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Human Galectin-9 Promotes the Expansion of HIV Reservoirs *in vivo* in Humanized Mice

Running title: Galectin-9 and HIV Reservoirs in *vivo*

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ABSTRACT

Objective: The human endogenous protein Galectin-9 (Gal-9) reactivates latently HIV-infected cells *in vitro* and *ex vivo*, which may allow for immune-mediated clearance of these cells. However, Gal-9 also activates several immune cells, which could negatively affect HIV persistence by promoting chronic activation/exhaustion. This potential "double-edged sword" effect of Gal-9 raises the question of the overall impact of Gal-9 on HIV persistence *in vivo*.

Design: We used the BLT (bone marrow, liver, thymus) humanized mouse model to evaluate the impact of Gal-9 on HIV persistence *in vivo* during antiretroviral therapy (ART).

Methods: Two independent cohorts of ART-suppressed HIV-infected BLT mice were treated with either recombinant Gal-9 or PBS control. Plasma viral loads and levels of tissue-associated HIV DNA and RNA were measured by qPCR. Immunohistochemistry and HIV RNAscope were used to quantify CD4$^+$ T, myeloid, and HIV RNA+ cells in tissues. T cell activation and exhaustion were measured by flow cytometry, and plasma markers of inflammation were measured by multiplex cytokine arrays.

Results: Gal-9 did not induce plasma markers of inflammation or T cell markers of activation/exhaustion *in vivo*. However, the treatment significantly increased levels of tissue-associated HIV DNA and RNA compared to controls (P=0.0007 and P=0.011, respectively, for cohort I and P=0.002 and P=0.005, respectively, for cohort II). RNAscope validated the Gal-9 mediated induction of HIV RNA in tissue-associated myeloid cells, but not T cells.

Conclusions: Our study highlights the overall adverse effects of Gal-9 on HIV persistence and the potential need to block Gal-9 interactions during ART-suppressed HIV infection.

Key Words: Galectin-9; HIV; humanized mice; BLT mice; HIV reservoirs.

INTRODUCTION

The persistence of HIV latently-infected cells, in blood and tissues, remains a barrier to a cure for HIV infection [1]. Persistent latently-infected cells do not express enough viral antigens to be eliminated by the immune system. Different strategies to reactivate these cells have been proposed, including using the human lectin Galectin-9 (Gal-9) [2, 3]. However, Gal-9 activates and expands several immune cells (including T and myeloid cells [4-9]), which can lead to several undesirable effects. Indeed, recent studies showed that Gal-9, which is rapidly and sustainably elevated during HIV infection [10], may contribute to the state of chronic immune activation and inflammation during HIV infection [7, 11, 12]. These mixed effects of Gal-9 raise the question of the overall effects of Gal-9 on HIV persistence. In this short report, we investigate the overall beneficial versus detrimental impact of Gal-9 on HIV persistence *in vivo,* during antiretroviral therapy (ART) suppression, using a humanized mouse model (the bone marrow-liver-thymus humanized (BLT) model).

METHODS

Generation of the BLT mice. Two independent cohorts of BLT mice were generated as previously described [13, 14], in accordance with The Wistar Institute Animal Care and Research Committee regulations (protocol# 201360). Briefly, 6-8 weeks old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, Jackson Laboratory) mice were pretreated with busulfan at 30mg/kg and were then implanted with human fetal thymic tissue fragments and fetal liver tissue fragments under the murine renal capsule. Following the surgery, mice were injected via the tail vein with CD34$^+$ hematopoietic stem cells isolated from human fetal liver tissues. Human fetal liver and thymus tissues were procured from Advanced Bioscience Resources (Alameda, CA). Twelve weeks post-surgery, human immune cell reconstitution in peripheral blood was determined using the Symphony flow cytometer (BD Biosciences, San Jose, CA) using the following antibodies: mCD45-AF700, hCD45-FITC, hCD3-BUV805, hCD4-BUV395, hCD8-PerCP-Cy5.5 and Fixable Viability Stain 510 (catalog# 560510, 555482, 612895, 563550, 565310, and 564406, respectively; BD Biosciences, San Jose, CA). Data were analyzed with FlowJo (FlowJo LLC, Ashland, OR).

HIV infection, ART suppression, and Gal-9 treatment. BLT mice from each cohort were randomly divided into two groups and were infected intravenously (IV) with 1×10^4 TCID$_{50}$ of HIV$_{SUMA}$. Peripheral blood was collected weekly for plasma viral load assay. Two weeks post-infection, mice were placed on a diet combined with ART (1,500mg/kg Emtricitabine, 1,560mg/kg Tenofovir-Disoproxil-Fumarate, and 600mg/kg Raltegravir). Five weeks post-ART, mice were treated with phosphate-buffered saline (PBS) control or 2 mg/kg recombinant Gal-9 for two weeks (intraperitoneal (IP) injections every other day; seven doses) during ART suppression. Mice were then euthanized, and blood and tissues were collected.

Measuring plasma viral load by qPCR. Plasma viral loads were measured as previously described [13, 14].

Measuring cell-associated HIV DNA and RNA by qPCR. A single-cell suspension was generated using the gentleMACS™ Octo Dissociator (San Diego, CA). DNA and RNA were extracted using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, catalog # 80224). Cell-associated HIV DNA and RNA were measured as previously described [15].

Immunohistochemistry and quantitative image analysis (QIA). Immunohistochemical staining and QIA were performed as previously described [16]. In brief, immunohistochemistry was performed using a biotin-free polymer approach (Golden Bridge International) on 5-μm tissue sections mounted on glass slides, which were dewaxed and rehydrated with double-distilled water. Multistaining of CD4/CD68/CD163 to quantify CD4$^+$ T cells was performed. This multistaining approach allows the intense staining of the macrophage/myeloid cell markers to mask the faint CD4 expressed on these cells and to distinctly identify CD4$^+$ T cells from myeloid lineage cells. Heat-induced epitope retrieval was performed by heating sections in 0.01% citraconic anhydride containing 0.05% Tween-20, then incubated with primary antibody to CD4 (Goat anti-CD4, R&D system ref: AF-379-NA), CD68 (Rabbit anti-CD68, Sigma ref: HPA048982) and CD163 (rabbit anti-CD163, Lifespan Biosciences ref: LS-B2661). All slides were scanned at high magnification (×200) using the AT2 System (Aperio Technologies), yielding high-resolution data from the entire tissue section. Representative regions of interest (500 × 500 μm) were identified, and high-resolution images were extracted from these whole-tissue scans. The percentage area of the positive cell zone was quantified using CellProfiler version 3.1.5.

HIV clade B lineage-specific in situ hybridization and phenotyping. HIV-1 in situ hybridization was performed as previously described [17]. RNAscope (probe HIV-B, ACD, ref: 416111) was combined with immunofluorescence assay (IFA) to identify the cells harboring vRNA. Slides were co-stained with CD4 or CD3 (rabbit anti-CD3, Thermo ref: RM-9107-S) and CD68+CD163 to distinguish CD4$^+$ T cells and myeloid cells. High magnification confocal images were collected from regions of interest using an Olympus FV10i confocal microscope using a 60x phase contrast oil-immersion objective (NA 1.35) imaging using sequential mode to separately capture the fluorescence from the different fluorochromes at an image resolution of 1024x1024 pixels. Cells harboring vRNA were quantified using Fiji.

Measuring markers of T cell activation and exhaustion by flow cytometry. Cell suspension was stained with the following antibodies: CD45-AL700 (clone: 2D1; Biolegend), CD8-FITC (clone: HIT8a; BD Biosciences), CD38-APC (clone: HIT2; BD Biosciences), HLA-DR-APC-H7 (clone: G46-6; BD Biosciences), CD4-V450 (clone: RPA-T4; BD Biosciences), PD-1-PE (clone: NAT105; Biolegend), and CD3-PE-CF594 (clone: UCHT1; BD Biosciences). Data were collected on a BD Biosciences LSRII flow cytometer (gating strategy is in Supplementary Figure 1, http://links.lww.com/QAD/C739).

Measuring plasma markers of inflammation. Markers of inflammation were measured using U-PLEX Biomarker Group 1 Assay from Meso Scale Discovery (MSD catalog # K15067L-2).

Statistical analysis. The Shapiro–Wilk test was used to test for data normality. Parametric T-tests were then used to analyze normally-distributed datasets, and non-parametric T-tests were used to analyze non-normally distributed datasets using Prism 9.0 (GraphPad).

RESULTS

Gal-9 treatment is tolerable *in vivo* but increases levels of tissue-associated HIV DNA and RNA during ART. We generated two independent cohorts of humanized mice (n=8 for cohort I and n=6 for cohort II) (**Fig 1A-B**). BLT mice were infected with HIV (HIV$_{SUMA}$ transmitted/founder virus) and then placed on ART two weeks post-infection. During ART suppression, mice were treated with either PBS or 2 mg/kg recombinant human Gal-9 for two weeks. We did not observe any signs of toxicity or weight loss (**Fig. 1C**) in the Gal-9 treated mice compared to controls, suggesting this concentration was generally tolerable in ART-suppressed HIV-infected BLT mice. Plasma HIV viral loads (**Fig. 1D**) indicate that, in both cohorts, the infection was successful and resulted in 5.56^{05} (median) and 6.77^{05} (average) HIV copies/ml plasma by the second week post-infection and that ART suppressed the virus to below the limit of detection. We next examined cell-associated HIV RNA and DNA levels in the liver, lung, and spleen. We found that Gal-9 treatment increased the levels of tissue-associated HIV DNA (*P*=0.0007) and RNA (*P*=0.0106) in cohort I. Consistently, in cohort II, Gal-9 increased tissue-associated HIV DNA (*P*=0.0019) and RNA (*P*=0.0047) (**Fig. 1E**).

Staining of liver and spleen demonstrated that levels of both CD4$^+$ T cells and myeloid cells in tissues were not significantly different between the two groups (**Fig. 1F**), excluding the possibility the Gal-9-mediated induction of cell-associated HIV DNA and RNA was due to different levels of human cells in tissues. In addition, we used RNAscope to quantify levels of HIV RNA+ CD4$^+$ T or myeloid cells in the liver and spleen (**Fig. 1G**) and validated that Gal-9 induces levels of HIV RNA in myeloid cells, but not T cells. Collectively, these data suggest that the overall impact of Gal-9 on HIV persistence is negative, and that Gal-9 may expand tissue-associated HIV reservoirs during ART.

Gal-9 treatment does not significantly induce markers of inflammation of T cell activation. Next, we examined whether Gal-9 exhibits its adverse effects on HIV persistence by inducing T cell activation/exhaustion or systemic inflammation. We first examined CD4$^+$ and CD8$^+$ T cell activation/exhaustion markers in blood and spleen. Gal-9 treatment did not significantly induce T cell activation (as measured by the co-expression of CD38 and HLA-DR) or exhaustion (as measured by PD-1 expression) in blood (**Fig. 2A**) or tissues (**Fig. 2B**). Next, we examined the plasma levels of several cytokines. While Gal-9 treatment reduced the levels of IL-33 (p=0.0321), it did not significantly alter the systemic levels of several cytokines involved in inflammation (**Fig.**

2C). These data suggest that Gal-9 does not induce generalized T cell activation or systemic inflammation.

DISCUSSION

A comprehensive understanding of the overall impact of viral and host factors that modulate HIV persistence is critical to developing curative strategies for HIV. Gal-9 is one of the endogenous host immune-modulatory factors that has been recently associated with opposing effects on HIV infection [10, 18, 19]. Several studies have highlighted the potential beneficial effects of Gal-9 during HIV infection. First, Gal-9 renders CD4+ T cells less susceptible to HIV infection via induction of the host restriction factor cyclin-dependent kinase inhibitor 1 (p21) [20]. Second, recombinant Gal-9 induces HIV transcription and reverses HIV latency *in vitro* and *ex vivo* [2, 3]. This ability of Gal-9 to induce latent HIV transcription suggested that it could be considered within the "shock and kill" HIV eradication framework [2, 3]. However, on the other hand, several other studies have highlighted the detrimental effects of Gal-9 during HIV infection. First, endogenous Gal-9 rapidly increases after HIV infection [10] and does not return to normal after suppressive ART [10]. These persistent elevated Gal-9 levels are associated with higher HIV transcription *in vivo* in the blood of HIV-infected ART-suppressed individuals [2], and several studies suggest that Gal-9 may contribute to the state of chronic immune activation and inflammation during HIV infection [7, 11, 12]. Second, Gal-9 increases HIV entry by inducing the CD4+ T cell-surface concentration of protein disulfide isomerase (PDI) [21]. Lastly, Gal-9 modulates HIV transcription by activating the TCR-downstream signaling pathways *in vitro* [4]. Due to these pleiotropic effects of Gal-9 on HIV, we sought to evaluate whether the overall impact of Gal-9 on HIV persistence is beneficial or detrimental *in vivo*.

Using the BLT humanized mouse model of HIV infection, we found that Gal-9 can directly expand HIV reservoirs and exhibit overall adverse effects on HIV persistence. These effects would limit the potential use of Gal-9 to reduce viral reservoirs by itself. However, future studies will be needed to investigate whether Gal-9, together with added immune effector strategies, would positively affect HIV persistence. Future studies will also be needed using larger animal models with a more intact immune system than the BLT humanized mouse model. In our study, we used tissues from two human donors; additional human donors will be needed to minimize potential biases from donor-to-donor variation. Finally, future studies will be needed to examine the lineages and the replication-competency of the HIV provirus expanded by Gal-9. Together, these investigations will be required to examine the mechanisms underlying our *in vivo* observations.

Our data show that the Gal-9-mediated expansion of HIV reservoirs is specific to myeloid cells. Gal-9 expands and activates myeloid cells [5-9]; these effects might lead to HIV reactivation and ongoing replication in this cell population, which may explain our results. Furthermore, Gal-9 can

impact many aspects of immune responses relevant to persistence. For example, Gal-9 exhibits several immunosuppressive activities [22-30], including the ability to increase the function of regulatory T cells (T-regs)[30] and impair natural killer (NK) cells' cytotoxicity [31]. The potential link between Gal-9-mediated impact on immune activation and functions, relevant to HIV persistence, warrants a broader investigation.

Endogenous Gal-9 is highly abundant *in vivo*, especially during HIV infection[10], and its sustained levels have been associated, during ART-suppressed HIV infection, with the state of chronic inflammation and immune activation central to the development of several HIV-associated co-morbidities [32-39]. Therefore, clarifying the mechanistic underpinnings of the overall adverse effects of elevated Gal-9 during ART-suppressed HIV infection may lead to the development of interventions to target Gal-9 (such as anti-Gal-9 antibodies and small molecule inhibitors targeting Gal-9 [40-42]) to improve immune functionality, reduce inflammation-associated co-morbidities, and reduce levels of HIV persistence, in the setting of viral suppression by ART.

AUTHOR CONTRIBUTIONS

Z.Y, L.B.G, T.N, L.C.N, L.J.M, and M.A-M designed the experiments. Z.Y, L.B.G, C.H, A.G, J.K, K.Y.H, and C.D carried out the experiments. Z.Y, L.B.G, C.D, L.J.M, M.A-M analyzed and interpreted data. Z.Y, L.B.G, L.J.M, and M.A-M wrote the manuscript, and all authors edited it.

COMPETING INTERESTS STATEMENT

Authors have no competing interests.

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Figure 1. Gal-9 treatment is tolerable *in vivo* but increases levels of tissue-associated HIV DNA and RNA during ART-suppressed HIV infection. (**A**) a schematic overview of the study design. (**B**) percentage of human CD45^{+}, CD3^{+}CD45^{+}, CD4^{+}CD3^{+}CD45^{+} cells measured in the peripheral blood of BLT mice at day -7 (n=14). Unpaired T tests. Mean and standard error of mean (SEM) are displayed. (**C**) mice weight over time in both cohorts. (**D**) plasma viral load over time in both cohorts (n=14). (**E**) Gal-9 treatment-induced levels of tissue-associated HIV DNA and HIV RNA (measured by qPCR) than controls in both cohorts. Data shown are only from tissues with detectable levels of cell-associated HIV DNA or RNA. Unpaired T tests. Mean and SEM are displayed. (**F**) immunohistochemistry and quantitative image analysis of CD4^{+} T and myeloid cells in the liver and spleen of both cohorts (n=14). Unpaired T tests. Mean and SEM are displayed. (**G**) Top: representative example of myeloid (blue) and CD4^{+} T (green) cells harboring HIV RNA (red) using RNAscope in paraformaldehyde (PFA) fixed liver tissues. Scale bar=100μm. Bottom: levels of HIV RNA+ CD4^{+} T or myeloid cells in the liver and spleen of mice from cohort II (n=6). Unpaired T tests. Mean and SEM are displayed.
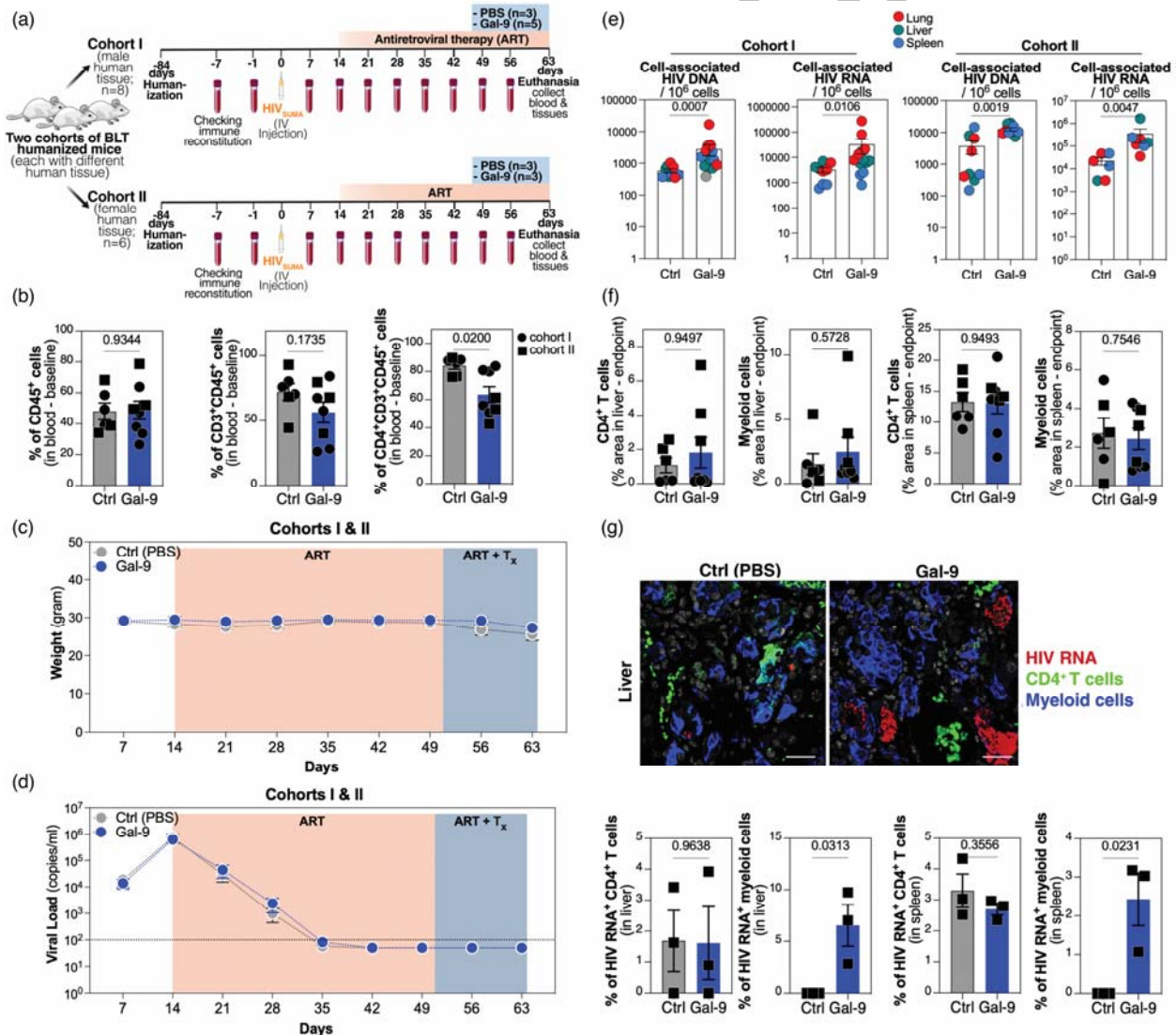
Figure 2. Gal-9 treatment does not significantly induce markers of inflammation of T cell activation. **(A-B)** impact of Gal-9 treatment on markers of CD4$^+$ and CD8$^+$ T cell activation (co-expression of CD38 and HLA-DR activation markers) and exhaustion (PD-1 expression) measured by flow cytometry in the blood (A; n=14) and spleen (B; n=8). Unpaired t-tests. The mean and standard mean of error (SEM) are displayed. **(C)** Impact of Gal-9 treatment on plasma levels of markers of systemic inflammation measured by multiplex arrays (n=8). Unpaired t-tests. The mean and standard mean of error (SEM) are displayed.