 6 h in a 96-well round-bottom plate. Protein transport inhibitor cocktail (eBioscience) was added after 1 h, and cells were stained for surface NK cell markers (CD56 and CD16) and intracellular IFN- γ (Cytotfix/Cytoperm; BD Biosciences).

RNA-Seq analysis. RNA from 5 to 10 million NK cells treated overnight under the indicated conditions was extracted using an RNeasy plus kit (Qiagen). Samples were prepared for Illumina sequencing following the manufacturer's protocol using the TruSeq stranded total RNA library prep kit with rRNA depletion using the Ribo-Zero human/mouse/rat kit. First-strand synthesis was completed using SuperScript III (catalog no. 18080044; Thermo Fisher), with an extension temperature of 50°C. Sequencing was performed using a NextSeq high-output kit, version 2.5 (150 cycles) (catalog no. 20024907; Illumina) on a NextSeq 500, with single indexing. The resulting sequence data were quality controlled using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC (95). Low-quality reads were trimmed using Trimmomatic (96). Differential gene expression was calculated with DESeq2 (97). Reactome pathway analysis was performed using analysis tools at <https://reactome.org> (98).

Flow cytometry. To evaluate STAT phosphorylation, cells were stained with antibodies against pSTAT1(Y701) (clone 4a), pSTAT3(Y705) (clone 4/P), and pSTAT5(Y694) (clone 47) (BD Biosciences) as previously described (43).

To evaluate NK cell activation, cells were washed in phosphate-buffered saline (PBS), incubated with LIVE/DEAD fixable aqua stain (Invitrogen) for 10 min, and washed, and then anti-human antibodies to the following proteins were used for surface staining: CD3-BV786 (clone SP34-2, catalog no. 563800; BD), CD56-BV605 (clone HCD56, catalog no. 318334; Biolegend), CD16-fluorescein isothiocyanate (FITC) (clone 3G8, catalog no. 555406; BD), CD69-allophycocyanin (APC)-Cy7 (clone FN50, catalog no. 310914; Biolegend), and CD25-phycoerythrin (PE) (clone BC96, catalog no. 12-0259-42; eBiosciences). To evaluate cytotoxic profiles, cells were washed in PBS, incubated with LIVE/DEAD fixable aqua stain (Invitrogen) for 10 min, and washed, and then anti-human antibodies to the following proteins were used for surface staining: CD3-BV786 (clone SP34-2, catalog no. 563800; BD) and CD56-BV605 (clone HCD56, catalog no. 318334; Biolegend). After surface staining, NK cells were fixed and permeabilized with Cytotfix/Cytoperm buffer (catalog no. 554722; BD Biosciences), followed by intracellular staining with antibodies to granzyme B-AF700 (clone GB11, catalog no. 560213; BD Biosciences), granzyme A-PE-Cy7 (clone CB9, catalog no. 507221; BD Biosciences), perforin-FITC (clone dG9, catalog no. 308104; Biolegend), granulysin-PE (clone DH2, catalog no. 308104; Biolegend), TRAIL-APC (clone N2B2, catalog no. 109310; Biolegend), and FASL-BV421 (clone DX2, catalog no. 11-0959-42; eBiosciences). For experiments with HIV-infected CD4 T cells, cells were stained using LIVE/DEAD fixable aqua stain (Invitrogen) and antibodies to the following proteins: CD3-BV786 (clone SP34-2, catalog no. 563800; BD), CD4-APC (S3.5, catalog no. MHCD0405; BD), CD56-BV605 (clone HCD56, catalog no. 318334; Biolegend), and p24/FITC (for intracellular staining) (clone KC57-FITC, catalog no. 6604665; Beckman Coulter).

Cells were analyzed on a BD LSR Fortessa X20 flow cytometer with FACSDiva software (Becton, Dickinson, Mountain View, CA) and analyzed using FlowJo (Tree Star, Inc., Ashland, OR).

Cytokine analysis. Supernatants were collected from each well and stored at -20°C until ready for analysis with a IFN- γ enzyme-linked immunosorbent assay (ELISA) kit (catalog no. ENEHIFNG2; Invitrogen) and CXCL-10 U-PLEX human IP-10 assay MSD (catalog no. K151UFK).

NK cell cytotoxicity assays. Flow cytometry-based assays were performed using overnight-activated NK cell cultures when K562-GFP (ATCC) cells were used as targets. Pretreated NK cells were washed and incubated in duplicates at 1:1 effector-to-target cell (E/T) ratios for 4 h in 96-well U-bottom plates. After incubation, cells were harvested, washed, and stained for flow cytometry. LIVE/DEAD fixable aqua stain (Invitrogen) was used for assessment of dead K562 cells. In parallel cultures, cells were stained to assess NK cell degranulation and intracellular production of IFN- γ . Anti-PE-CD107a antibody (clone H4A) was added to the cocultures, and after 1 h of incubation, eBioscience protein transport inhibitor cocktail (eBioscience) was added for an additional 3 h. After incubation, cells were harvested, washed, stained with LIVE/DEAD fixable aqua stain (Invitrogen), and surface stained with anti-CD56-BV605 antibody (Biolegend) in PBS plus 3% FBS for 20 min on ice in the dark. Cells were then fixed and permeabilized with Cytotfix/Cytoperm buffer (BD Biosciences) and washed with Perm/Wash buffer (BD Biosciences), followed by intracellular staining with anti-TNF- α -APC-Cy7 and anti-IFN- γ -PE-Cy7 antibodies (Biolegend) for 30 min at 4°C. After washing, cells were resuspended in buffer for cytometric analysis. The percentage of direct killing for each treatment was calculated with the following formula: (% of live K562 cells with NK cells) - (% of live K562 cells without NK cells).

The DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) cell cytotoxicity assay was performed to measure cytotoxicity against cancer cell lines A2780, U87, OCILy1, and OCILy10 according to the manufacturer's protocol with a few modifications (catalog no. AD0116; PerkinElmer). Adherent cell lines A2780 and U87 were washed 5 times after labeling with BATDA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid]. OCILy1 and OCILy10 were washed 3 times after labeling with BATDA. The percentage of specific killing was calculated with the following formula: $100 \times [(BATDA \text{ release with NK cells}) - (BATDA \text{ release with no NK cells})] / [(maximum \text{ BATDA release}) - (BATDA \text{ release with no NK cells})]$.

For experiments using HIV_{NL4.3}-infected CD4 T cells as targets, infected cultured central memory CD4 T cells were generated as previously described until day 13 (99), washed, and cocultured overnight with overnight-pretreated NK cells in a 96-well U-bottom plate. Cells were washed and stained for flow cytometry analysis as described above. NK cell-mediated elimination of autologous transmitted/founder (TF) virus CH058-infected cells was evaluated by flow cytometry as previously described (100). Briefly, CD4 T cells were isolated from rested human PBMCs and activated with phytohemagglutinin-L (PHA-L) and

rhIL-2. Activated CD4 T cells were then infected with vesicular stomatitis virus G (VSV-G)-pseudotyped transmitted/founder (TF) virus CH058, as described previously (100). Autologous NK cells were isolated from resting PBMCs and treated for 24 h with 100 μ M HODHBt or an equivalent volume of DMSO with or without 100 ng/mL of rhIL-15. Twenty-four hours postinfection, infected CD4 T cells were stained with a viability dye (AquaVivid; ThermoFisher Scientific) and a cell proliferation dye (eFluor670; eBioscience) and used as target cells. Autologous purified NK cells, stained with another cellular marker (cell proliferation dye eFluor450; eBioscience), were added at different NK cell/target ratios (1:4, 1:2, and 1:1) in 96-well V-bottom plates (Corning, Corning, NY). The plates were subsequently centrifuged for 1 min at 300 \times *g* and incubated at 37°C, 5% CO₂ for 5 h before being fixed in a 2% PBS–formaldehyde solution. Infected cells were identified by intracellular staining for HIV-1 p24. Samples were acquired on an LSR II flow cytometer (BD Biosciences), and data analysis was performed using FlowJo version X.0.7 (Tree Star). For experiments using CIML NK cells, naive CD4 T cells were activated with anti-CD3/anti-CD28 antibody for 3 days and infected with the HIV_{AD8} molecular clone. At day 7, HIV-infected cells were cocultured overnight with CIML NK cells in a 96-well U-bottom plate. Cells were washed and stained for flow cytometry analysis as described above. In each case, the percentage of direct killing was calculated with the following formula: (% of p24⁺ cells in targets) – (% of p24⁺ cells in targets plus effectors)/(% of p24⁺ cells in targets) by gating on live target cells. For the results shown in Fig. 4, data were normalized as follows (% of p24⁺ cells in targets plus effectors treated with rhIL-15 in combination with either DMSO or HODHBt) – (% of p24⁺ cells in targets plus effectors treated with DMSO or HODHBt alone).

Statistics. Statistical analyses were performed using GraphPad Prism 9.0 software (GraphPad Software). The statistical analysis used is indicated in each figure legend. A *P* value of less than 0.05 was considered significant (*, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001). All the data with error bars are presented as mean values \pm standard deviations.

Study approval. Volunteers 17 years and older at the Gulf Coast Regional Blood Center served as blood participants. White blood cell concentrates (buffy coat) prepared from a single unit of whole blood by centrifugation were purchased.

Ethics statement. Written informed consent was obtained from all study participants, and the research adhered to the ethical guidelines of CRCHUM and was reviewed and approved by the CRCHUM Institutional Review Board (Ethics Committee approval number CE 16.164-CA). The research adhered to the standards indicated by the Declaration of Helsinki. All participants were adults and provided informed written consent prior to enrollment, in accordance with Institutional Review Board approval.

Data availability. Data have been deposited to NCBI SRA under accession number [PRJNA753488](https://www.ncbi.nlm.nih.gov/sra/PRJNA753488).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 5.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 4.5 MB.

SUPPLEMENTAL FILE 3, XLSX file, 1.1 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.02 MB.

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A.B. and A.B.M. conceived and designed the experiments. A.B.M., C.L., and J.R. performed experiments. M.G., C.R.Y.C., and K.B.C. provided reagents. A.B.M., J.R., A.F., and A.B. analyzed the data and wrote the manuscript. B.N. and K.A.C. performed the RNA-Seq bioinformatic analysis. The authors read and approved the manuscript.

A.B. and A.B.M. have a patent application on the use of HODHBt to enhance immune responses. The rest of the authors declare no conflict of interest.

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