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In Vivo Activation of Human NK cells by Treatment with an IL-15 Superagonist Potently Inhibits Acute *In Vivo* HIV-1 Infection in Humanized Mice

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Running Head: NK Cell Activation Inhibits *In Vivo* HIV Infection

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Abstract

Natural killer (NK) cells with anti-HIV-1 activity may inhibit HIV-1 replication and dissemination during acute HIV-1 infection. We hypothesized that the NK cell capacity to suppress acute *in vivo* HIV-1 infection would be augmented by activating them via treatment with an IL-15 superagonist, IL-15 bound to soluble IL-15Rα, an approach that potentiates human NK cell-mediated killing of tumor cells. *In vitro* stimulation of human NK cells with a recombinant IL-15 superagonist significantly induced their expression of cytotoxic effector molecules granzyme B and perforin, their degranulation upon exposure to K562 cells as indicated by cell-surface expression of CD107a and their capacity to lyse K562 cells and HIV-1-infected T cells. The impact of IL-15 superagonist-induced activation of human NK cells on acute *in vivo* HIV-1 infection was investigated using hu-spl-PBMC-NSG mice, NOD-SCID-IL2rγ$^{-/-}$ mice intrasplenically injected with human PBMCs, which develop productive *in vivo* infection after intrasplenic inoculation with HIV-1. IL-15 superagonist treatment potently inhibited acute HIV-1 infection in hu-spl-PBMC-NSG mice even when delayed until three days after intrasplenic HIV-1 inoculation. Removal of NK cells from the human PBMCs prior to intrasplenic injection into NSG mice completely abrogated IL-15 superagonist-mediated suppression of *in vivo* HIV-1 infection. Thus, the *in vivo* activation of NK cells, integral mediators of the innate immune response, by treatment with an IL-15 superagonist increases their anti-HIV activity and enables them to potently suppress acute *in vivo* HIV-1 infection. These results indicate that *in vivo* activation of NK cells may represent a new immunotherapeutic approach to suppress acute HIV-1 infection.

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47 **Importance**

48 Epidemiological studies have indicated that NK cells contribute to the control of
49 HIV-1-infection and *in vitro* studies have demonstrated that NK cells can selectively kill
50 HIV-1-infected cells. We demonstrated that *in vivo* activation of NK cells by treatment
51 with an IL-15 superagonist that potently stimulates the anti-tumor activity of NK cells
52 markedly inhibited acute HIV-1 infection in humanized mice, even when activation of the
53 NK cells by IL-15 superagonist treatment is delayed until 3 days after HIV-1 inoculation.
54 NK cell-depletion from the PBMCs prior to their intrasplenic injection abrogated the
55 suppression of *in vivo* HIV-1-infection observed in humanized mice treated with the IL-
56 15 superagonist, demonstrating that the activated human NK cells were mediating IL-15
57 superagonist-induced inhibition of acute HIV-1 infection. Thus, *in vivo*
58 immunostimulation of NK cells, a promising therapeutic approach for cancer therapy,
59 may represent a new treatment modality for HIV-1-infected individuals particularly in the
60 earliest stages of infection.
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66 **Introduction**

67 The crucial role of the HIV-specific T cell and antibody response mounted by the
68 adaptive immune system to control HIV-1 infection is well established (1). However,
69 during acute infection viremia is not controlled because it takes several weeks after the
70 initiation of infection for the adaptive immune response to activate and clonally expand
71 sufficient HIV-1-specific T cells and B cells to suppress HIV-1 infection (2). This delay in
72 the mobilization of the adaptive immune response permits HIV-1 to rapidly replicate and
73 disseminate during the acute phase of infection leading to the production of high plasma
74 viral loads which are associated with an adverse disease course (3, 4). Early control of
75 HIV-1 replication can have a beneficial impact on subsequent disease course as
76 evidenced by the ability of some individuals whose viremia was suppressed by
77 combination antiretroviral therapy (cART) during acute infection to achieve long-term
78 infection control despite lacking protective HLA B alleles (5-7). Prior to the development
79 of an effective HIV-1-specific adaptive immune response, NK cells, crucial innate
80 immune effector cells which are large granular cytotoxic lymphocytes, are rapidly
81 activated and expanded and may contribute to controlling the initial phase of HIV-1
82 replication (8, 9). Infection induces changes in the cellular expression of ligands
83 recognized by NK cell receptors which enables NK cells to specifically identify and kill
84 virally infected cells to control and/or abort viral infections prior to the initiation of
85 antigen-specific responses (10). One mechanism by which HIV-1-infected cells become
86 susceptible to killing by NK cells is through the reduction in their surface expression of
87 MHC Class I molecule expression mediated by HIV-1 Nef as a means of evading killing
88 by HIV-1-specific CD8+ cytotoxic T cells (11). Further support for the role of NK cells in

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89 controlling HIV-1 replication and improving clinical outcomes is provided by several
90 genetic population studies linking slower HIV-1 disease progression with the expression
91 of specific allotypes of killer cell immunoglobulin-like receptors (KIRs) and their
92 respective HLA-class I ligands (10). However, the correlates of protective HIV-1-specific
93 immunity conferred by NK cells are not well characterized and there is no direct
94 evidence that *in vivo* stimulation of NK cell activity can enhance their capacity to inhibit
95 acute *in vivo* HIV-1 infection.

96 After infection, NK cells are rapidly activated by IL-15, a multifunctional cytokine
97 produced by activated dendritic cells and macrophages, which enables NK cells to
98 generate protective responses capable of clearing viral infections (12-14). In contrast to
99 IL-2 and other cytokines which are secreted and circulate until they bind directly to their
100 cognate receptors on target cells, IL-15 secreted by dendritic cells and macrophages
101 binds to the IL-15-specific receptor alpha chain (IL-15Rα/CD215) embedded on their
102 cell-surfaces to form a membrane-bound IL-15:IL-15Rα complex. This complex is then
103 presented in *trans* to bind to the IL-15Rβ (IL-2Rβ/CD122) and IL-15Rɣ (IL-2Rɣ/CD132)
104 chains expressed by adjacent NK cells and $CD8^+$ T cells which activate them (13, 15).
105 Alternatively, IL-15:IL-15Rα complexes are cleaved from dendritic cell and macrophage
106 membranes and are released into the serum, where they circulate as stable
107 heterodimers which display greater *in vivo* bioactivity and half-life (4 hours vs. 30
108 minutes) than secreted IL-15 monomers (16) to potently stimulate NK cells and/or $CD8^+$
109 T cells to eliminate virally infected cells (17). Consequently, the functional activity of
110 recombinant IL-15 can be greatly augmented by complexing it with soluble recombinant
111 IL-15Rα and thereby converting IL-15 from an agonist to a superagonist (6). To replicate

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the increased functional activity of the IL-15:IL-15Rα complex, we constructed a fusion protein, IL-15N72D:IL-15RαSu/Fc (IL-15 superagonist), which consists of two linked IgG1 Fc domains, each fused to an IL-15R α-chain 65-aa binding region spanning the Sushi (Su) domain (18). The Sushi domain is then bound to IL-15N72D, an IL-15 molecule with the aspartic acid at position 72 mutated to asparagine which increases its affinity for the human IL-15Rβ-chain and its biological activity by ~5-fold as compared to native human IL-15 (19). As a consequence of these structural modifications, the IL-15 superagonist, identified as ALT-803 for Phase I studies evaluating its efficacy as a candidate cancer therapeutic, exhibits ~25-fold higher biological activity and >35-fold longer serum half-life (~25 hours vs. ~40 minutes) than soluble IL-15 and potently stimulates *in vivo* NK cell and $CD8^+$ T cell proliferation in mice (20). In the current study, we investigated whether the capacity of NK cells to inhibit acute HIV-1 infection could be stimulated by the *in vivo* administration of the IL-15 superagonist to humanized mice days after inoculation with HIV-1. For these experiments we used a humanized mouse model, hu-spl-PBMC-NSG mice, generated by intrasplenically injecting NOD-SCID IL2rγ$^{-/-}$ (NSG) mice with activated human peripheral blood mononuclear cells (PBMCs), which develop rapid and robust HIV-1 infection after intrasplenic inoculation with HIV-1.

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129 **Materials and Methods**

130 *Generation of HIV-1 infectious molecular clones.* Virus stocks were generated by
131 transient transfection of 293T cells with a plasmid, NL-LucR.T2A-JR-CSF.ecto, which
132 encodes an infectious HIV-1 molecular clone whose Env protein ectodomain sequence
133 is derived from the JR-CSF Env and which expresses the *Renilla reniformis* luciferase
134 (LucR) gene (HIV-LucR) as previously described (21). LucR has a short cellular half-life
135 of approximately 3 hours (22), so its expression reflects active replication. HIV-LucR,
136 which was engineered to express the LucR reporter gene and the heterologous JR-CSF
137 *env* gene in *cis* with all of the HIV-1 open reading frames, continues to express LucR
138 over multiple cycles of replication after inoculation permitting highly sensitive and
139 specific detection of active HIV-1 replication for several weeks after inoculation (21).
140 The infectious titer of the HIV-LucR virus [5- to 10 x 10^7 infectious units (IU)/ml] was
141 determined by limiting dilution infection of TZM-bl cells as described (21).

142 *Generation of the IL-15 superagonist fusion complex.* The expression vector
143 pMSGV-IL-15RαSu/Fc expressing the IL-15RαSu/Fc fusion gene was constructed by
144 overlap PCR amplification of DNA templates encoding the Su domain of human IL-15Rα
145 (aa1–65 of human IL-15Rα) and the human IgG1 Fc fragment and was ligated into the
146 pMSGV-1 expression vector as described (23). To bind to the two IL-15 binding sites in
147 the IL-15RαSu/Fc fusion protein, we used IL-15N72D, human IL-15 mutated to replace
148 the native aspartic acid at position 72 with an asparagine. This IL-15 mutein displays a
149 ~5-fold higher levels of biological activity than the native IL-15 due to its increased
150 affinity and binding to the human IL-15Rβ-chain (19). The complex of IL-15RαSu/Fc
151 bound to IL-15N72D (IL-15 superagonist) was generated by co-transfecting expression

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vectors encoding IL-15RαSu/Fc and IL-15N72D into CHO cells and purifying the soluble the IL-15 superagonist fusion protein complex using a two-step affinity and ion exchange chromatography-based process as described (20).

Measurement of the in vivo capacity of the IL-15 superagonist to inhibit in vivo HIV-1 infection. NOD-SCID IL2rγ$^{-/-}$ (NSG) mice (Jackson Laboratories, Bar Harbor, ME) were bred and maintained under isolation and biocontainment conditions as described (24). The *in vivo* anti-HIV-1 activity of the IL-15 superagonist was determined using humanized mice generated through a modification of an *in vivo* adoptive transfer system we developed (25). PBMCs isolated from HIV-1 naïve donors were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (10 µg/ml), glutamine (2 mM) and HEPES (10 mM) and activated with phytohemagglutinin (PHA) (4 µg/ml) and IL-2 (100 U/ml) for 24 hours, washed and resuspended (~8 x 10^7 cells/ml) in sterile cold PBS. NSG mice were intrasplenically injected with the activated human PBMCs (~8 x 10^6 cells) to generate hu-spl-PBMC-NSG mice. Some of the hu-spl-PBMC-NSG mice were challenged *in vivo* with HIV-1 by intrasplenic injection with 100 µL of HIV-LucR virus (1 x 10^8 IU/ml) in parallel with intrasplenic injection of the activated PBMCs. One or three days after intrasplenic HIV-1 challenge, the mice were treated with one intravenous dose of the IL-15 superagonist (0.2 mg/kg) which provides the mice with a serum C_{max} of ~25 nM. For some experiments, the PBMCs were depleted of $CD8^+$ T cells or NK cells (>95% depletion) prior to their injection into the mice by immunomagnetic sorting using anti-human CD8 or anti-human CD56 microbeads (Miltenyi Biotec, Cambridge, MA). The level of HIV-1 infection that developed in the transferred human PBMCs in the spleens of the hu-spl-

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PBMC-NSG mice was quantified by measuring the LucR activity in the mouse splenic lysates using the Renilla Luciferase Assay System (Promega, Madison, WI) as described (26). To visualize and quantify *in vivo* HIV-1 infection using bioluminescent imaging, mice were imaged with the IVIS Spectrum imager (Caliper LifeSciences, Hopkinton, MA) after intravenous injection (5 µg/mouse) of the bioluminescence substrate RediJect Coelenterazine h (Caliper Life Sciences) and the images were analyzed using the Wizard bioluminescent selection tool for automatic wavelength and exposure detection. The bioluminescent and gray-scale images were overlaid using the LivingImage 4.0 software package to create a pseudocolor image that represents bioluminescence intensity. The bioluminescent intensity in the mouse spleens was quantified using the LivingImage 4.0 software package and reported as photon counts/second.

Flow cytometric analysis for the evaluation of cellular phenotype and perforin and granzyme B expression. Splenocytes isolated from the mice were stained with APC-labeled anti-human CD45 monoclonal antibody (mAb), FITC-labeled anti-human CD3 mAb, phycoerythrin (PE)-Cy7-labeled anti-human CD4, Pacific Blue-labeled anti-human CD8 mAb, PE-labeled anti-human CD56/CD16 mAb and APC-labeled anti-human CD16 mAb (all from BioLegend, San Diego, CA) and were analyzed using an LSRII (BD Biosciences, San Jose, CA) and FlowJo software (Treestar, Ashland, OR) as described (24). The effect of IL-15 superagonist treatment on perforin and granzyme B expression in NK cells was determined by treating human PBMCs from seronegative donors with the indicated concentration of IL-15 superagonist for two days. The PBMCs were washed, stained with APC-CY7-labeled anti-human CD3 mAb and PE-labeled anti-

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human CD56 mAb, incubated in fixation and permeabilization buffers, intracellularly stained with FITC-conjugated anti-human granzyme B mAb and with PerCP-Cy5.5-labeled perforin (BioLegend) and washed. The mean fluorescent intensity (MFI: geometric mean) of granzyme B and perforin expression by the gated $CD3^{-}CD56^{+}$ NK cells was determined by analysis on a FACSverse with FACSuite software (BD Biosciences).

Measurement of NK cell cytotoxic function directed against K562 cells and HIV-1-infected ACH2 cells. The capacity of the IL-15 superagonist treatment to stimulate NK cell degranulation was examined by quantifying expression of CD107a using flow cytometry after incubation of PBMCs harvested from HIV-negative donors with K562 cells as described (27). In parallel, we determined the effect of IL-15 superagonist stimulation on the functional cytolytic activity of NK cells by quantifying their capacity to lyse K562 target cells using a modification of a published technique (28). Briefly, K562 cells target cells were labeled with CellTrace™ Violet (Invitrogen-Life Technologies, Grand Island, NY). Human PBMCs either untreated or treated with the IL-15 superagonist (10 nM) were mixed with CellTrace™ Violet-labeled K562 target cells at the indicated effector:target (E:T) ratio in complete media and incubated at 37˚C with 5% CO_2 for 3 days. The K562 target cell viability was assessed by analysis of propidium iodide positive staining of the CellTrace™ Violet-labeled K562 target cells on a BD FACSVerse and cytotoxicity was calculated as the percentage of dead target cells in samples co-cultured with effector cells.

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To determine the capacity of the IL-15 superagonist to stimulate NK cell-mediated lysis of HIV-1-infected cells, PBMCs were harvested from HIV-negative donors and cultured (5 x 10^5 cells/well) with the indicated concentration of the IL-15 superagonist for 2 days. ACH2 cells, T cells latently infected with a single integrated HIV-1 provirus which display minimal HIV-1 replication unless stimulated with PMA and/or TNFα, were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (29). ACH2 cells were used as target cells and were either not activated or were stimulated with PMA (2 ng/ml) and TNFα (2 ng/ml) for 24 hours to stimulate HIV-1 production as described (30). After the effector cells were cultured for 48 hours with the indicated dose of the IL-15 superagonist, the effector cells were co-cultured with unactivated or activated ACH2 cells at an E:T ratio of 25:1 for 24 hours as described (31). Lysis of target cells was then measured using the fluorescence-based CytoTox-One Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's instructions. Background fluorescence was determined by adding unstimulated target ACH2 cells (2 x 10^4 cells/well) to effector PBMCs (5 X 10^5 cells/well) that were either treated or untreated with the IL-15 superagonist at an E:T ratio of 25:1. The percent of specific cell lysis was determined by subtracting background florescence from experimental florescence and dividing by the maximum LDH release from target cells, determined by lysing (2 x 10^4 cells/well) stimulated ACH2 cells, and then multiplying the resulting number by 100.

*Statistical analysis of data***.** GraphPad Prism statistical software was used for statistical analysis with an independent two-tailed Students *t*-test. Differences were considered statistically different for *P* values < 0.05.

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Study approval. All the studies were performed under protocols approved by the Institute for Animal Studies and the Institutional Review Board at the Albert Einstein College of Medicine in compliance with the human and animal experimentation guidelines of the United States Department of Health and Human Services.

Results

The in vitro cytolytic activity of human NK cells is induced by IL-15 superagonist treatment. The IL-15 superagonist, IL-15N72D:IL-15RαSu/Fc, is a homodimer of two engineered IgG1 Fc domains fused with the IL-15Rα binding domain each of which is bound to a mutated IL-15 molecule, IL-15N72D, which activates IL-15Rβɣ chain-expressing NK cells by mimicking the trans-presentation of IL-15 by the soluble IL-15:IL-15Rα complex (Figure 1A). Based on its capacity to potently activate the cytotoxic activity of NK cells and $CD8^+$ T cells, this IL-15 superagonist, also identified as ALT-803, has shown promise as an immunostimulatory agent to treat cancer (32). We investigated whether activation of NK cells by treatment with this IL-15 superagonist would increase their cytotoxic activity to enable them to eliminate HIV-1 infected cells and inhibit the *in vivo* acquisition of HIV-1 infection. The predominant killing pathway utilized by NK cells to kill target cells they identify as malignant or infected is by exocytosis of preformed granules containing lytic proteins, mainly perforin and granzyme B, into a cytotoxic synapse formed with the target cells (33). Because increasing cellular levels of perforin and granzyme B increases the cytotoxic capacity of NK cells (34), we examined whether IL15R-agonist treatment increased the cytolytic capacity of NK cells by inducing perforin and granzyme B production. Human PBMCs

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266 from six seronegative donors were treated with the IL-15 superagonist or untreated for
267 two days and then analyzed by flow cytometry to quantify the intracellular expression of
268 perforin and granzyme B by the NK cells. As compared to NK cells in the untreated
269 PBMCs, the NK cells in the human PBMCs treated with the IL15R-agonist for two days
270 displayed significantly higher levels of perforin (Figure 1B) and significantly higher levels
271 of granzyme B (Figure 1C) in a dose responsive fashion. CD107a is a marker of NK cell
272 degranulation, and its upregulation on cell surfaces is induced by interaction with MHC-
273 negative target cells and correlates with NK cell lysis of target cells and cytokine
274 secretion (27). To evaluate the capacity of IL-15 superagonist-stimulation to increase
275 the cytolytic activity of NK cells, we incubated human PBMCs isolated from
276 seronegative donors with K562 cells, a highly sensitive *in vitro* target for NK cells, and
277 examined the effect of treatment with the IL-15 superagonist on NK cell-surface-
278 expression of CD107a. In the absence of K562 cells, incubation of the PBMCs with the
279 IL-15 superagonist (1,000 pM) did not significantly increase cell-surface CD107a
280 expression by the NK cells (~5% CD107a positive, data not shown). When the PBMCs
281 were incubated with the target K562 cells, cell-surface CD107a expression increased by
282 5-fold (~26%), and treatment of the PBMCs with the IL-15 superagonist significantly
283 increased their cell-surface CD107a expression by an additional 2.3-fold (~62%) in a
284 dose responsive manner (Figure 1D). Increased cell-surface expression of CD107a
285 induced by treatment the IL-15 superagonist was associated with an increase in the *in*
286 *vitro* capacity of the NK cells in the PBMCs to lyse K562 cells (Figure 1E), the standard
287 NK-sensitive target cell for human NK cell assays (28). In addition, NK cell stimulation
288 by IL-15 superagonist was also indicated by its induction of the early activation marker

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CD69 (data not shown). Taken together, these data demonstrate that the IL-15 superagonist enhances the *in vitro* cytotoxic capability of human NK cells, possibly via upregulating their expression of the effector molecules granzyme B and perforin and increasing their activation state.

To determine whether IL-15 superagonist-stimulation increased the capacity of NK cells to kill HIV-1-infected cells, we used ACH2 cells, a latently infected cell line that can be induced to produce HIV-1 after PMA and/or TNF-α treatment (29) and which has previously been used as target cells to evaluate HIV-1-specific NK cell-mediated ADCC activity (31). Unstimulated PMBCs and IL-15 superagonist-stimulated PBMCs were incubated with unactivated ACH2 cells or PMA/TNF-α-stimulated ACH2 cells and lysis of ACH2 cells was quantified by measuring the amount of LDH released into the culture supernatant. Human PBMCs treated with the IL-15 superagonist (100 nM) displayed a ~8-fold increased lysis of stimulated ACH2 cells ($p<0.0001$) as compared to unstimulated PBMCs (Figure 1F).

In vivo HIV-1 infection is potently suppressed by treatment of hu-spl-PBMC-NSG mice with the IL-15 superagonist. We next examined whether the increased *in vitro* NK cell cytotoxic activity induced by the IL-15 superagonist correlated with an increased capacity of IL-15 superagonist-activated NK cells to inhibit acute *in vivo* HIV-1 infection. For these studies we used a humanized mouse model that supports *in vivo* HIV-1 infection, hu-spl-PBMC-NSG mice, NSG mice intrasplenically injected with human PBMCs, which we previously used to evaluate HIV-1-specific CD8+ T cell activity (25). The spleens of these mice continue to be populated with human PBMCs including NK cells and support HIV-1 infection for at least 1 month after intrasplenic injection. This

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312 model also permits us to identify effector cells responsible for anti-HIV-1 activity by
313 removing specific cell populations from the human PBMCs prior to intrasplenically
314 injecting them into the mouse spleens. As the infectious inoculum, we used HIV-LucR, a
315 replication-competent molecular HIV clone expressing the HIV-1_{JR-CSF} Env protein and a
316 *Renilla* luciferase reporter gene, which enables HIV-1 infection to be quantified by
317 directly measuring the luciferase activity of the infected cells (21). HIV-LucR virus has
318 been well-established as an infectious inoculum that can be used to evaluate the
319 capacity of antibodies, NK cells and CD8 T cells to inhibit HIV-1 infection (35-42). One
320 day after hu-spl-PBMC-NSG mice were infected with HIV-1 by intrasplenic injection with
321 HIV-LucR, one group of mice was intravenously injected with a dose of the IL-15
322 superagonist. Five days later, HIV-1 infection was quantified by determining the level of
323 expression of the luciferase reporter gene in the mouse spleens. Splenic lysates of the
324 IL-15 superagonist-treated mice displayed a greater than 99% reduction in luciferase
325 activity (average = 804 RLU) as compared to untreated mice (average = 827,829 RLU),
326 indicating that the IL-15 superagonist treatment delivered one day after direct
327 intrasplenic challenge with HIV-1 significantly suppressed ($p<0.001$) acute HIV-1
328 infection (Figure 2A). The use of an infectious HIV-1 expressing a LucR reporter gene
329 enabled us to directly visualize *in vivo* infection by intravital bioluminescent imaging and
330 observe suppression of acute HIV-1 infection by activation of NK cells by the IL-15
331 superagonist. Hu-spl-PBMC-NSG mice were infected with HIV-1 by intrasplenic
332 injection with HIV-LucR. One day later the mice were treated either with the IL-15
333 superagonist or PBS and four days later, the level of *in vivo* HIV-1 infection was
334 visualized and quantified by bioluminescent imaging of LucR activity using the IVIS

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335 Spectrum intravital imaging system. Direct visualization demonstrated that the level of
336 bioluminescence in the spleens of the IL-15 superagonist-treated hu-spl-PBMC-NSG
337 mice was markedly reduced in the IL-15 superagonist-treated hu-spl-PBMC-NSG mice
338 as compared to untreated mice (Figure 2B). Quantification of the bioluminescent signal
339 by measurement of total photon flux demonstrated that IL-15 superagonist treatment
340 significantly reduced bioluminescence due to HIV-LucR infection (Figure 2C).

341 To examine the effect of acute HIV-1 infection and treatment with IL-15
342 superagonist on the human T cell and NK cell populations in the hu-spl-PBMC-NSG
343 mouse spleens, one group of hu-spl-PBMC-NSG mice were inoculated with HIV-LucR
344 and one day later some of the HIV-LucR-inoculated mice and HIV-LucR-uninoculated
345 mice were intravenously injected with a dose of the IL-15 superagonist. Six days later,
346 the human $CD3^{-}CD56^{+}$ NK cell and $CD3^{+}$ T cell populations (Figure 3A) and $CD4^{+}$ and
347 $CD8^{+}$ T cell populations (Figure 3B) in the mouse spleens were evaluated by flow
348 cytometry. These results demonstrated that one week after intrasplenic injection, the
349 hu-spl-PBMC-NSG mouse spleens were well-populated with human NK cells, CD4+ T
350 cells and CD8+ T cells which was not markedly altered as a consequence of HIV-LucR-
351 inoculation or IL-15 superagonist treatment.

352 We next investigated whether activation of NK cells by the IL-15 superagonist
353 could inhibit the acquisition of HIV-1 infection even when the NK cells were activated by
354 IL-15 superagonist administered three days after inoculation with HIV-1. Hu-spl-PBMC-
355 NSG mice were intrasplenically challenged with HIV-LucR and three days later some
356 mice were treated with a dose of the IL-15 superagonist. Five days after treatment, the
357 level of HIV-1 infection in the hu-spl-PBMC-NSG mouse spleens was quantified and

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was significantly reduced ($p<0.005$) in the IL-15 superagonist-treated mice by almost 95% as compared to untreated mice (Figure 4A). We extended those findings by examining whether the potent suppressive effect mediated by IL-15 superagonist treatment was sustained by quantifying the level of HIV-1 infection in the mouse spleens at 2 weeks and at 3 weeks after treatment. When hu-spl-PBMC-NSG mice were treated three days after intrasplenic injection with HIV-LucR with an intravenous injection of the IL-15 superagonist, the development of HIV-1 infection in the humanized mouse spleens was significantly inhibited ($p<0.05$) by greater than 90% when evaluated at day 14 and 21 post-treatment as compared to untreated mice (Figure 4B). The continued reduction in the level of HIV-1 infection by over 97% at 21 days after inoculation suggested that IL-15 superagonist treatment initiated 3 days after HIV-1 inoculation eliminated cells initially infected with HIV-1 and thereby potently suppressed the establishment of primary HIV-1 infection. However, when activation of NK cells by the IL-15 superagonist is delayed by not initiating IL-15 superagonist treatment until 5 days after HIV-1 inoculation, establishment of primary HIV-1 infection was no longer significantly inhibited (data not shown).

NK cells mediate IL-15 superagonist-induced inhibition of in vivo HIV-1 infection. To confirm that NK cells activated by the IL-15 superagonist were responsible for inhibiting *in vivo* HIV-1 infection, we depleted by immunomagnetic sorting either the population of $CD8^+$ T cells or the population of NK cells from the PBMC prior to intrasplenic injection (Figure 5A). NSG mice were intrasplenically injected with either activated unfractionated PBMCs, $CD8^+$ T cell-depleted PBMCs, or NK cell-depleted PBMCs and in parallel infected with HIV-1 by intrasplenic injection with HIV-LucR. One

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day later, some groups of mice were either untreated or treated with one intravenous dose of the IL-15 superagonist and five days later the level of acute HIV-1 infection was quantified by determining the level of expression of the luciferase reporter gene in the mouse splenocytes. While the depletion of $CD8^+$ T cells from the PBMCs had only minimal effects on reducing the IL-15 superagonist-mediated inhibition of HIV-1 infection, NK cell-depletion of human PBMCs completely abrogated ($p<0.0001$) IL-15 superagonist-mediated inhibition of *in vivo* HIV-1 infection (Figure 5B).

Human NK cells activated by in vivo IL-15 superagonist treatment eliminate HIV-1 infected cells in hu-spl-PBMC-NSG mouse spleens. To determine whether treatment with the IL-15 superagonist during acute HIV-1 infection induced *in vivo* activation of human NK cells in the hu-spl-PBMC-NSG, we examined its effect on expression of CD69, a functional marker for NK cell activation that correlates with the expression of the degranulation marker, CD107a (43, 44). Hu-spl-PBMC-NSG mice were infected with HIV-1 by intrasplenic injection with HIV-LucR. One day later, one group of mice was treated with the IL-15 superagonist. Two days after IL-15 superagonist treatment, flow cytometric analysis demonstrated that the $CD3^-/CD56^+/CD16^+$ human NK cells in the spleens of humanized mice displayed an almost three-fold increase ($p<0.001$) in CD69 expression as compared to human NK cells in spleen of untreated hu-spl-PBMC-NSG mice (Figure 6). We next investigated whether *in vivo* IL-15 superagonist activation of NK cells increased their capacity to kill HIV-1-infected cells in hu-spl-PBMC-NSG mice with established *in vivo* infection. Hu-spl-PBMC-NSG mice were infected by intrasplenic injection of HIV-LucR. After five days, which provided sufficient time for *in vivo* infection to develop, one group of mice was treated with an intravenous injection of the IL-15

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superagonist. One day after IL-15 superagonist treatment, the level of HIV-1 infection in the mouse spleens, as determined by measurement of LucR activity, was reduced by greater than 90%, as compared to LucR levels in the spleens of untreated mice (Figure 7). Taken together, these results indicated that *in vivo* treatment with an IL-15 superagonist activated human NK cells, which increased their *in vivo* cytolytic activity and enabled them to kill HIV-1-infected cells and suppress acute HIV-1 infection.

Discussion

To our knowledge, this study is the first reported demonstration that *in vivo* activation of NK cells can inhibit acute HIV-1 infection. The NK cells were activated by *in vivo* treatment with a superagonist of IL-15, a multifunctional cytokine which plays a crucial role in NK cell and $CD8^+$ T cell development, activation, proliferation, differentiation and functional activity (12, 15, 45). PBMCs treated *in vitro* with the IL-15 superagonist displayed increased NK cell activity as indicated by their increased expression of perforin and granzyme B as well as cell-surface CD107a upon exposure to K562 cells and by their augmented capacity to lyse K562 cells, an NK cell-sensitive target, and activated ACH2 cells, an HIV-1 producing T cell line. Activation of the human NK cells by *in vivo* treatment with the IL-15 superagonist after intrasplenic injection of human PBMCs into the NSG mice was indicated by their increased expression of CD69, a marker associated with NK cell activation (43, 44). Acute *in vivo* HIV-1 infection was potently inhibited by IL-15 superagonist treatment of NSG mice intrasplenically injected with unfractionated human PBMCs or with $CD8^+$ T cell-depleted PBMCs. In contrast, when NK cells were depleted from the human PBMCs prior to their intrasplenic injection

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into the NSG mice, acute HIV-1 infection was not inhibited by treatment with the IL-15 superagonist. This demonstrated that human NK cells were the crucial effector cells activated by *in vivo* treatment with the IL-15-agonist that mediated inhibition of acute *in vivo* HIV-1 infection. Taken together, our data indicate that *in vivo* activation of human NK cells by treatment with the IL-15 superagonist, even when delayed until 3 days after HIV-1 inoculation, suppressed the establishment of productive infection. It is likely that this is due to NK cell-mediated elimination of a large fraction of the cells initially infected with HIV-1 during the acute infection, which markedly reduced the population of HIV-1-infected cells and subsequent level of productive HIV-1 infection. This was supported by our demonstration that one day after treatment with the IL-15 superagonist, the level of HIV-1 infection in the spleens of hu-spl-PBMC-NSG mice with established HIV-1 infection was reduced by greater than 90%. While treatment with the IL-15 superagonist initiated 5 days after inoculation markedly reduced the number of HIV-1-infected cells measured one day later (i.e. measured at day 6 after inoculation as shown in Figure 7), sustained suppression of infection was not observed in these mice as it was in mice treated with the IL-15 superagonist within 3 days after inoculation. This decline in sustainable inhibition of HIV-1 inhibition may be due to the additional two-day deferral in treatment which provides HIV-1 with more time to spread sufficiently to a critical number of infected cells that escape NK cell-mediated killing and disseminate infection. Thus, administration of the IL-15 superagonist at day 3 or day 5 after inoculation can potently inhibit HIV infection, but sustained suppression of HIV infection only occurs when the IL-15 superagonist is administered within 3 days of exposure. NK cells contribute to the control of HIV-1 replication in HIV-1-infected individuals as indicated by the capacity of

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450 HLA-B Bw4-80I-activated NK cells that express the activating killer immunoglobulin-like
451 NK cell receptor (KIR3DS1) to potently inhibit HIV-1 replication (46) and the delayed
452 progression of HIV-1 disease observed in individuals expressing HLA-B Bw4-80I, the
453 ligand for KIR3DL1 receptors which are highly expressed on NK cells (47). NK cells
454 constitutively express inhibitory and activating receptors capable of identifying and
455 rapidly lysing virally infected cells (48). Consequently, even during initial exposure to
456 HIV-1 and prior to the development of HIV-specific adaptive immune responses, NK
457 cells can recognize and eliminate HIV-1 infected cells whose HLA Class I MHC
458 molecules are downregulated by HIV-1 Nef (48). The capacity of some NK cells to
459 recognize and kill HIV-1-infected cells may be limited by the selective Nef-mediated
460 reduction of only HLA-A and HLA-B molecules with continued expression of HLA-C and
461 HLA-E molecules (49). It is possible that IL-15 superagonist treatment may activate
462 other NK cells which are capable of effectively killing HIV-infected $CD4^+$ T cells despite
463 their continued expression of HLA-C and HLA-E because this subpopulation of NK cells
464 does not express inhibitory receptors specific for HLA-C and HLA-E molecules (11).
465 Activated NK cells may also identify and kill HIV-1 infected cells by alternative
466 mechanisms including the detection of stress molecules whose expression is induced
467 by viral infection (50, 51). In addition, activated NK cells may also inhibit the spread of
468 acute HV-1 infection by secreting CC chemokines, such as RANTES, MIP1-α and
469 MIP1-β, which block HIV-1 binding to CCR5 (52).

470 These results extend the reports of previous studies which first suggested a role
471 for IL-15 in treating HIV-1 infection based on its capacity to potently activate $CD8^+$ T
472 cells and NK cells (53), enhance the *in vitro* function and survival of HIV-specific $CD8^+$ T

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473 cells (54), delay recurrence of HIV-1 viremia after antiretroviral therapy interruption (55)
474 and prevent HIV-1 transmission during breast feeding (56). While IL-15-induced
475 stimulation of NK cells and CD8+ T cells may have the beneficial effect of inhibiting HIV-
476 1 replication during acute infection as shown in our study, during chronic infection IL-15-
477 mediated activation of $CD4^+$ T cells may potentially have the opposite and deleterious
478 effect of increasing their susceptibility to HIV-1 infection. This was indicated in studies of
479 acutely SIV-infected macaques treated with IL-15 whose memory $CD4^+$ T cells
480 displayed increased susceptibility to SIV infection (57) and who subsequently displayed
481 increased viral set points and accelerated progression of disease (58). It is possible that
482 our observation that the IL-15 superagonist potently inhibited HIV-1 infection in the hu-
483 spl-PBMC-NSG mice is due to its ~25-fold higher biological activity and >35-fold longer
484 serum half-life than soluble IL-15 (20). The greater potency of the IL-15 superagonist
485 as compared to IL-15 may enable it to stimulate a more potent anti-HIV-1-immune
486 response than the recombinant IL-15 used to treat the macaques and outweigh the
487 potential deleterious effect of IL-15-mediated activation of $CD4^+$ T cells, particularly
488 during acute infection. It is also possible that IL-15, particularly when delivered as a
489 complex with the IL-15Rα chain, has different effects on macaque NK cells and T cells
490 than on human NK cells and T cells.

491 Multiple studies have demonstrated that immunostimulation of $CD8^+$ T cells and
492 NK cells by the IL-15:IL-15Rα complex stimulates the *in vivo* clearance of tumors and
493 has led to the initiation of Phase I trials for their use to treat cancer (59). While studies in
494 mice have indicated that $CD8^+$ T cells were the crucial effector cells mediating IL-15-
495 induced clearance of tumors (20, 60), our results demonstrate that activated NK cells

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were the crucial effector cells activated by the IL-15 superagonist to inhibit acute HIV-1 infection. The functional activity of NK cells is impaired during chronic HIV-1 infection (9), and it is possible that this impairment may be reversed by treatment with the IL-15 superagonist. Further studies in humanized mice infected with HIV-1 and treated with the IL-15 superagonist, should enable us to further delineate the effects of NK cell activation mediated by IL-15 superagonist treatment on inhibiting *in vivo* HIV-1 infection. Treatment of SHIV-infected monkeys with broadly neutralizing HIV-specific antibodies improved the function of their HIV-specific $CD8^+$ T cells and lowered their viral-load set points, suggesting that neutralizing antibody therapy could increase anti-viral immune responses to enable better control of SHIV infection (61). It is possible that NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) may contribute to the reported reduction in rebound viremia in humanized mice treated with broadly neutralizing HIV-specific antibodies (62). Thus, an intriguing possibility is that increasing NK cell activity by treatment with the IL-15 superagonist may further facilitate the anti-HIV-1 activity of neutralizing antibody therapy by augmenting ADCC activity. Further studies in humanized mice infected with HIV-1 and treated with the IL-15 superagonist, should enable us to further delineate the effects of IL15R-agonist-mediated NK cell activation on directly inhibiting *in vivo* HIV-1 infection and further suppressing HIV-1 infection through ADCC when combined with broadly neutralizing HIV-specific antibody therapy.

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769 **Figure Legends**

770

771 **Figure 1. The IL-15 superagonist treatment of human PBMCs increases their**
772 **cytotoxic activity. (A)** Schematic structure of the IL-15 superagonist. **(B)** Intracellular
773 perforin expression and **(C)** intracellular granzyme B expression in $CD3^{-}CD16^{+}CD56^{+}$
774 cells were determined by flow cytometry after human PBMCs from six seronegative
775 donors were incubated for 2 days with the indicated concentration of the IL-15
776 superagonist and the data represents the mean +/- SEM of MFI values obtained from
777 the six donors. **(D)** After human PBMCs were stimulated with the indicated
778 concentration of the IL-15 superagonist for 16 hours, they were co-incubated with K562
779 target cells (5:1 E:T ratio) for 4 hours and CD107a expression by $CD3^{-}CD56^{+}$NK cells
780 was determined by flow cytometry. The results are expressed as the mean ± STE
781 percentage of CD107a positive $CD3^{-}CD56^{+}$ NK cells from PBMCs isolated from 4
782 normal donors. **(E)** Human PBMCs were cultured with K562 cells at the indicated E:T
783 ratio with or without the IL-15 superagonist (10 nM). After 3 days, the fraction of dead
784 K562 cells was quantified by flow cytometry. **(F)** Human PBMCs were cultured with
785 activated ACH2 cells with or without the indicated dose of the IL-15 superagonist. After
786 24 hours, the percent cell lysis of target cells was measured using the CytoTox-One
787 Homogeneous Membrane Integrity Assay. Data represents at least three replicates per
788 group with mean ± SEM of the group. * $P < 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, Student's *t*
789 test.

790 **Figure 2. Treatment of hu-spl-PBMC-NSG mice with the IL-15 superagonist**
791 **inhibited acute *in vivo* HIV-1 infection.** NSG mice were intrasplenically injected with

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activated human PBMCs (~8 x 10^6 cells) and with HIV-LucR (1 x 10^7 IU) and one day later were either untreated or intravenously treated with the IL-15 superagonist (0.2 mg/kg). **(A)** Five days after HIV-LucR inoculation, the level of HIV-1 infection in the treated mice was evaluated by measuring LucR activity in splenic lysates. **(B)** Four days after HIV-LucR inoculation, *in vivo* infection in the mice was evaluated by intravenously injecting them with a luciferase bioluminescent substrate and then scanning the mice with the IVIS Spectrum imager to measure the bioluminescence intensity in the spleens. Pseudocolor images of the bioluminescent intensity measured as photon counts/second overlaid with gray-scale images of a control uninoculated mouse and IL-15 superagonist or PBS-treated mice are shown. **(C)** Quantification of the total flux of the bioluminescent signals detected by the IVIS Spectrum imager reported as photons/second in IL-15 superagonist or PBS-treated mouse spleens. ** $P \leq 0.01$, Student's *t* test. The experimental protocol is shown at the bottom of the Figure.

Figure 3. Effect of *in vivo* IL-15 superagonist treatment and/or HIV-1 infection on the population of human NK cells and T cells in hu-spl-PBMC-NSG mouse spleens. NSG mice were intrasplenically injected with activated human PBMCs (~8 x 10^6 cells). In parallel, some of these mice were also inoculated by intrasplenic injection of HIV-LucR (1 x 10^7 IU). One day later, some of the uninoculated and HIV-LucR-inoculated mice were intravenously treated with the IL-15 superagonist (0.2 mg/kg). Six days later, the spleens were harvested and after gating on the live human $CD45^+$ population, the fraction of splenocytes that were **(A)** NK cells ($CD3^-CD56^+CD16^+$) or T cells ($CD3^+$) and **(B)** $CD8^+$ T cells or $CD4^+$ T cells was determined by flow cytometry.

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814 The mean value ± SEM of each group of hu-spl-PBMC-NSG mice which were HIV-
815 LucR-inoculated and/or IL-15 superagonist-treated as indicated is shown.

816 **Figure 4. IL-15 superagonist-treatment of hu-spl-PBMC-NSG mice administered**
817 **three days after inoculation inhibited acute *in vivo* HIV-1 infection.** Three days
818 after NSG mice were intrasplenically injected with activated human PBMCs (~8 x 10^6
819 cells) and HIV-LucR (1 x 10^7 IU), the mice were either untreated or treated with one
820 intravenous dose of the IL-15 superagonist (0.2 mg/kg). **(A)** Five days after HIV-LucR
821 inoculation, the level of HIV-1 infection in the IL-15 superagonist-treated mice and
822 untreated mice were evaluated by measuring LucR activity in splenic lysates. **(B)** HIV-1
823 infection was quantified by measuring LucR activity in the splenic lysates in another
824 experimental group at 14 days after inoculation and in another experimental group 21
825 days after inoculation after HIV-LucR inoculation. A dot plot showing the LucR value
826 from each mouse is shown with mean ± SEM of the group. * $P < 0.05$, Student's *t* test.
827 The experimental protocol is shown at the bottom of the Figure.

828 **Figure 5. NK cells mediate IL-15 superagonist-inhibition of HIV-1 infection in hu-**
829 **spl-PBMC-NSG mice.** Activated PBMCs that were unfractionated or were depleted of
830 $CD8^+$ T cells or NK cells by immunomagnetic sorting were intrasplenically injected into
831 NSG mice (~ 8 x 10^6 cells) in parallel with HIV-LucR (1 x 10^7 IU). One day later, the
832 mice were treated with an intravenous dose of the IL-15 superagonist or PBS. **(A)** The
833 effectiveness of the depletion of $CD8^+$ T cells or $CD56^+$ NK cells from the PBMCs prior
834 to injection was assessed by flow cytometry after staining with antibodies to human
835 CD3, CD8 and CD56. **(B)** Six days after intrasplenic injection, HIV-1 infection in the
836 spleens of the mice treated as indicated was quantified by measuring LucR activity in

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837 the splenic lysates. A dot plot showing the LucR value from each mouse is shown with
838 mean ± SEM of the group. **** $P < 0.0001$, Student's *t* test

839 **Figure 6. *In vivo* activation of human NK cells by the IL-15 superagonist in the**
840 **spleens of HIV-1-infected hu-spl-PBMC-NSG mice.** NSG mice were intrasplenically
841 injected with human PBMCs (~8 x 10^6 cells) and in parallel inoculated with HIV-LucR (1
842 x 10^7 IU) and then either untreated or treated with one intravenous dose of the IL-15
843 superagonist (0.2 mg/kg) one day after infection. Two days later, the harvested
844 splenocytes were analyzed by flow cytometry for expression of human CD3, CD56,
845 CD16 and CD69. After gating on the human $CD3^-/CD56^+/CD16^+$ NK cell population, the
846 fraction of NK cells expressing CD69 was determined. The data point for each mouse is
847 shown with mean ± SEM of the group. *** $P < 0.001$, Student's *t* test. The experimental
848 protocol is shown at the bottom of the Figure.

849 **Figure 7. *In vivo* activation of human NK cells by the IL-15 superagonist**
850 **stimulates elimination of HIV-1-infected cells in the spleens of HIV-1-infected hu-**
851 **spl-PBMC-NSG mice.** Five days after NSG mice were intrasplenically injected with
852 human PBMCs (~8 x 10^6 cells) and in parallel inoculated with HIV-LucR (1 x 10^7 IU),
853 they were either untreated or treated with one intravenous dose of the IL-15
854 superagonist (0.2 mg/kg). One day later, HIV-1 infection in the mouse spleens was
855 quantified by measuring LucR activity in the splenic lysates. A dot plot showing the
856 LucR value from each mouse is shown with mean ± SEM of the group. ** $P < 0.01$,
857 Student's *t* test. The experimental protocol is shown at the bottom of the Figure.
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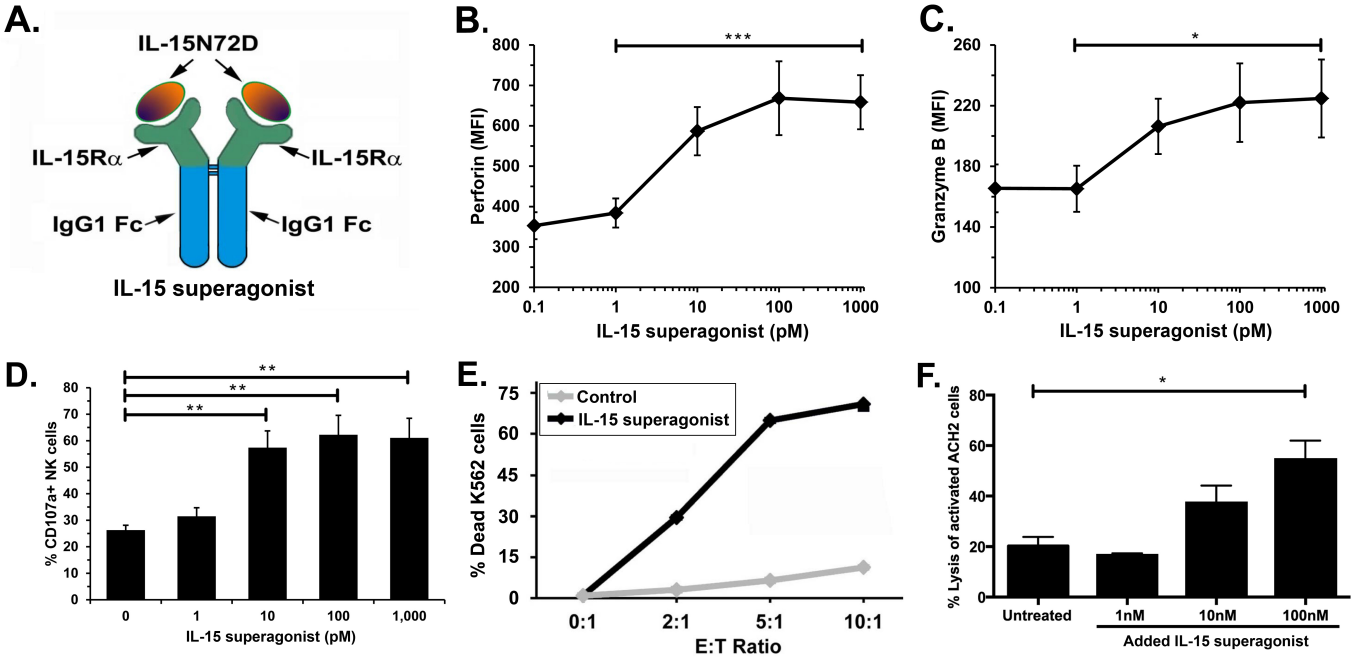

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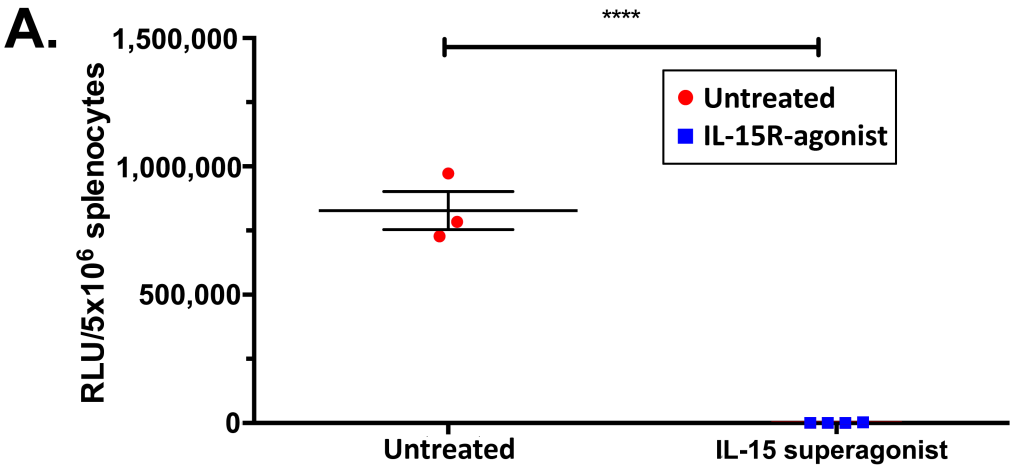

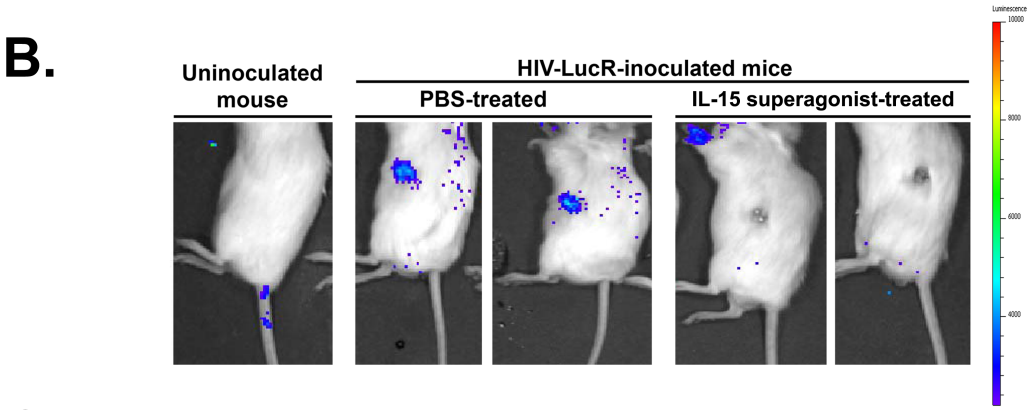

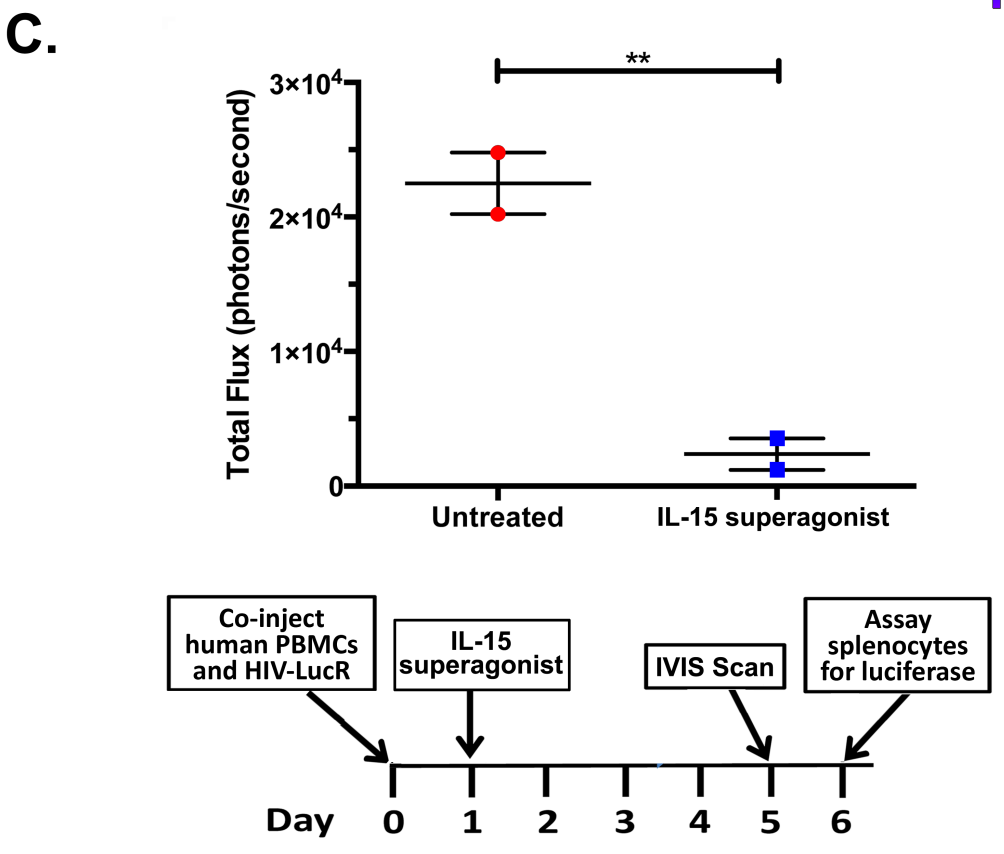

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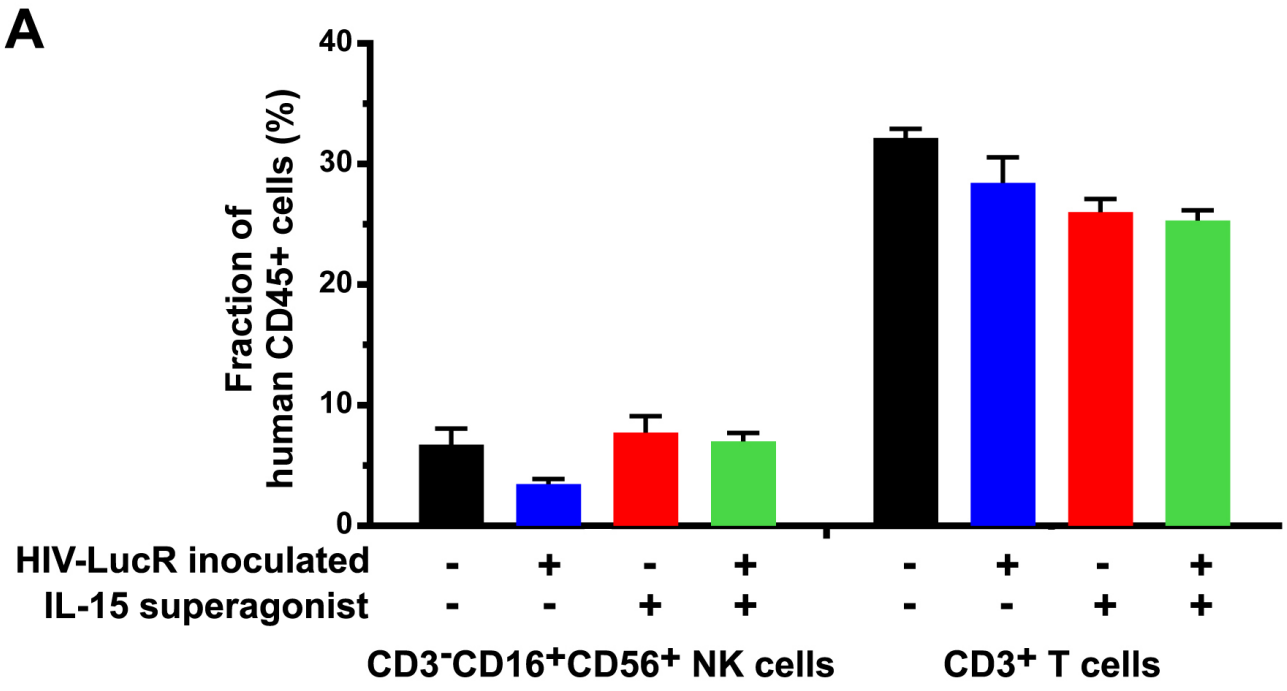

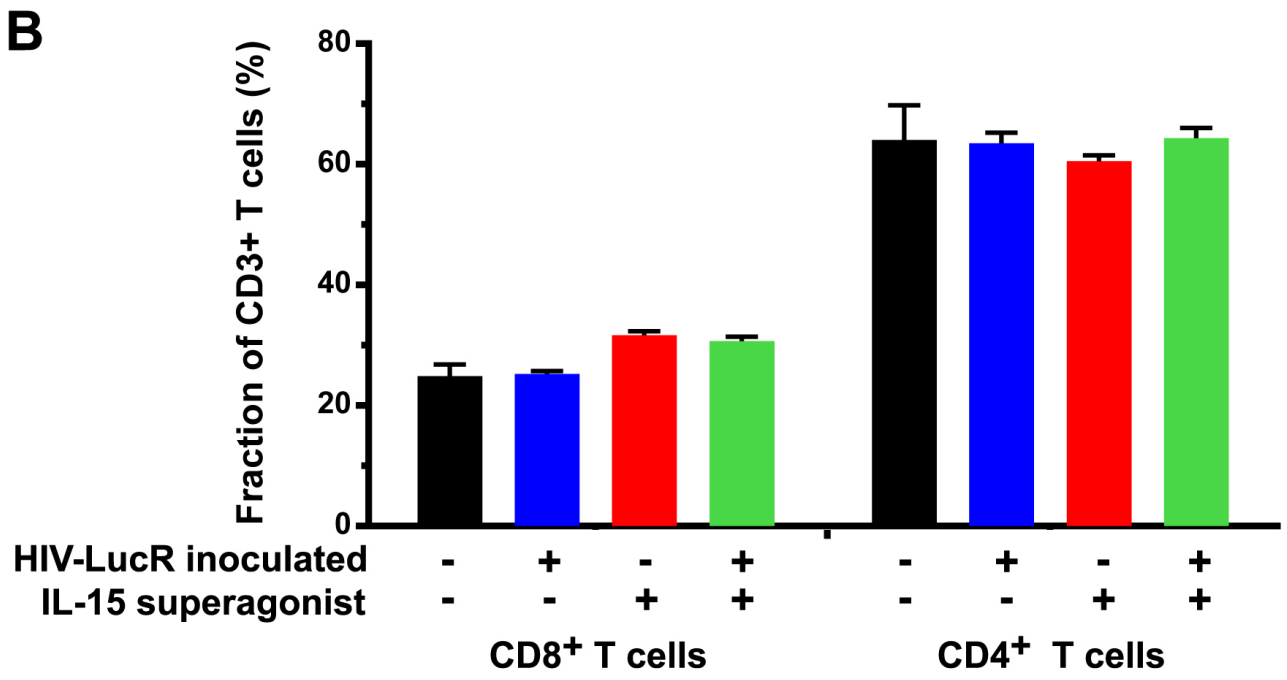

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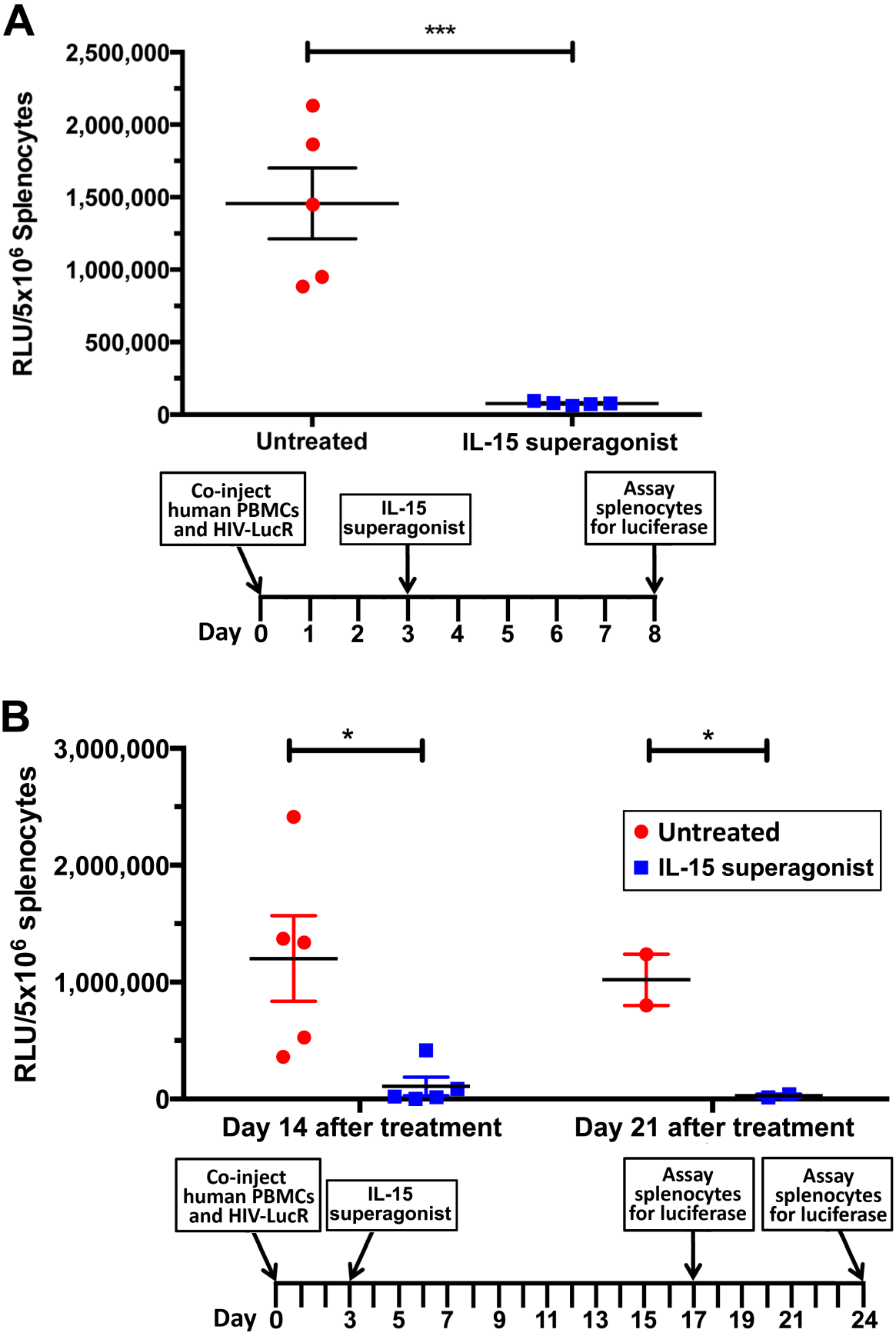

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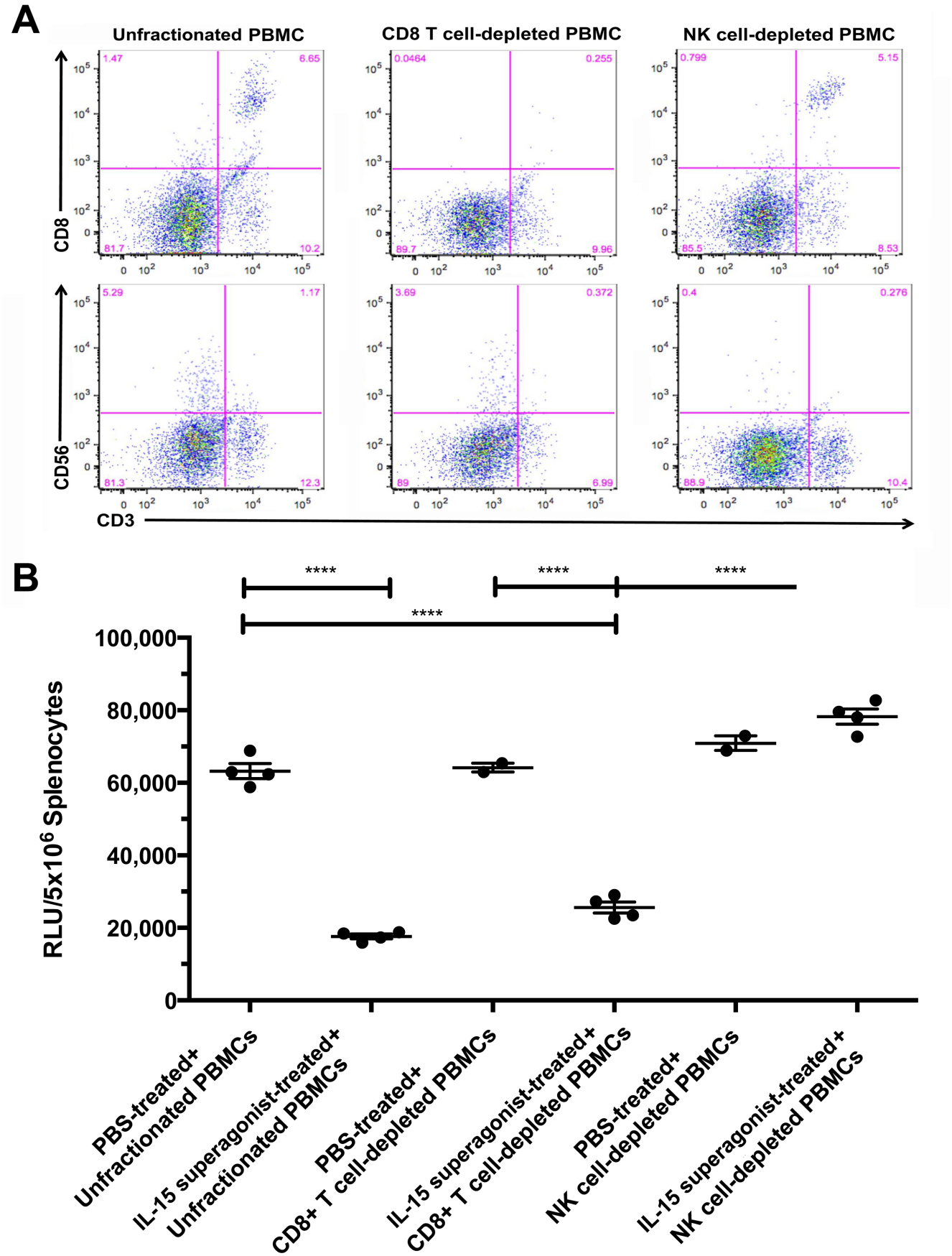

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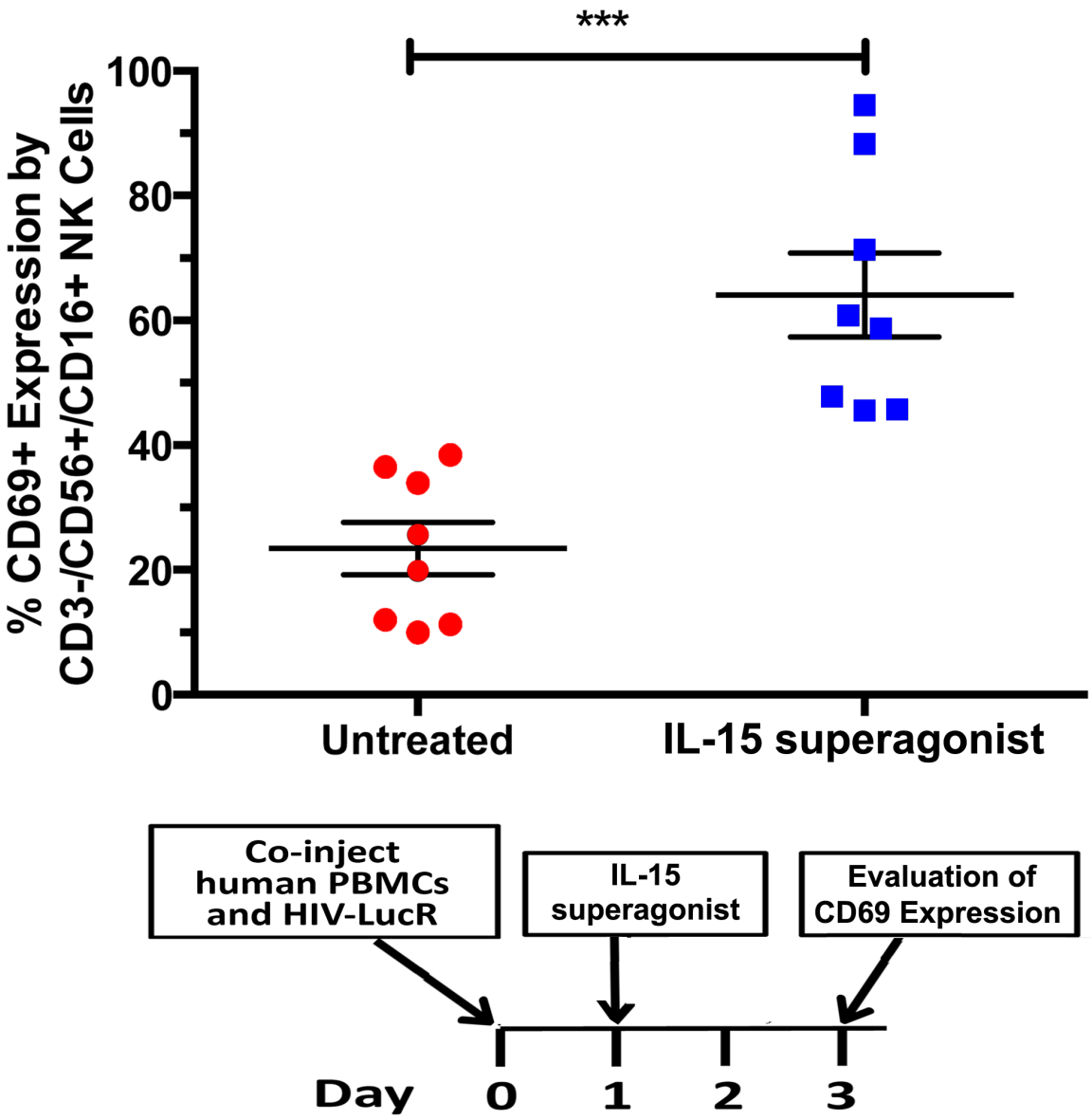

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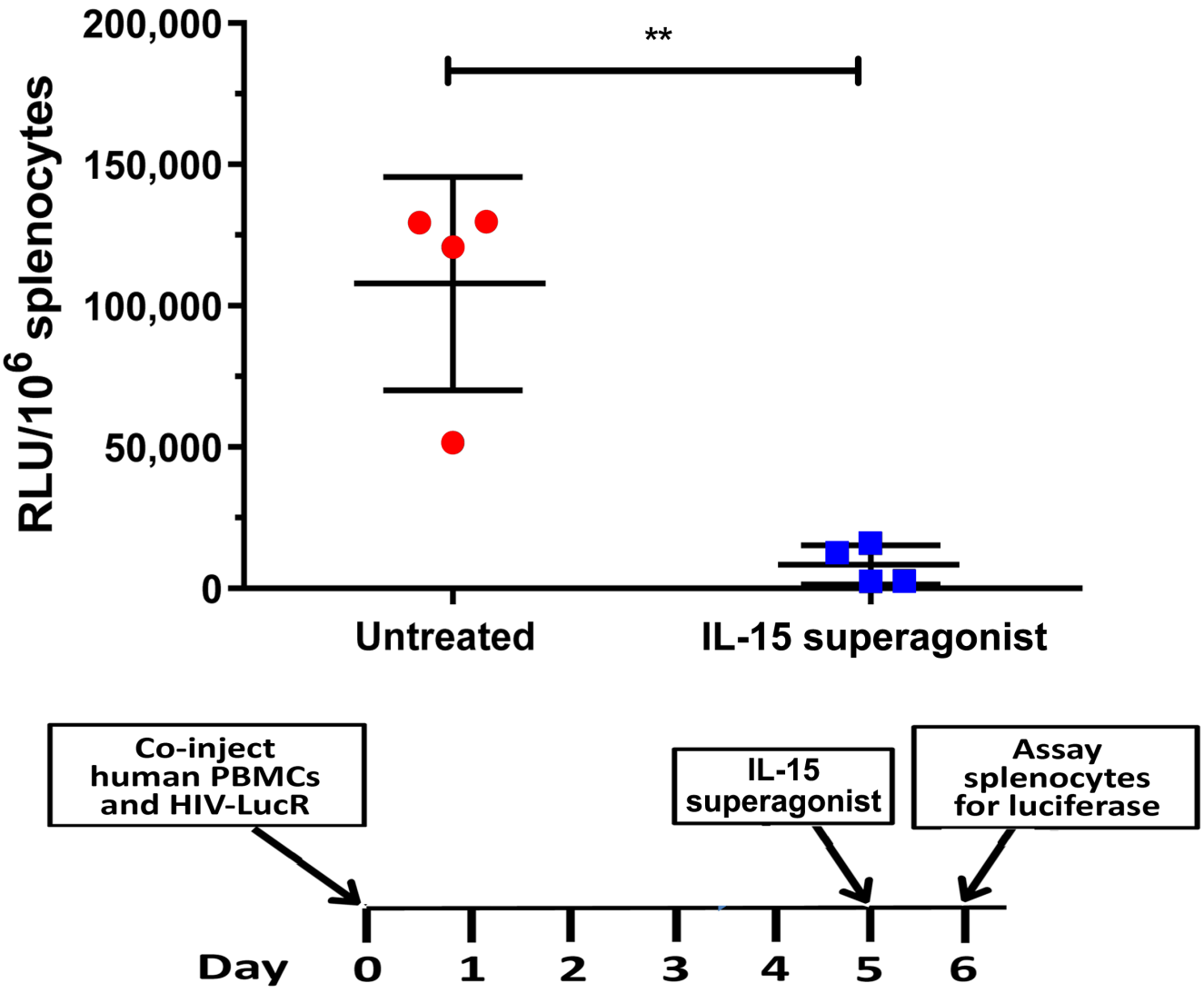

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