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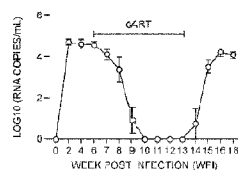
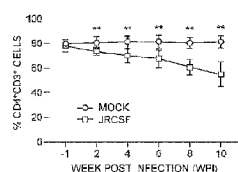
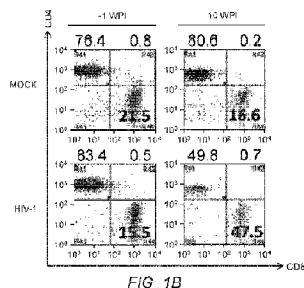
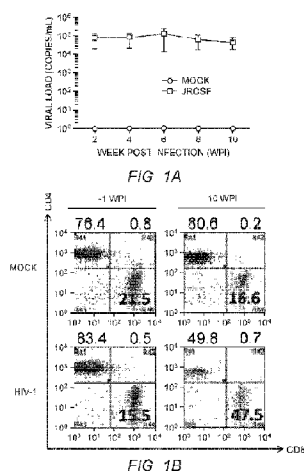
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(54) Title: IMMUNE MODULATION METHODS TO REACTIVATE HIV-1 RESERVOIR

(57) Abstract: The present invention relates to methods for reactivating latent HIV-1 and treating HIV-1 infection in a subject by depleting T regulatory cells. The invention further relates to animal models with depleted T regulatory cells and methods of screening for HIV-1 therapeutics.





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Immune Modulation Methods to Reactivate HIV-1 Reservoir

RELATED APPLICATIONS

[0001] The present application claims the benefit, under 35 U.S.C. § 119(e), of U.S. Provisional Application No. 62/160,834, filed March 13, 2015, the content of which is incorporated by reference herein in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under AI077454 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates to methods for reactivating latent HIV-1 and treating HIV-1 infection in a subject by depleting T regulatory cells. The invention further relates to animal models with depleted T regulatory cells and methods of screening for HIV-1 therapeutics.

BACKGROUND OF THE INVENTION

[0004] HIV-1 persistence during effective combination anti-retroviral therapy (cART) is a most critical problem in curing HIV-1 infection in patients (Siliciano *et al.*, *Nat. Med.* 9:727 (2003); Siliciano *et al.*, *Cold Spring Harb. Perspect. Med.* 1:a007096 (2011)). The “cART-resistant HIV reservoirs” are not clearly defined. This latent reservoir which harbor latent but replication competent proviruses (Finzi *et al.*, *Science* 278:1295 (1997)) is established within days of acute infection (Chun *et al.*, *Proc. Natl. Acad. Sci. USA* 94:13193 (1997); Whitney *et al.*, *Nature* 512, 74 (2014)). Based on mathematical modeling, it may take scores of decades of cART treatment for the patient to eradicate the HIV-1 reservoir (Finzi *et al.*, *Science* 278:1295 (1997); Siliciano *et al.*, *Nat. Med.* 9:727 (2003)).

[0005] It has been proposed that reducing the HIV-1 reservoirs or curing HIV-1 requires reactivation of the latent/low-replicating reservoir and subsequent killing of HIV-1 infected cells (Choudhary *et al.*, *Annu. Rev. Pharmacol. Toxicol.* 51:397 (2011)).

However, the HIV-1 reservoirs *in vivo* remain poorly defined and it is not clear how to efficiently reactivate the latent HIV-1 *in vivo*. Recent reports suggested that pharmacological reagents can activate latent HIV-1 gene expression *in vitro* (Archin *et al.*, *Nature* 487:482 (2012); Shirakawa *et al.*, *Trends Microbiol.* 21:277 (2013)). However these reagents alone are unlikely to efficiently activate HIV-1 reservoirs and lead to the elimination of the HIV-1 reservoir *in vivo*. A recent report has suggested that low-persistent replicating HIV-1 in lymphoid tissues plays a critical “reservoir” role in HIV-1 persistence during effective cART (Lorenzo-Redondo *et al.*, *Nature* 530:51 (2016)). New approaches with immune activation are necessary to efficiently reactivate the HIV-1 reservoirs *in vivo* (Bullen *et al.*, *Nat. Med.* 20:425 (2014)).

[0006] Humanized mice that have been transplanted with human hematopoietic stem cells, lymphoid tissue or peripheral blood cells in immunodeficient mice, have been used to study human infectious disease (Bility *et al.*, *J. Gastroenterol. Hepatol.* 28 Suppl 1:120 (2013); Leung *et al.*, *Eur. J. Immunol.* 43:2246 (2013); Zhang *et al.*, *Cell. Mol. Immunol.* 9:237 (2012)). Several mutant mouse strains have been used for generating humanized mice to date. NOD-*rag1*^{-/-}*Il2rg*^{null} (NRG) mouse lacks mouse T, B and NK cells, which support increased human cell engraftment. NRG humanized mice, therefore, is a validated and robust small animal model for studying HIV-1 infection, immunopathogenesis and latency *in vivo* (Bournazos *et al.*, *Cell* 158:1243 (2014); Diskin *et al.*, *J. Exp. Med.* 210:1235 (2013); Halper-Stromberg *et al.*, *Cell* 158:989 (2014); Horwitz *et al.*, *Proc. Natl. Acad. Sci. USA* 110:16538 (2013); Klein *et al.*, *Nature* 492:118 (2012)).

[0007] Regulatory T (Treg) cells, which suppress T cell activation and HIV-1 replication, are susceptible to HIV-1 infection due to their expression of HIV-1 coreceptor CCR5 and preferential activation of HIV-1 LTR (Holmes *et al.*, *Immunol. Res.* 41:248 (2008); Jiang *et al.*, *Blood* 112:2858 (2008); Oswald-Richter *et al.*, *PLoS Biol.* 2:E198 (2004)). It has been reported that Treg cells are important for acute viral replication and spread during early infection (Jiang *et al.*, *Blood* 112:2858 (2008)) via Vpr-dependent mechanisms (Sato *et al.*, *PLoS Pathog.* 9:e1003812 (2013)). However, Treg cells likely play different roles depending on the stage of HIV-1 infection (Holmes *et al.*, *Immunol. Res.* 41:248 (2008)). A recent report suggests that Treg cells may inhibit

HIV-1 replication in activated CD4⁺ T cells via a cyclic adenosine mono-phosphate (cAMP)-dependent mechanism (Moreno-Fernandez *et al.*, *Blood* 117:5372 (2011)), suggesting that Treg may contribute to HIV latency in T cells. The precise role of Treg cells in HIV-1 latency during HIV-1 chronic infection remains unknown.

[0008] The present invention addresses previous shortcomings in the art by providing methods for reactivating virus during chronic HIV-1 infection.

SUMMARY OF THE INVENTION

[0009] The present invention takes advantage of the relationship between T regulatory cells and HIV-1 reservoirs in infected subjects. HIV-1 reservoirs can be reactivated by Depletion of T regulatory cells results in reactivation of HIV-1 reservoirs, leading to more effective treatment of HI-1 infection and associated diseases and disorders.

[0010] Accordingly, in one aspect, the invention relates to a method of reactivating latent HIV-1 in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby reactivating latent HIV-1.

[0011] Another aspect of the invention relates to a method of reducing HIV-1 reservoirs in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby reducing HIV-1 reservoirs.

[0012] A further aspect of the invention relates to a method of treating HIV-1 infection in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby treating HIV-1 infection.

[0013] An additional aspect of the invention relates to a method for identifying a compound suitable for treatment of HIV-1 infection, the method comprising providing an animal model of HIV-1 infection in which T regulatory cells have been depleted, delivering a candidate compound to the animal, and measuring HIV-1 levels in the animal, wherein a decrease in HIV-1 levels compared to an animal that has not received the candidate compound identifies the candidate compound as a compound suitable for treatment of HIV-1 infection.

[0014] These and other aspects of the invention are set forth in more detail in the description of the invention below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figures 1A-1D show that humanized mice support persistent HIV-1 infection and respond to cART with cART-resistant HIV-1 reservoirs. (A) Plasma viral load (copies/mL, y axis) measured over time (weeks, x axis) in HIV-1_{JRCSF}-infected humanized mice. (B) Representative flow cytometry plots of CD4 (y axis) vs. CD8 (x axis) on CD3⁺ human cells. (C) CD4⁺ T cell frequency (% in human CD3⁺ cells, y axis) measured over time (weeks, x axis). The mean with standard deviations from MOCK (n=6) and HIV-1 (n=6) was shown in each panel. (D) Plasma viral load (genome copies/mL, y axis) measured over time (weeks, x axis) in HIV-infected humanized mice under cART from 7-13 wpi, and after stopping cART from 13-18 wpi (n=7). The detection limit of HIV-1 in the blood is 100 copies/ml. The experiments were repeated twice. The data from each experiment was merged and summarized. Error bars display standard deviations. * P < 0.05.

[0016] Figures 2A-2F show that Treg cell depletion increases HIV-1 replication in humanized mice. HBSS or ONTAK[®] was injected at 11 wpi in humanized mice infected with HIV-1_{JRCSF}. CD4 T cells were analyzed for the expression of CD25 by flow cytometry. (A) Representative flow cytometry plots of CD4(y axis) vs CD25 (x axis) are shown. (B) Relative frequency of CD4⁺CD25^{hi} Treg cells is summarized (HIV-1 (n=6) and HIV-1+ONTAK[®] (n=7)). (C) Plasma viral load (copies/mL, y axis) measured at 1 week (hatched bar) and 2 weeks (black bar) after ONTAK[®] injection in HIV-infected humanized mice. (D) Representative flow cytometry plots of CD4 (y axis) vs. HIV-1 p24 (x axis) on CD3⁺CD8⁻ human cells in the blood (PB), lymph node (LN) and spleen (Sp) are shown. (E) The frequency of p24⁺ cells on CD3⁺CD8⁻ cells of PB, LN and Sp in MOCK (n=3), HIV-1 (n=6) and HIV-1+ONTAK[®] (n=7) group is shown. The experiments were repeated twice. The data from each experiment was merged and summarized. Error bars display standard deviations. * P < 0.05. (F) Immunohistochemical (IHC) staining of HIV-1 p24 (brown) in Sp and LN from MOCK, HIV-1 and HIV-1+ONTAK[®] group.

[0017] Figures 3A-3C show ONTAK[®] does not affect the frequency of non-Treg population. HBSS or ONTAK[®] was injected at 11 wpi in humanized mice infected with

HIV-1_{JRCSF}. CD4⁺ and CD8⁺ T cells in the blood were analyzed for the expression of CD25 by flow cytometry. Relative frequency of CD4⁺CD25^{int} T cells (A), CD8⁺ T cells (B) and CD8⁺CD25⁺ T cells (C) are shown.

[0018] Figures 4A-4C show ONTAK[®] efficiently depletes CD4⁺CD25⁺FoxP3⁺ Treg cells in HIV/cART mice. HBSS or ONTAK[®] was injected in humanized mice infected with HIV-1_{JRCSF} and treated with cART. (A) CD4⁺ T cells (CD3⁺CD8⁻ T cells to include human CD4 T cells whose surface CD4 have been down-regulated by HIV-1 infection) from spleens (SP) or bone marrow (BM) were analyzed for the expression of CD25 and FoxP3 by flow cytometry. Relative frequency of CD4⁺CD25^{int} T cells; (B) Summary data of Treg depletion in the spleen; and (C) depletion of Treg cells in the bone marrow are shown.

[0019] Figures 5A-5D show specific Treg cell depletion induces HIV-1 reactivation under cART in humanized mice. (A) Plasma viral load (copies/mL, y axis) measured over time (weeks, x axis) in HIV-1_{JRCSF}-infected humanized mice under cART (AZT, NVP and 3CT; 6-13.3 wpi) with ONTAK[®] (n=12, treated at 12 wpi) or control (n=10) treatment. (B) Spleen cell-associated HIV-1 viral mRNA, (C) cell-associated HIV DNA copy number (copies/human cells) and (D) Relative HIV-1 mRNA levels over HIV proviral DNA levels in the spleen in each group (MOCK; n=3, HIV-1; n=5, HIV-1+cART; n=9, HIV-1+cART+ONTAK[®]; n=9) are shown. The experiments were repeated three times with three donor tissues. Error bars display standard deviations. *, p < 0.05.

[0020] Figures 6A-6C show that ONTAK[®] does not increase the relative HIV proviral DNA in cART mice. HBSS or ONTAK[®] was injected in humanized mice infected with HIV-1 and treated with cART. Bone marrow cells were analyzed for cell-associated HIV mRNA relative to human CD4 (A) or cell-associated HIV proviral DNA. ONTAK[®] significantly increases cell-associated HIV RNA, but not cell-associated HIV DNA. Relative HIV mRNA (A), HIV DNA (B) and HIV/RNA/DNA(C) are shown.

[0021] Figure 7 shows sequence analysis of rebounded viruses reveals no RTi-resistant mutations. HIV-1 Gag-Pol sequences from plasma rebound viruses upon ONTAK[®] treatment under cART. JRCSF represents the sequence from input virus. 1 to

4 represent the sequences from 4 different mice with plasma rebounded viruses. Shaded residues indicate RT inhibitors-associated mutations.

[0022] Figures 8A-8E show Treg cell depletion reactivates latent HIV-1 in memory T cells in humanized mice. The frequencies of p24⁺ cells in T cells, plasmacytoid dendritic cells (pDCs), monocytes and myeloid DCs from spleen (Sp, A) and bone marrow (BM, B) in each group are shown. Each dot represents a single mouse (MOCK, n=3; HIV-1, n=5; HIV+cART, n=9; HIV-1+cART+ONTAK[®], n=9). (C) HIV-1 p24 in the spleen was detected by immunofluorescence staining with anti-human CD3, anti-HIV-1 p24 and DAPI. Representative results from each group were shown in merged picture (top) and in single pictures (3 at the bottom of each merged picture). A high magnification picture was shown (inset) for the HIV-1+cART+ONTAK[®] group. (D) CD4 naïve T (T_N, CD3⁺CD8⁻CD45RA⁺CCR7⁺) or memory T (T_M, CD3⁺CD8⁻CD45RA⁻) cell subset was analyzed for p24 expression. Representative flow cytometry plots of CD4 (y axis) vs HIV-1 p24 (x axis) on CD4 naïve T cells (T_N, upper panels) and memory T cells (T_M, bottom panels) in each group were shown. The numbers indicate the frequency of p24⁺ cells (%). (E) The frequencies of p24⁺ cells in T_N (left panels) and T_M (right panels) subsets from spleens are shown. Each dot represents a single mouse (MOCK, n=3; HIV-1, n=4; HIV+cART, n=10; HIV-1+cART+ONTAK[®], n=10). The experiments were repeated twice. The data from each experiment was merged and summarized. Error bars display standard deviations. **, p < 0.01.

[0023] Figures 9A-9B show analysis of T HIV-1 reactivation among memory CD4 T cell subsets by Treg depletion. (A) CD4 central memory T (T_{CM}, CD3⁺CD8⁻CD45RA⁻CCR7⁺) or effector memory T (T_{EM}, CD3⁺CD8⁻CD45RA⁺CCR7⁻) cell subset was analyzed for p24 expression by flow cytometry. Gating on flow cytometry plot for T_{CM} and T_{EM} cells are shown in left panel. Representative flow cytometry plots of CD4 (y axis) vs HIV-1 p24 (x axis) on T_{CM} (upper panels) and T_{EM} (bottom panels) in each group are shown. The numbers indicate the frequency of p24⁺ cells (%). (B) The frequencies of p24⁺ cells in T_{CM} (left panels) and T_{EM} (right panels) subsets from spleen and bone marrow are summarized. Each dot represents a single mouse (MOCK, n=3; HIV-1, n=4; HIV+cART, n=10; HIV-1+cART+ONTAK[®], n=10). Error bars display standard deviations. **, p < 0.01 (one-way ANOVA; bonfferoni's post-hoc).

[0024] Figure 10 shows poly I:C (TLR3 ligand) treatment activates the cART-resistant HIV reservoir.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The present invention will now be described in more detail with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0026] Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a complex comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

[0027] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

[0028] Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right, unless specifically indicated otherwise. Nucleotides and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by either the one-letter code, or the three letter code, both in accordance with 37 C.F.R. §1.822 and established usage.

[0029] Except as otherwise indicated, standard methods known to those skilled in the art may be used for cloning genes, amplifying and detecting nucleic acids, and the like. Such techniques are known to those skilled in the art. *See, e.g., Sambrook et al.,*

Molecular Cloning: A Laboratory Manual 2nd Ed. (Cold Spring Harbor, NY, 1989); Ausubel *et al.* Current Protocols in Molecular Biology (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

[0030] All publications, patent applications, patents, patent publications and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

[0031] As used in the description of the invention and the appended claims, the singular forms “a,” “an,” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0032] Also as used herein, “and/or” refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (“or”).

[0033] The term “about,” as used herein when referring to a measurable value such as an amount of polypeptide, dose, time, temperature, enzymatic activity or other biological activity and the like, is meant to encompass variations of $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, $\pm 0.5\%$, or even $\pm 0.1\%$ of the specified amount.

[0034] The transitional phrase “consisting essentially of” means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim, “and those that do not materially affect the basic and novel characteristic(s)” of the claimed invention. *See, In re Herz*, 537 F.2d 549, 551-52, 190 USPQ 461, 463 (CCPA 1976) (emphasis in the original); *see also* MPEP § 2111.03.

[0035] The term “about,” as used herein when referring to a measurable value such as an amount of polypeptide, dose, time, temperature, enzymatic activity or other biological activity and the like, is meant to encompass variations of $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, $\pm 0.5\%$, or even $\pm 0.1\%$ of the specified amount.

[0036] The term “reactivate,” as used herein, refers to the activation of latent HIV-1 proviruses present in resting CD4⁺ T cells.

[0037] The term “latent,” as used herein, refers to replication competent HIV-1 proviruses present in resting CD4⁺ T cells.

[0038] The term “reservoir,” as used herein, refers to the latent but replication competent HIV-1 proviruses present in resting CD4⁺ T cells.

[0039] The term “T regulatory cell” or Treg cell,” as used herein, refers to cells that suppress the immune responses of other cells. T regulatory cells are generally CD3⁺CD4⁺CD25⁺Foxp3⁺ and CD127⁻.

[0040] An “effective” amount as used herein is an amount that provides a desired effect.

[0041] A “therapeutically effective” amount as used herein is an amount that provides some improvement or benefit to the subject. Alternatively stated, a “therapeutically effective” amount is an amount that will provide some alleviation, mitigation, or decrease in at least one clinical symptom in the subject. Those skilled in the art will appreciate that the therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

[0042] By the terms “treat,” “treating,” or “treatment,” it is intended that the severity of the subject's condition is reduced or at least partially improved or modified and that some alleviation, mitigation or decrease in at least one clinical symptom is achieved.

[0043] One aspect of the invention relates to a method of reactivating latent HIV-1 in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby reactivating latent HIV-1.

[0044] Another aspect of the invention relates to a method of reducing HIV-1 reservoirs in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby reducing HIV-1 reservoirs.

[0045] A further aspect of the invention relates to a method of treating HIV-1 infection and/or a disease or disorder associated with HIV-1 infection in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby treating HIV-1 infection. The method may be used to prevent, delay, or slow the progression of HIV-1 infection to AIDS related complex (ARC) or AIDS in a subject, *e.g.*, a human subject, or to treat a subject with ARC or AIDS.

[0046] A further aspect of the invention relates to agents that deplete T regulatory cells in a subject for use in a method of reactivating latent HIV-1 in a subject in need thereof, a method of reducing HIV-1 reservoirs in a subject in need thereof, or a method of treating HIV-1 infection and/or a disease or disorder associated with HIV-1 infection in a subject in need thereof.

[0047] Another aspect of the invention relates to agents that deplete T regulatory cells in a subject for use in the manufacture of a medicament for reactivating latent HIV-1 in a subject in need thereof, reducing HIV-1 reservoirs in a subject in need thereof, or treating HIV-1 infection and/or a disease or disorder associated with HIV-1 infection in a subject in need thereof.

[0048] The depletion of T regulatory cells may be carried using any method known in the art. In some embodiments, depleting T regulatory cells comprises delivering to the subject an effective amount of denileukin diftitox (ONTAK[®]). In other embodiments, depleting T regulatory cells comprises delivering to the subject an effective amount of an antibody that specifically binds to CD25. In other embodiments, depleting T regulatory cells comprises delivering to the subject an effective amount of a toll like receptor (TLR) ligand, *e.g.*, a TLR3 ligand, *e.g.*, poly I:C.

[0049] In certain embodiments, the T regulatory cells are depleted by at least 50%, *e.g.*, at least 60%, 70%, 80%, 90%, 95%, or more.

[0050] In some embodiments, the methods of the invention further comprise delivering to the subject a HIV-1 therapeutic agent. Any HIV-1 therapeutic agent known in the art may be used. In certain embodiments, the HIV-1 therapeutic agent is an antiretroviral agent, *e.g.*, an antiretroviral agent selected from the group consisting of reverse transcriptase inhibitors, protease inhibitors, viral integration inhibitors, viral entry inhibitors, viral maturation inhibitors, iRNA agents, antisense RNA, vectors expressing iRNA agents or antisense RNA, PNA, antiviral antibodies and any combination thereof. Such agents are well known in the art and are described, for example in U.S. Patent No. 8,497,251, incorporated by reference herein in its entirety. In certain embodiments, the antiretroviral agent is selected from the group consisting of AZT, 3TC, ddI, ddC, 3TC, saquinavir, indinavir, ritonavir, nelfinavir, nevirapine, efavirenz, and combinations thereof.

[0051] In some embodiments, the methods of the invention further comprise delivering to the subject a combination or cocktail of HIV-1 therapeutic agents as is known in the art, such as combination anti-retroviral therapy (cART) or highly active antiretroviral therapy (HAART), *e.g.*, at least two or three different drugs from at least two

different classes selected from reverse transcriptase inhibitors, protease inhibitors, viral integration inhibitors, viral entry inhibitors, and viral maturation inhibitors.

[0052] In some embodiments, the subject is a subject in need of treatment, *e.g.*, a subject that has or is suspected of having and HIV-1 infection or has been diagnosed with a disease or disorder associated with HIV-1 infection, *e.g.*, ARC or AIDS. In some embodiments, the subject is an animal model of HIV-1 infection, *e.g.*, a rodent such as a mouse or a primate such as a monkey.

[0053] As a further aspect, the invention provides pharmaceutical formulations and methods of administering the same to carry out the methods of the invention. The pharmaceutical formulation may comprise any of the reagents discussed above in a pharmaceutically acceptable carrier.

[0054] By “pharmaceutically acceptable” it is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material can be administered to a subject without causing any undesirable biological effects such as toxicity.

[0055] The formulations of the invention can optionally comprise medicinal agents, pharmaceutical agents, carriers, adjuvants, dispersing agents, diluents, and the like.

[0056] The compounds of the invention can be formulated for administration in a pharmaceutical carrier in accordance with known techniques. *See, e.g.*, Remington, *The Science And Practice of Pharmacy* (21th Ed. 2005). In the manufacture of a pharmaceutical formulation according to the invention, the compound (including the physiologically acceptable salts thereof) is typically admixed with, *inter alia*, an acceptable carrier. The carrier can be a solid or a liquid, or both, and is preferably formulated with the compound as a unit-dose formulation, for example, a tablet, which can contain from 0.01 or 0.5% to 95% or 99% by weight of the compound. One or more compounds can be incorporated in the formulations of the invention, which can be prepared by any of the well-known techniques of pharmacy.

[0057] A further aspect of the invention is a method of treating subjects *in vivo*, comprising administering to a subject a pharmaceutical composition comprising a compound of the invention in a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is administered in a therapeutically effective amount. Administration of the compounds of the present invention to a human subject or an

animal in need thereof can be by any means known in the art for administering compounds.

[0058] The formulations of the invention include those suitable for oral, rectal, topical, buccal (*e.g.*, sub-lingual), vaginal, parenteral (*e.g.*, subcutaneous, intramuscular including skeletal muscle, cardiac muscle, diaphragm muscle and smooth muscle, intradermal, intravenous, intraperitoneal), topical (*i.e.*, both skin and mucosal surfaces, including airway surfaces), intranasal, transdermal, intraarticular, intrathecal, and inhalation administration, administration to the liver by intraportal delivery, as well as direct organ injection (*e.g.*, into the liver, into the brain for delivery to the central nervous system, into the pancreas, or into a tumor or the tissue surrounding a tumor). In some embodiments, the formulation is delivered to the site of tissue damage (*e.g.*, fibrosis) or inflammation. The most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular compound which is being used.

[0059] In certain embodiments, the inhibitor is administered via one or more of oral administration, injection, and a surgically implanted pump. In some embodiments, the administration is via intravenous injection, intraportal delivery, or direct liver injection.

[0060] For injection, the carrier will typically be a liquid, such as sterile pyrogen-free water, pyrogen-free phosphate-buffered saline solution, bacteriostatic water, or Cremophor EL[R] (BASF, Parsippany, N.J.). For other methods of administration, the carrier can be either solid or liquid.

[0061] For oral administration, the compound can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. Compounds can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that can be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, edible white ink and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured

as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance.

[0062] Formulations suitable for buccal (sub-lingual) administration include lozenges comprising the compound in a flavored base, usually sucrose and acacia or tragacanth; and pastilles comprising the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

[0063] Formulations of the present invention suitable for parenteral administration comprise sterile aqueous and non-aqueous injection solutions of the compound, which preparations are preferably isotonic with the blood of the intended recipient. These preparations can contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions can include suspending agents and thickening agents. The formulations can be presented in unit\dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

[0064] Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously described. For example, in one aspect of the present invention, there is provided an injectable, stable, sterile composition comprising a compound of the invention, in a unit dosage form in a sealed container. The compound or salt is provided in the form of a lyophilizate which is capable of being reconstituted with a suitable pharmaceutically acceptable carrier to form a liquid composition suitable for injection thereof into a subject. The unit dosage form typically comprises from about 10 mg to about 10 grams of the compound or salt. When the compound or salt is substantially water-insoluble, a sufficient amount of emulsifying agent which is pharmaceutically acceptable can be employed in sufficient quantity to emulsify the compound or salt in an aqueous carrier. One such useful emulsifying agent is phosphatidyl choline.

[0065] Formulations suitable for rectal administration are preferably presented as unit dose suppositories. These can be prepared by admixing the compound with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

[0066] Formulations suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which can be used include petroleum jelly, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

[0067] Formulations suitable for transdermal administration can be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration can also be delivered by iontophoresis (*see*, for example, Tyle, *Pharm. Res.* 3:318 (1986)) and typically take the form of an optionally buffered aqueous solution of the compound. Suitable formulations comprise citrate or bis/tris buffer (pH 6) or ethanol/water and contain from 0.1 to 0.2M of the compound.

[0068] The compound can alternatively be formulated for nasal administration or otherwise administered to the lungs of a subject by any suitable means, *e.g.*, administered by an aerosol suspension of respirable particles comprising the compound, which the subject inhales. The respirable particles can be liquid or solid. The term "aerosol" includes any gas-borne suspended phase, which is capable of being inhaled into the bronchioles or nasal passages. Specifically, aerosol includes a gas-borne suspension of droplets, as can be produced in a metered dose inhaler or nebulizer, or in a mist sprayer. Aerosol also includes a dry powder composition suspended in air or other carrier gas, which can be delivered by insufflation from an inhaler device, for example. *See* Ganderton & Jones, *Drug Delivery to the Respiratory Tract*, Ellis Horwood (1987); Gonda (1990) *Critical Reviews in Therapeutic Drug Carrier Systems* 6:273-313; and Raeburn *et al.*, *J. Pharmacol. Toxicol. Meth.* 27:143 (1992). Aerosols of liquid particles comprising the compound can be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. *See, e.g.*, U.S. Patent No. 4,501,729. Aerosols of solid particles comprising

the compound can likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

[0069] Alternatively, one can administer the compound in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

[0070] Further, the present invention provides liposomal formulations of the compounds disclosed herein. The technology for forming liposomal suspensions is well known in the art. When the compound is in the form of an aqueous-soluble material, using conventional liposome technology, the same can be incorporated into lipid vesicles. In such an instance, due to the water solubility of the compound, the compound will be substantially entrained within the hydrophilic center or core of the liposomes. The lipid layer employed can be of any conventional composition and can either contain cholesterol or can be cholesterol-free. When the compound of interest is water-insoluble, again employing conventional liposome formation technology, the compound can be substantially entrained within the hydrophobic lipid bilayer which forms the structure of the liposome. In either instance, the liposomes which are produced can be reduced in size, as through the use of standard sonication and homogenization techniques. The liposomal formulations containing the compound disclosed herein, can be lyophilized to produce a lyophilizate which can be reconstituted with a pharmaceutically acceptable carrier, such as water, to regenerate a liposomal suspension.

[0071] In the case of water-insoluble compounds, a pharmaceutical composition can be prepared containing the water-insoluble compound, such as for example, in an aqueous base emulsion. In such an instance, the composition will contain a sufficient amount of pharmaceutically acceptable emulsifying agent to emulsify the desired amount of the compound. Particularly useful emulsifying agents include phosphatidyl cholines and lecithin.

[0072] In addition to compound, the pharmaceutical compositions can contain other additives, such as pH-adjusting additives. In particular, useful pH-adjusting agents include acids, such as hydrochloric acid, bases or buffers, such as sodium lactate, sodium acetate, sodium phosphate, sodium citrate, sodium borate, or sodium gluconate. Further, the compositions can contain microbial preservatives. Useful microbial preservatives include methylparaben, propylparaben, and benzyl alcohol. The microbial preservative is

typically employed when the formulation is placed in a vial designed for multidose use. Other additives that are well known in the art include, *e.g.*, detackifiers, anti-foaming agents, antioxidants (*e.g.*, ascorbyl palmitate, butyl hydroxy anisole (BHA), butyl hydroxy toluene (BHT) and tocopherols, *e.g.*, α -tocopherol (vitamin E)), preservatives, chelating agents (*e.g.*, EDTA and/or EGTA), viscomodulators, tonicifiers (*e.g.*, a sugar such as sucrose, lactose, and/or mannitol), flavorants, colorants, odorants, opacifiers, suspending agents, binders, fillers, plasticizers, lubricants, and mixtures thereof. The amounts of such additives can be readily determined by one skilled in the art, according to the particular properties desired.

[0073] The additive can also comprise a thickening agent. Suitable thickening agents can be those known and employed in the art, including, *e.g.*, pharmaceutically acceptable polymeric materials and inorganic thickening agents. Exemplary thickening agents for use in the present pharmaceutical compositions include polyacrylate and polyacrylate co-polymer resins, for example poly-acrylic acid and poly-acrylic acid/methacrylic acid resins; celluloses and cellulose derivatives including: alkyl celluloses, *e.g.*, methyl-, ethyl- and propyl-celluloses; hydroxyalkyl-celluloses, *e.g.*, hydroxypropyl-celluloses and hydroxypropylalkyl-celluloses such as hydroxypropyl-methyl-celluloses; acylated celluloses, *e.g.*, cellulose-acetates, cellulose-acetatephthallates, cellulose-acetatesuccinates and hydroxypropylmethyl-cellulose phthallates; and salts thereof such as sodium-carboxymethyl-celluloses; polyvinylpyrrolidones, including for example poly-N-vinylpyrrolidones and vinylpyrrolidone co-polymers such as vinylpyrrolidone-vinylacetate co-polymers; polyvinyl resins, *e.g.*, including polyvinylacetates and alcohols, as well as other polymeric materials including gum traganth, gum arabicum, alginates, *e.g.*, alginic acid, and salts thereof, *e.g.*, sodium alginates; and inorganic thickening agents such as atapulgite, bentonite and silicates including hydrophilic silicon dioxide products, *e.g.*, alkylated (for example methylated) silica gels, in particular colloidal silicon dioxide products. Such thickening agents as described above can be included, *e.g.*, to provide a sustained release effect. However, where oral administration is intended, the use of thickening agents as aforesaid will generally not be required and is generally less preferred. Use of thickening agents is, on the other hand, indicated, *e.g.*, where topical application is foreseen.

[0074] In particular embodiments, the compound is administered to the subject in a therapeutically effective amount, as that term is defined above. Dosages of pharmaceutically active compounds can be determined by methods known in the art, *see, e.g.,* Remington, *The Science And Practice of Pharmacy* (21th Ed. 2005). The therapeutically effective dosage of any specific compound will vary somewhat from compound to compound, and patient to patient, and will depend upon the condition of the patient and the route of delivery. In one embodiment, the compound is administered at a dose of about 0.001 to about 10 mg/kg body weight, *e.g.,* about 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg/kg. In some instances, the dose can be even lower, *e.g.,* as low as 0.0005 or 0.0001 mg/kg or lower. In some instances, the dose can be even higher, *e.g.,* as high as 20, 50, 100, 500, or 1000 mg/kg or higher. The present invention encompasses every sub-range within the cited ranges and amounts.

[0075] A further aspect of the invention relates to the use of animal models of HIV-1 infection in which T regulatory cells have been depleted to screen for compounds suitable for treatment of HIV-1 infection. This method takes advantage of a model in which latent HIV-1 has been or can be reduced, permitting an analysis of the efficacy of a compound under these desirable conditions.

[0076] Thus, one aspect of the invention relates to a method for identifying a compound suitable for treatment of HIV-1 infection, the method comprising providing an animal model of HIV-1 infection in which T regulatory cells have been depleted, delivering a candidate compound to the animal, and measuring HIV-1 levels in the animal, wherein a decrease in HIV-1 levels compared to an animal that has not received the candidate compound identifies the candidate compound as a compound suitable for treatment of HIV-1 infection.

[0077] The T regulatory cells may be depleted by any of the methods described above.

[0078] In some embodiments, the animal is a mouse, *e.g.,* a humanized mouse model.

[0079] Anti-HIV activity of a therapeutic agent may be measured by any method known in the art. Any *in vitro* or *in vivo* assay known in the art to measure HIV infection, production, replication or transcription can be used to test the efficacy of a

therapeutic of the invention. For example, but not by way of limitation, viral infection assays, CAT or other reporter gene transcription assays, HIV infection assays, or assays for viral production from cells latently infected with HIV (for example, but not limited to, by the method described by Chun *et al.*, *Nature* 387:183-188 (1977)) can be used to screen for and test potential inhibitors the virus.

[0080] The compounds that may be tested in the model may be a wide range of molecules and is not a limiting aspect of the invention. Compounds include, for instance, a polyketide, a non-ribosomal peptide, a polypeptide, a polynucleotide (for instance an siRNA, antisense oligonucleotide or ribozyme), other organic molecules, or a combination thereof. The sources for compounds to be screened can include, for example, chemical compound libraries, fermentation media of Streptomyces, other bacteria and fungi, and extracts of eukaryotic or prokaryotic cells.

[0081] The present invention is more particularly described in the following examples that are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLE 1

Experimental Procedures

[0082] **Construction of humanized NRG-hu mice:** Approval for the animal work was obtained from the University of North Carolina (UNC) Institutional Animal Care and Use Committee (IACUC). NRG-hu HSC mice were constructed as previously reported (Jiang *et al.*, *Blood* 112:2858 (2008); Li *et al.*, *PLoS Pathog.* 10:e1004291 (2014); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014); Zhang *et al.*, *Blood* 117:6184 (2011); Zhang *et al.*, *Blood* 109:2978 (2007); Zhang *et al.*, *Cell Mol. Immunol.* 9:237 (2012)). Greater than 90% of the transplanted mice were stably reconstituted with human leukocytes in the blood (>10% at 12-14 weeks of age or after transplant). Humanized mice in each cohort reconstituted from the same human donor fetal liver tissue had similar levels of engraftment. All mice were housed in the DLAM facility at UNC-Chapel Hill.

[0083] **HIV-1 infection of humanized mice:** Humanized mice were infected by intravenous (i.v.) injection with HIV-1JRCSF (CCR5 tropic) stocks (10 ng p24/mouse in

50µl) or with mock stocks in control mice (Jiang *et al.*, *Blood* 112:2858 (2008); Li *et al.*, *PLoS Pathog.* 10:e1004291 (2014); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014); Zhang *et al.*, *Blood* 117:6184 (2011); Zhang *et al.*, *Blood* 109:2978 (2007)).

[0084] cART regimens in NRG-hu HSC mice: Humanized mice received cART (Choudhary *et al.*, *J. Virol.* 83:8254 (2009); Li *et al.*, *PLoS Pathog.* 10:e1004291 (2014)). Two regimens were used in this study. Both regimens showed similar suppression of HIV-1 replication. As indicated in **Table 2**, some experiments used a combination of Zidovudine (AZT, 50mg/kg), Nevirapine (NVP, 33mg/kg) and Lamivudine (3TC, 25mg/kg), by daily treatment through intraperitoneal (i.p.) injection. In other experiments, HIV-infected mice were treated with AZT in drinking water (0.6mg/ml), and daily injection of GSK364735C (Integrase inhibitor, 8mg/kg, i.p.). Whole blood from humanized mice receiving cART were analyzed for drug concentrations and HIV-1 genomes.

[0085] Treg cell depletion by denileukin diftitox (DAB389IL-2, or ONTAK[®]) in humanized mice: Denileukin diftitox (ONTAK[®]; UNC hospital pharmacy, Chapel Hill, NC) injection was performed as previously reported (Jiang *et al.*, *Blood* 112:2858 (2008); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014)). For *in vivo* treatment of humanized mice with stable persistent HIV-1 replication, mice were injected intraperitoneally with ONTAK[®] (31µg/kg, diluted in HBSS). HBSS-injected, HIV-1_{JRCSE} infected mice were used as control.

[0086] Quantification of HIV viral load in plasma: Peripheral blood (PB) was taken with EDTA as an anti-coagulant at indicated time points after HIV infection. Plasma was prepared by centrifugation and stored at -80°C until assay. Viral RNA was isolated from the plasma. HIV viral load (sensitivity > 400 copies/ml) was measured by qRT-PCR as described previously (Jiang *et al.*, *Blood* 112:2858 (2008); Li *et al.*, *PLoS Pathog.* 10:e1004291 (2014); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014); Zhang *et al.*, *Blood* 109:2978 (2007)).

[0087] Flow cytometry: Leukocytes from PB, Lymph Node (LN), Bone Marrow (BM) and Spleen (Sp) were counted by Guava Easy Cytes (Millipore). Cell surface and intracellular markers were stained with different antibodies using BD Fix/Perm Kit (BD), followed by 1% paraformaldehyde fixation and analyzed by CyAn ADP (DAKO).

[0088] IHC and immunofluorescence staining of spleens: Spleens were harvested from NRG-hu mice, fixed with 10% formalin (Fisher, Fair Lawn, NJ), embedded in paraffin and cut into 5µm tissue sections. IHC staining procedure was performed as described previously (Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014)).

Immunofluorescence staining was performed by following procedure. Antigen retrieval was performed by incubation in Diva Decloacker (Biocare Medical, Concord, CA) for 30 min at 95°C, followed by slow cooling down for one hour. The tissue section was blocked with Background Sniper (Biocare Medical, Concord, CA). The sections were then stained with the primary antibodies: rabbit monoclonal anti-human CD3 (Life Span Bio Sciences, Seattle, WA; 1:100 dilution) and mouse monoclonal anti-HIV-1 p24 (Dako, 1:5 dilution) diluted in blocking buffer (PBS, 0.05% Tween 20, 5% goat serum), and secondary antibodies: Alexa Fluor 594 Donkey Anti-Mouse IgG (Life Technologies, Eugene, OR) and Alexa Fluor 488 Donkey Anti-Rabbit IgG (Life Technologies, Eugene, OR). The sections were stained with DAPI and mounted with an anti-fade mounting media (Abcam Cambridge, MA). Sections were analyzed by confocal microscopy (Zeiss LSM 700 Confocal Laser Scanning Microscope).

[0089] Statistical analysis: Unpaired 2-tailed Student t tests and 1-way analysis of variance with the Bonferroni multiple comparison test were performed using GraphPad Prism (GraphPad Software, San Diego, CA) (Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014)). P value of < 0.05 was considered statistically significant. All data were reported as mean ± SD (Standard deviation).

EXAMPLE 2

HIV-1 Persistent Infection and cART-resistant Reservoirs Humanized Mice

[0090] Humanized mouse models have been established for studying HIV-1 infection, pathogenesis and therapy *in vivo* (Berges *et al.*, *Virology* 397:100 (2010); Choudhary *et al.*, *J. Virol.* 83:8254 (2009); Jiang *et al.*, *Blood* 112:2858 (2008); Li *et al.*, *PLoS Pathog.* 10:e1004291 (2014); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014); Sun *et al.*, *J. Exp. Med.* 204:705 (2007); Zhang *et al.*, *Blood* 109:2978 (2007); Zhang *et al.*, *Cell Mol. Immunol.* 9:237 (2012)). Humanized mice were infected with HIV-1 and analyzed for

CD4⁺ T cell frequency and plasma viral load over time. Plasma viral replication was detected for more than 20 weeks post infection (wpi) with stable plasma viremia (FIGS 1A-1D). CD4⁺ T cell in the blood began to decrease progressively over time (FIGS. 1B-1C). To model HIV-1 cure therapeutic strategies, cART regimens were established in the humanized mouse model to stably suppress HIV-1 replication below detection limit either by viremia in blood (<500 HIV genome copies/ml) or by immunohistochemistry (IHC) in lymphoid tissues. Humanized mice were infected with HIV-1 and started cART at 6 wpi when stable plasma HIV-1 viremia had been detected (FIG. 1D). The viral replication was efficiently suppressed and reached undetectable levels at 3-4 wpi upon cART. Complete suppression of viral replication was able to persist during cART. As seen in human patients, HIV-1 replication rebounded rapidly after cART was stopped (FIG. 1D and (Choudhary *et al.*, *J. Virol.* 86:114 (2012); Choudhary *et al.*, *J. Virol.* 83:8254 (2009); Denton *et al.*, *J. Virol.* 86:630 (2012); Marsden *et al.*, *J. Virol.* 86, 339 (2012))). Therefore, HIV-1 infection in humanized mice is a relevant and robust model for studying HIV-1 persistent infection, immunopathogenesis and cART-resistant reservoirs *in vivo*.

EXAMPLE 3

Treg Cells Suppress Virus Replication During Chronic HIV-1 Infection *In Vivo*

[0091] It has been previously reported that ONTAK[®] injection specifically depletes Treg cells from peripheral blood mononuclear leukocytes and lymphoid organs (Jiang *et al.*, *Blood* 112:2858 (2008); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014)). To confirm that ONTAK[®] injection specifically depletes Treg cell population in the humanized mice, the change of Treg cell frequency upon ONTAK[®] injection was analyzed. HIV-1-infected humanized mice were injected with ONTAK[®] and each human T cell population was analyzed by flow cytometry. ONTAK[®] was shown to specifically deplete CD4⁺CD25^{hi} Treg cells but not CD4⁺CD25^{int} or CD8⁺CD25⁺ T cells in humanized mice chronically infected with HIV-1 at 11 wpi (FIGS. 2A-2B, and FIGS. 3A-3C). As expected, Treg cell depletion increased the frequency of HLA-DR⁺/CD38⁺CD8⁺ T cells in humanized mice (Jiang *et al.*, *Blood* 112:2858 (2008); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014)). To

determine if Treg cell depletion affects HIV-1 replication during chronic HIV-1 infection, plasma HIV-1 viral load was measured in chronically HIV-1-infected humanized mice after ONTAK[®] injection. We found that plasma HIV-1 viral load was significantly increased at 1 or 2 weeks after ONTAK[®] injection (**FIG. 2C**). HIV-1 p24 expression in CD3⁺CD8⁺ T cells after ONTAK[®] injection was also analyzed by flow cytometry. In HIV-1 infected humanized mice, HIV-1 p24 was mostly expressed in CD3⁺CD8⁺CD4⁺ T cells (**FIG. 2D**). HIV-1 replication in CD3⁺CD8⁺ T cells was significantly enhanced in all lymphoid organs at 2 weeks after Treg cell depletion (**FIG. 2E**). IHC staining also revealed that HIV-1 p24 expression in lymphoid tissues was significantly increased after ONTAK[®] injection (**FIG. 2F**). Taken together, these data suggest that Treg cell depletion leads to host immune activation and enhanced HIV-1 replication during chronic HIV-1 infection.

EXAMPLE 4

Treg Cells Suppress Virus Reactivation From HIV-1 Reservoirs

[0092] It was hypothesized that Treg cells also contribute to the establishment and/or maintenance of HIV-1 reservoirs during cART. To investigate the role of Treg cells in HIV-1 reservoir maintenance, Treg cells were depleted in the cART-receiving mice with ONTAK[®] to specifically deplete CD25^{hi}FoxP3⁺ Treg cells (**FIGS. 2A-2B, FIGS. 3A-3C and 4A-4C**). Interestingly, Treg cell depletion significantly induced HIV-1 rebound even under effective cART (**FIG. 5A**). We also detected higher HIV-1 p24⁺ cells in lymphoid tissues (**FIGS. 5B-5D**). Cell-associated HIV-1 RNA and proviral DNA levels were also measured in the lymphoid tissue by qRT-PCR, demonstrating that cell-associated HIV-1 RNA but not proviral DNA levels were significantly increased after Treg cell depletion under effective cART in the spleen (**FIGS. 5B-5D**) and in the bone marrow (**FIGS. 6A-6C**). The lack of HIV proviral DNA increase indicates that the elevated HIV replication by ONTAK[®] was not due to cART failure and HIV-1 infection of new cells. When the sequence of HIV-1 *pol* gene of the rebounded viruses was analyzed, no mutations associated with ART-resistance were detected (**FIG. 7**). These data suggest that the HIV-1 viruses were reactivated from HIV-1 reservoir (latent or low

replicating) cells by Treg cell depletion, and Treg cells are involved in maintaining HIV-1 reservoirs during cART.

EXAMPLE 5

HIV-1 Is Reactivated From Memory CD4⁺ T Cell Depletion

[0093] cART-resistant HIV-1 reservoir cells were analyzed in different lymphoid tissues. It was discovered that human T cells were the major cell type in the spleen and bone marrow that showed reactivated HIV-1 (FIGS. 8A-B). Immunofluorescence staining of lymphoid tissues was performed to define the HIV-1 “reservoir” cells with reactivated HIV-1 gene expression after Treg cell depletion. Both HIV-infected myeloid cells (p24⁺/CD3⁻) were detected as well as T cells (p24⁺/CD3⁺) in the spleen of HIV-1 infected mice, whereas no significant p24⁺ cells were detectable in cART-treated mice. Interestingly, all p24⁺ cells in Treg cell-depleted, cART-treated mice were CD3⁺ T cells (FIG. 8C), suggesting that T cells latently infected with HIV-1 were the main HIV-1 reservoir target cells responding to Treg cell depletion under cART. Flow cytometry analysis also revealed that CD4⁺ memory T cells (CD3⁺CD8⁻CD45RA⁻, T_M), but not naive T cells (CD3⁺CD8⁻CCR7⁺CD45RA⁺, T_N), were the major target cells in the spleen and bone marrow (FIGS. 8D-E) as reported in human patients (Chun *et al.*, *Proc. Natl. Acad. Sci. USA* 94:13193 (1997); Finzi *et al.*, *Science* 278:1295 (1997); Wong *et al.*, *Science* 278:1291 (1997)). Both CD4⁺ effector memory T cells (CD3⁺CD8⁻CCR7⁻CD45RA⁻, T_{EM}) and central memory T cells (CD3⁺CD8⁻CCR7⁺CD45RA⁻, T_{CM}) appeared to harbor reactivated HIV-1 (FIGS. 9A-B). These data suggest that T cells, especially memory T cells, are the major HIV-1 reservoir cells for HIV-1 reactivation induced by Treg cell depletion.

EXAMPLE 6

Activation of cART-Resistant HIV Reservoir by TLR3 Ligand

[0094] Mice were infected with HIV-JRCSF and treated with cART (768 mg/kg raltegravir, 249.6 mg/kg tenofovir disoproxil, and 166.4 mg/kg emtricitabine daily) from

week 4.5 to week 11.5. Poly I:C (a TLR3 ligand) was given at week 7.5 and week 10.5. HIV genome at indicated time point was detected by real-time PCR. The results show that TLR ligands such as Poly I:C can activate cART-resistant HIV reservoir *in vivo* during cART treatment (**FIG. 10**). TLR ligands activate innate immune responses.

[0095] It has been previously reported that ONTAK[®] injection specifically depletes Treg cells from peripheral blood mononuclear leukocytes and lymphoid organs (Jiang *et al.*, *Blood* 112:2858 (2008); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014)). To confirm that ONTAK[®] injection specifically depletes Treg cell population in the humanized mice, the change of Treg cell frequency upon ONTAK[®] injection was analyzed. HIV-1-infected humanized mice were injected with ONTAK[®] and each human T cell population was analyzed by flow cytometry. ONTAK[®] was shown to specifically deplete CD4⁺CD25^{hi} Treg cells but not CD4⁺CD25^{int} or CD8⁺CD25⁺ T cells in humanized mice chronically infected with HIV-1 at 11 wpi (**FIGS. 2A-2B**, and **FIGS. 3A-3C**). As expected, Treg cell depletion increased the frequency of HLA-DR⁺/CD38⁺CD8⁺ T cells in humanized mice (Jiang *et al.*, *Blood* 112:2858 (2008); Nunoya *et al.*, *J. Infect. Dis.* 209:1039 (2014)). To determine if Treg cell depletion affects HIV-1 replication during chronic HIV-1 infection, plasma HIV-1 viral load was measured in chronically HIV-1-infected humanized mice after ONTAK[®] injection. We found that plasma HIV-1 viral load was significantly increased at 1 or 2 weeks after ONTAK[®] injection (**FIG. 2C**). HIV-1 p24 expression in CD3⁺CD8⁻ T cells after ONTAK[®] injection was also analyzed by flow cytometry. In HIV-1 infected humanized mice, HIV-1 p24 was mostly expressed in CD3⁺CD8⁻CD4⁻ T cells (**FIG. 2D**). HIV-1 replication in CD3⁺CD8⁻ T cells was significantly enhanced in all lymphoid organs at 2 weeks after Treg cell depletion (**FIG. 2E**). IHC staining also revealed that HIV-1 p24 expression in lymphoid tissues was significantly increased after ONTAK[®] injection (**FIG. 2F**). Taken together, these data suggest that Treg cell depletion leads to host immune activation and enhanced HIV-1 replication during chronic HIV-1 infection.

[0096] The experiments have shown that Treg cell depletion leads to increased HIV-1 replication and its reactivation from latently infected T cells *in vivo* using a humanized mouse model. Established humanized mouse model supported long-term (>20 weeks) HIV-1 persistent infection with progressive CD4 T cell depletion and immune activation

(FIGS. 1A-C), suggesting that the model reproduced HIV-1 infection and immunopathogenesis in human patients. The cART regimens stably suppressed HIV-1 replication to undetectable levels in this model (FIG. 1D). Upon cART interruption, HIV-1 replication rebounded rapidly to pre-treatment levels (FIG. 1D), suggesting that this model was useful for studying cART-resistant HIV-1 reservoirs *in vivo*. Using this model, it was found that viral replication was significantly increased during chronic HIV-1 infection upon specific Treg cell depletion (FIGS. 2A-2F and Table 1). More interestingly, it was found that Treg cell depletion significantly increased HIV-1 reactivation (FIG. 5), associated with reactivation of cART-resistant CD4⁺ memory reservoir T cells (FIG. 4). These data suggest that Treg cells suppress HIV-1 replication during chronic infection and inhibit its reactivation from cART-resistant CD4⁺ memory T reservoir cells.

Table 1. ONTAK[®] treatment of humanized mice with chronic HIV-1 infection.

Mice	TXP	Blood		Spleen		Plasma HIV-1 VL (copies/mL)		
		CD4 ^a	CD8 ^a	CD4 ^a	CD8 ^a	-1 wk ^b	1 wk ^b	2 wk ^b
MOCK	HBSS	83.7	14.8	74.0	23.4	ND ^c	ND	ND
MOCK	HBSS	81.1	16.7	72.6	24.1	ND	ND	ND
MOCK	HBSS	72.8	23.1	61.2	33.5	ND	ND	ND
JRCSF	HBSS	41.3	52.9	41.7	46.5	2.93E+04	1.86E+04	1.31E+04
JRCSF	HBSS	62.4	33.1	48.1	43.0	3.08E+05	2.90E+05	5.14E+05
JRCSF	HBSS	15.9	82.5	9.0	89.3	2.84E+04	0.00E+00	2.10E+04
JRCSF	HBSS	49.5	47.5	40.1	59.7	2.58E+04	7.61E+04	1.04E+05
JRCSF	HBSS	52.5	44.6	43.8	53.1	7.53E+04	6.83E+04	1.34E+05
JRCSF	HBSS	57.5	40.1	47.4	50.1	3.01E+04	1.71E+04	5.09E+04
JRCSF	ONTAK [®]	9.9	86.4	21.9	71.0	3.21E+05	2.33E+05	5.18E+05
JRCSF	ONTAK [®]	13.1	79.6	22.9	65.5	9.98E+04	8.26E+04	5.43E+05
JRCSF	ONTAK [®]	67.3	28.1	56.1	37.0	9.38E+05	6.74E+05	7.64E+05
JRCSF	ONTAK [®]	61.8	35.4	48.5	47.9	3.29E+04	1.84E+05	2.26E+05
JRCSF	ONTAK [®]	60.2	37.0	48.9	48.2	1.03E+05	5.61E+04	1.01E+05
JRCSF	ONTAK [®]	40.1	57.4	34.1	62.9	1.72E+04	8.20E+04	6.40E+04
JRCSF	ONTAK [®]	48.8	48.1	42.1	55.0	4.49E+04	3.77E+04	1.42E+05

^a Percentage of CD4⁺ or CD8⁺ from human CD45⁺ CD3⁺ cells.

^b -1wk; 1 week before, 1 or 2wk; 1 or 2 weeks after ONTAK[®].

^c ND; not detectable (<400 copies/ml).

[0097] Two of the most critical questions in the effort to cure HIV-1 infection are identification and reactivation of cART-resistant HIV-1 reservoir cells (Siliciano *et al.*, *Cold Spring Harb. Perspect. Med.* 1:a007096 (2011)). The data here shows that, using a highly relevant humanized mouse model with persistent HIV-1 infection and cART-resistant HIV-1 reservoirs, Treg cells contribute to suppressing immune activation and HIV-1 replication. More importantly, Treg cells inhibit HIV-1 reactivation and help to maintain HIV-1 latency. Therefore, modulating Treg cell activity is an efficient way to reactivate cART-resistant HIV-1 reservoirs.

[0098] Humanized mouse models have been proven to be robust and highly relevant to study HIV-1 infection, persistence and therapy (Berges *et al.*, *Retrovirology* 3:76 (2006); Brainard *et al.*, *J. Virol.* 83:7305 (2009); Duyne *et al.*, *Curr. HIV Res.* 9:595 (2011); Hofer *et al.*, *PLoS Pathog.* 6:e1000867 (2010); Sun *et al.*, *J. Exp. Med.* 204:705 (2007); Watanabe *et al.*, *Blood* 109:212 (2007); Zhang *et al.*, *Blood* 109:2978 (2007); Zhang *et al.*, *Cell Mol. Immunol.* 9:237 (2012)). With regards to HIV-1 latency and reservoir reactivation, it has been reported that various cART regimens can efficiently suppress ongoing HIV-1 infection in humanized mice (Choudhary *et al.*, *J. Virol.* 86:114 (2012); Choudhary *et al.*, *J. Virol.* 83:8254 (2009); Denton *et al.*, *J. Virol.* 86:630 (2012); Marsden *et al.*, *J. Virol.* 86, 339 (2012)). Upon cART cessation, HIV-1 rapidly rebounds to pre-treatment levels and the cART-resistant HIV-1 reservoir is associated with latently infected memory T cells (**FIGS. 1A-1D** and Choudhary *et al.*, *J. Virol.* 83:8254 (2009); Denton *et al.*, *J. Virol.* 86:630 (2012)). Using humanized mice, the role of Treg cells during chronic HIV-1 infection and in maintaining cART-resistant HIV-1 reservoirs *in vivo* was defined here (**FIGS. 3 and 4 and Table 2**). These findings shed light on elucidating how HIV-1 establishes latency and how to efficiently reactivate cART-resistant HIV-1 reservoirs *in vivo*. In addition, this study suggests that modulating Treg cell activity may be an efficient way to activate HIV-1 for future HIV-1 cure effort (Katlama *et al.*, *Lancet* 381:2109 (2013)).

Table S2. Humanized mice with suppressed HIV-1 infection under cART treatment and mice with rebounded viral load after ONTAK[®] treatment

Group	Spleen (termination)		^a HIV VL (copies/ml)	^b HIV gag p24+ on CD3+CD8- T cell (%)		^c cART
	CD4 ^c	CD8 ^c	Plasma	spleen	Bone marrow	
MOCK	66.99	26.30	ND ^d	ND	ND	Naive
MOCK	60.25	30.89	ND ^d	ND	ND	Naive
MOCK	71.10	23.50	ND ^d	ND	ND	Naive
MOCK	63.94	30.05	ND ^d	ND	ND	Naive
MOCK	76.44	16.78	ND ^d	ND	ND	Naive
JRCSF	55.38	34.63	21800.03	22.94	12.04	Naive
JRCSF	54.77	35.51	13803.84	9.4	15.67	Naive
JRCSF	29.68	42.30	28183.83	4.4	8.7	Naive
JRCSF	76.67	15.00	11220.18	6.3	7.71	Naive
JRCSF	39.46	48.99	34041.05	11.75	14.91	Naive
JRCSF	34.73	58.35	52351.73	4.16	4.34	Naive
JRCSF	73.4	20.42	16351.79	11.4	3.95	Naive
JRCSF	69.29	25.01	75932.03	12.93	6.69	Naive
JRCSF+cART	51.30	25.96	ND ^d	ND	1.4	3NRTi
JRCSF+cART	43.73	44.44	ND ^d	ND	2.1	3NRTi
JRCSF+cART	57.78	35.34	ND ^d	ND	ND	3NRTi
JRCSF+cART	64.46	22.02	ND ^d	ND	2.5	3NRTi
JRCSF+cART	33.88	59.97	ND ^d	2.73	3.68	3NRTi
JRCSF+cART	74.48	18.09	ND ^d	1.83	3.51	3NRTi
JRCSF+cART	61.42	30.11	ND ^d	ND	ND	3NRTi
JRCSF+cART	52.15	34.06	ND ^d	ND	ND	3NRTi
JRCSF+cART	76.63	17.2	ND ^d	ND	2	3NRTi
JRCSF+cART	60.27	35.94	ND ^d	ND	ND	AZT+Pi+INTi
JRCSF+cART	50.46	43.46	ND ^d	1.49	2.37	AZT+Pi+INTi
JRCSF+cART	57.11	39.61	ND ^d	ND	1.5	AZT+Pi+INTi
JRCSF+cART+ONTAK [®]	42.86	42.86	1258.92	2.2	5.81	3NRTi

JRCSF+cART+ONTAK [®]	60.34	34.16	8984.92	1.10	7.26	3NRTi
JRCSF+cART+ONTAK [®]	53.55	29.98	1045.71	2.00	7.16	3NRTi
JRCSF+cART+ONTAK [®]	72.45	21.2	13803.8	ND	1.9	3NRTi
JRCSF+cART+ONTAK [®]	45.83	0.9	772398.2	12.3	15.5	3NRTi
JRCSF+cART+ONTAK [®]	57.14	23.81	28183.8	3.6	5.6	3NRTi
JRCSF+cART+ONTAK [®]	35.74	3.61	2901087.3	17.1	16.5	3NRTi
JRCSF+cART+ONTAK [®]	56.79	35.93	28183.8	ND	4.1	3NRTi
JRCSF+cART+ONTAK [®]	65.85	25.59	11134.65	4.5	2.3	3NRTi
JRCSF+cART+ONTAK [®]	61.62	30.58	7460.96	3.85	1.37	AZT+Pi+INTi
JRCSF+cART+ONTAK [®]	61.46	33.91	6542.37	7.53	4.55	AZT+Pi+INTi
JRCSF+cART+ONTAK [®]	52	43.19	630.93	2.26	1.3	AZT+Pi+INTi

[0099] The molecular mechanisms by which Treg cells suppress HIV-1 reactivation are still unclear. Suppression of HIV gene expression through suppressing T cell activation is probably involved. A recent report has shown that the CD39/Adenosine pathway in Treg cells can suppress HIV-1 replication in HIV-1 infected T cells (Moreno-Fernandez *et al.*, *Blood* 117:5372 (2011); Nikolova *et al.*, *PLoS Pathog.* 7:e1002110 (2011)). Treg cells also use cAMP for suppressing effector T cell function (Bopp *et al.*, *J. Exp. Med.* 204:1303 (2007)). Treg-mediated suppression of HIV-1 replication and effector T cell function seems to involve cell contact-dependent transfer of cAMP as a mechanism. Thus the CD39/Adenosine pathway may be involved in Treg-mediated suppression of HIV-1 reactivation from cART-resistant reservoirs. Recent reports also suggest that proliferation of HIV-1 reservoir cells is due to HIV integration in cancer-related genes (Maldarelli *et al.*, *Science* 345:179 (2014); Wagner *et al.*, *Science* 345:570 (2014)) or latency in long-lived cells like T memory stem cells (Buzon *et al.*, *Nat. Med.* 20:139 (2014)) may contribute to the HIV-1 reservoir formation and maintenance. Since Treg cells suppress T cell activation and proliferation (Schmidt *et al.*, *Front. Immunol.* 3:51 (2012)), depletion of Treg cells may contribute to both HIV-1 reactivation and to elevated expansion of these HIV-1 reservoir cells.

[0100] The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That which is claimed is:

1. A method of reactivating latent HIV-1 in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby reactivating latent HIV-1.
2. A method of reducing HIV-1 reservoirs in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby reducing HIV-1 reservoirs.
3. A method of treating HIV-1 infection in a subject in need thereof, comprising depleting T regulatory cells in the subject, thereby treating HIV-1 infection.
4. The method of any one of claims 1-3, wherein depleting T regulatory cells comprises delivering to the subject an effective amount of denileukin diftitox.
5. The method of any one of claims 1-3, wherein depleting T regulatory cells comprises delivering to the subject an effective amount of an antibody that specifically binds to CD25.
6. The method of any one of claims 1-5, wherein the T regulatory cells are depleted by at least 70%.
7. The method of any one of claims 1-6, wherein the T regulatory cells are depleted by at least 90%.
8. The method of any one of claims 1-7, further comprising delivering to the subject a HIV-1 therapeutic agent.
9. The method of claim 8, wherein the HIV-1 therapeutic agent is an antiretroviral agent.

10. The method of claim 9, wherein the antiretroviral agent is selected from the group consisting of reverse transcriptase inhibitors, protease inhibitors, viral integration inhibitors, viral entry inhibitors, viral maturation inhibitors, iRNA agents, antisense RNA, vectors expressing iRNA agents or antisense RNA, PNA, antiviral antibodies and any combination thereof.
11. The method of claim 9, wherein the antiretroviral agent is selected from the group consisting of AZT, 3TC, ddI, ddC, 3TC, saquinavir, indinavir, ritonavir, nelfinavir, nevirapine, efavirenz, and combinations thereof.
12. The method of any one of claims 1-11, wherein the subject is a human.
13. The method of any one of claims 1-11, wherein the subject is an animal model of HIV-1 infection.
14. A method for identifying a compound suitable for treatment of HIV-1 infection, the method comprising providing an animal model of HIV-1 infection in which T regulatory cells have been depleted, delivering a candidate compound to the animal, and measuring HIV-1 levels in the animal, wherein a decrease in HIV-1 levels compared to an animal that has not received the candidate compound identifies the candidate compound as a compound suitable for treatment of HIV-1 infection.
15. The method of claim 14, wherein the T regulatory cells have been depleted by delivering to the animal an effective amount of denileukin diftitox.
16. The method of claim 14, wherein the T regulatory cells have been depleted by delivering to the animal an effective amount of an antibody that specifically binds to CD25.
17. The method of any one of claims 14-16, wherein the T regulatory cells have been depleted by at least 70%.

18. The method of any one of claims 14-17, wherein the T regulatory cells have been depleted by at least 90%.
19. The method of any one of claims 14-18, wherein the animal is a mouse.
20. The method of any one of claims 14-19, wherein the animal model is a humanized mouse model.

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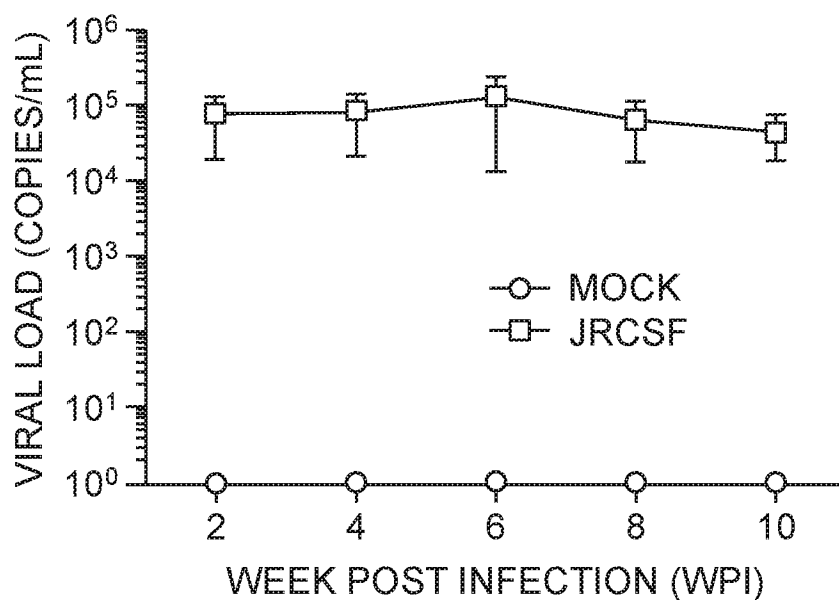


FIG. 1A

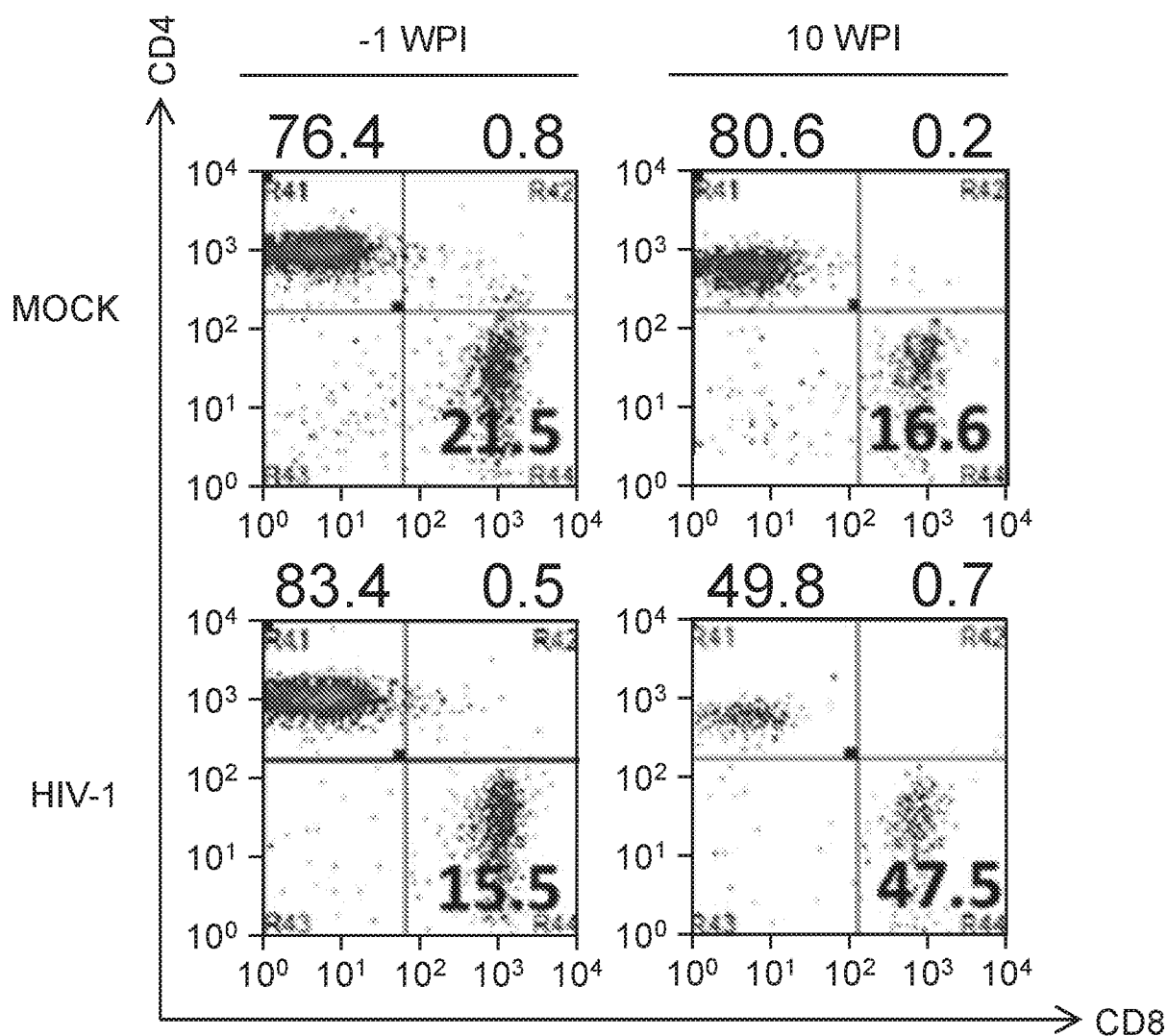


FIG. 1B

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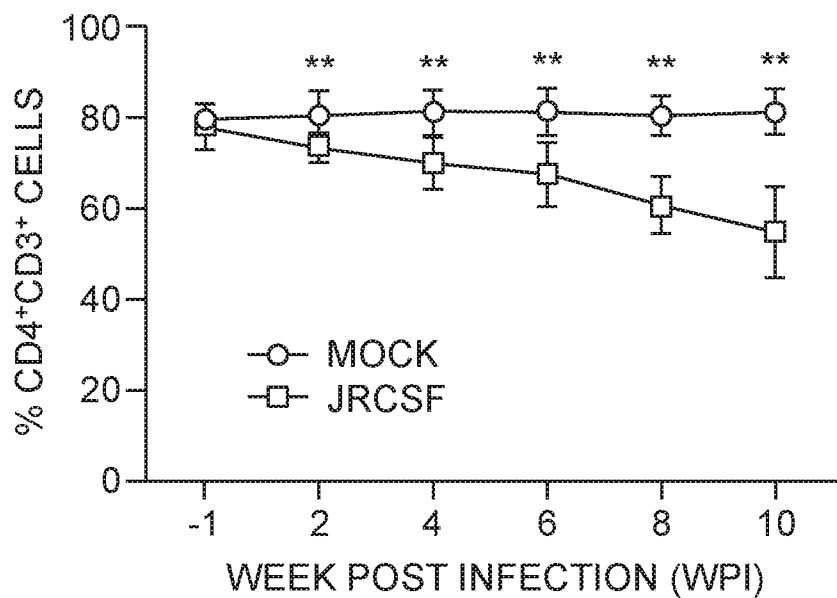


FIG. 1C

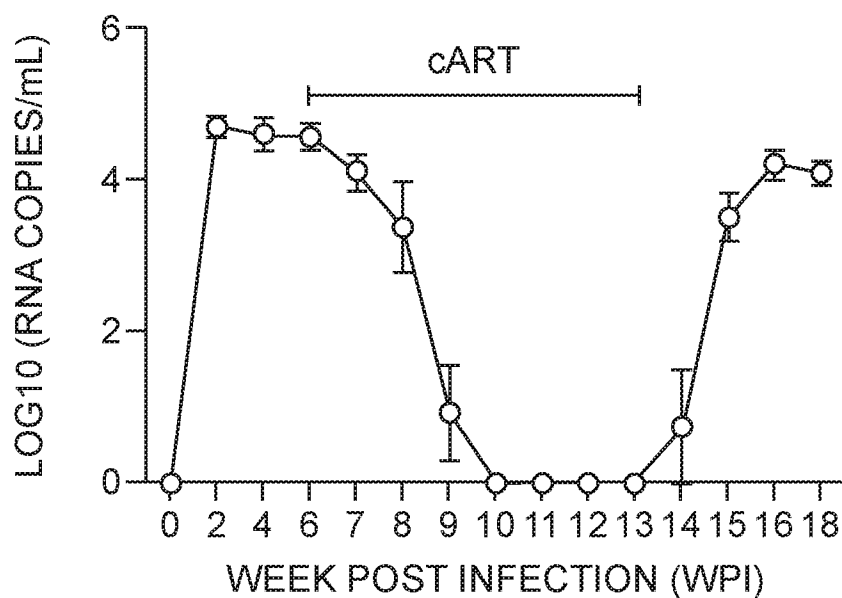


FIG. 1D

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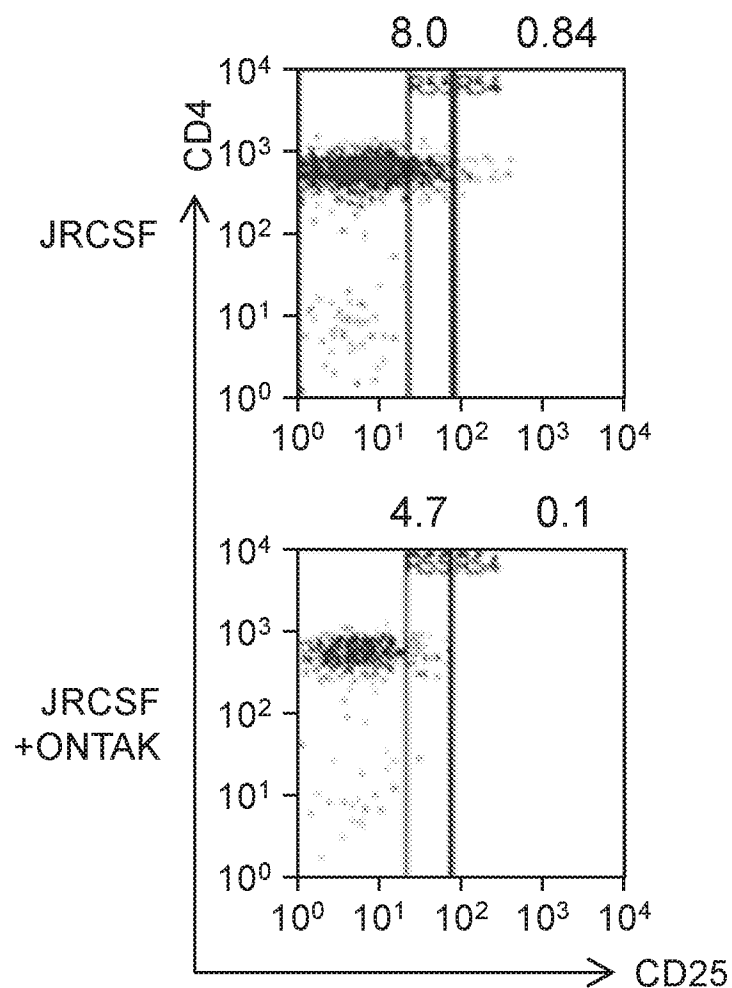


FIG. 2A

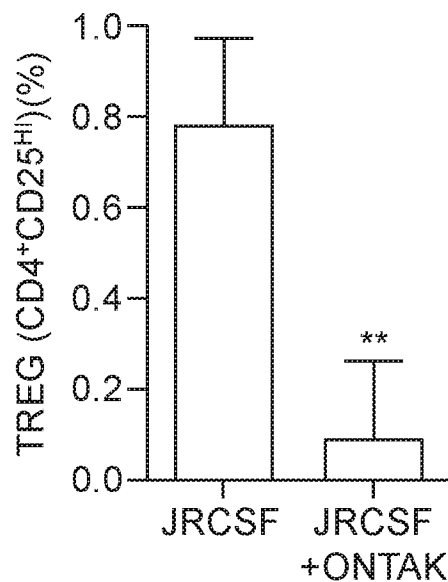


FIG. 2B

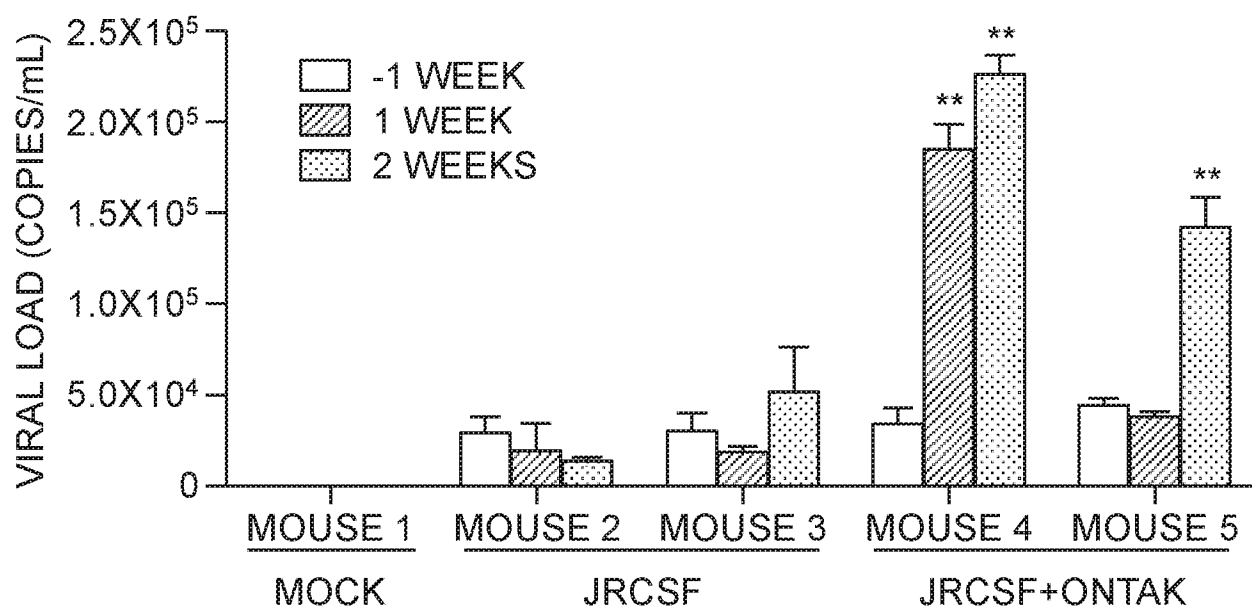
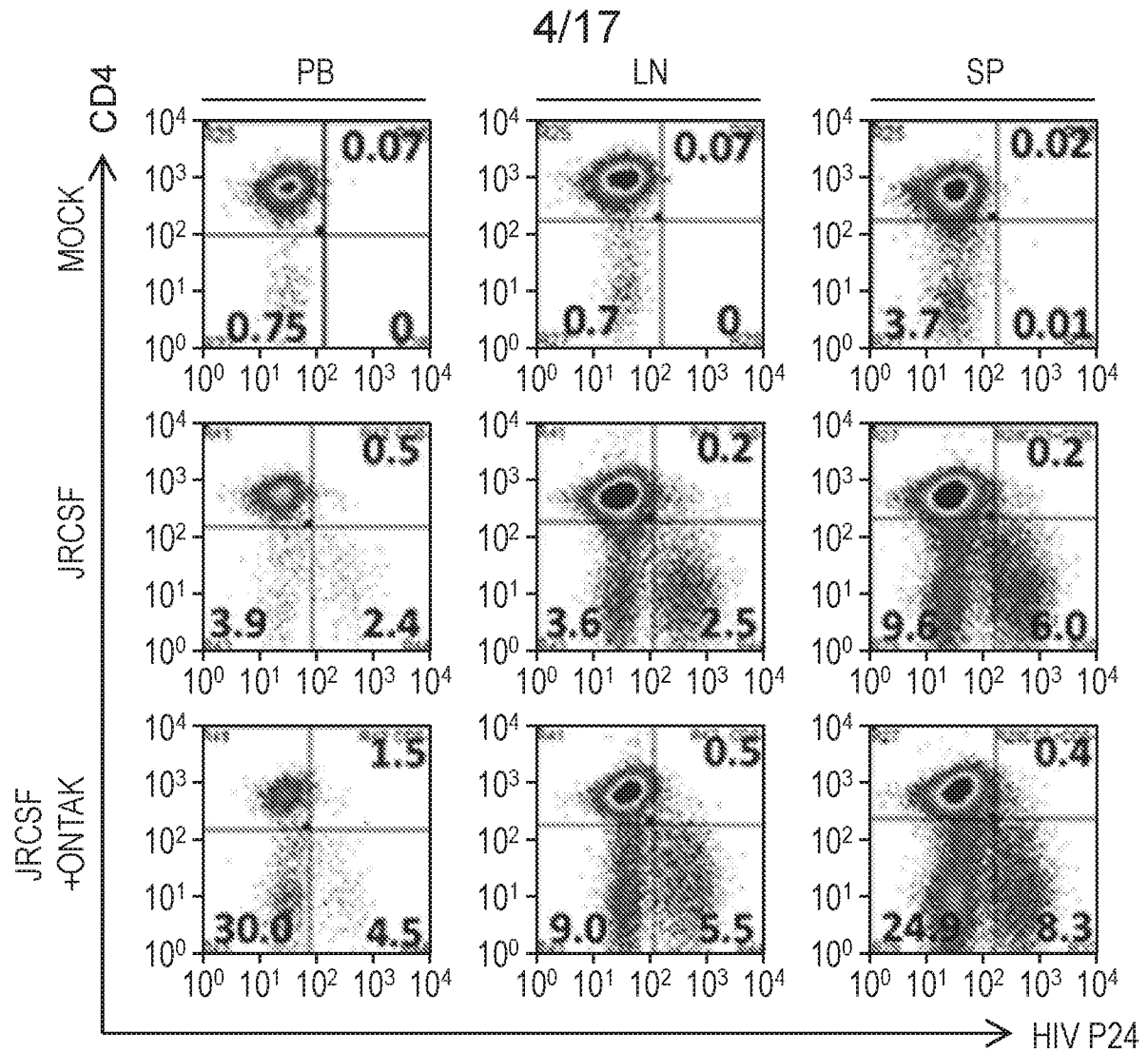
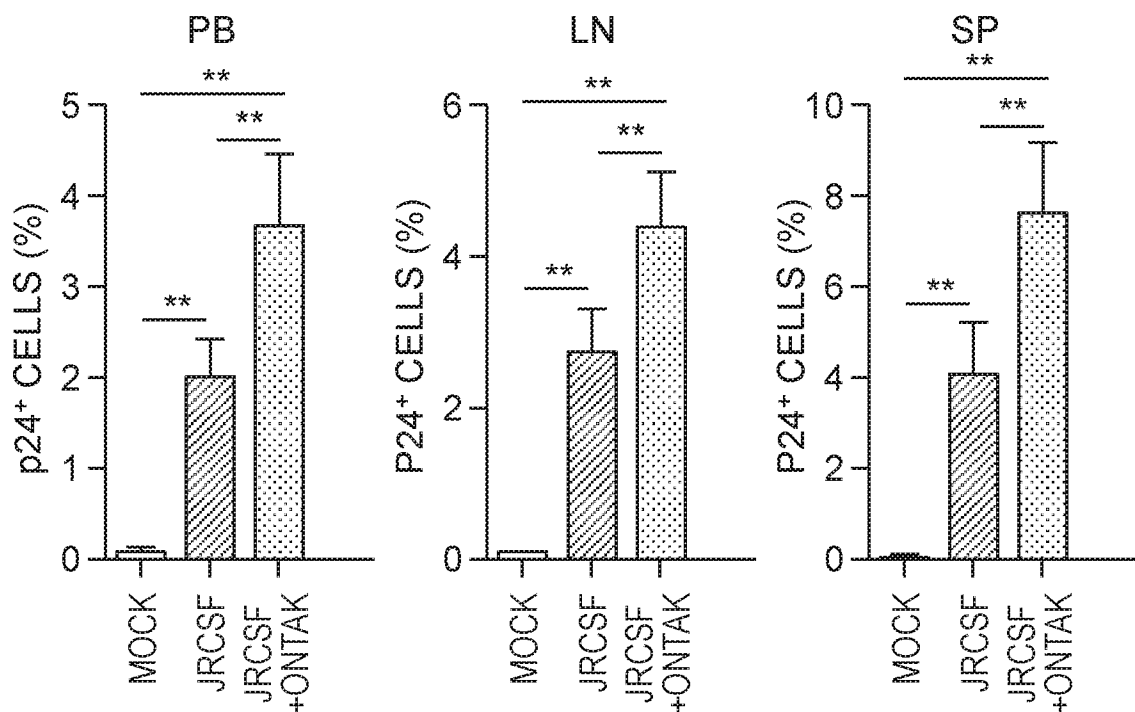


FIG. 2C

**FIG. 2D****FIG. 2E**

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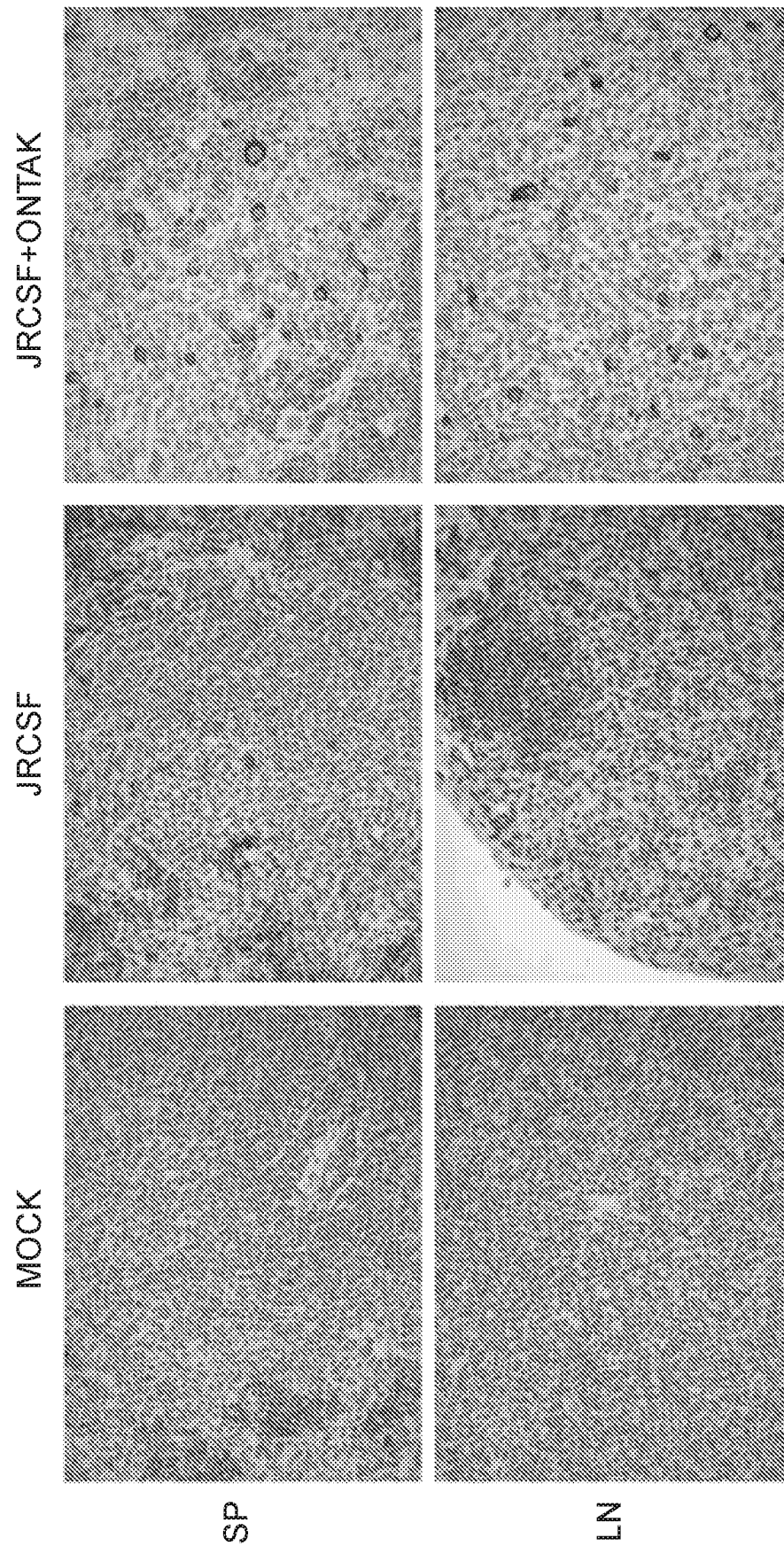
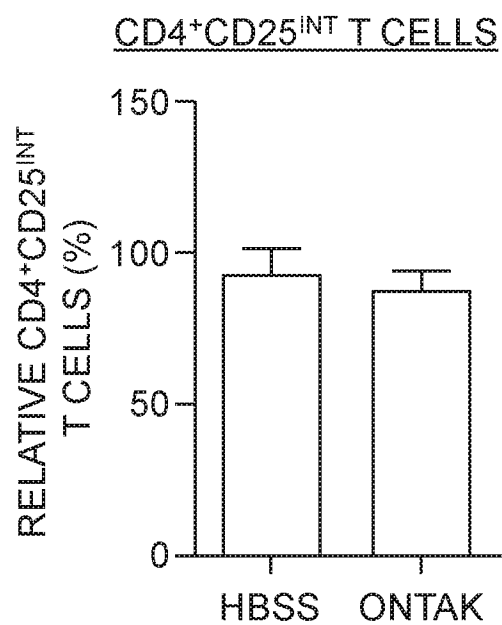
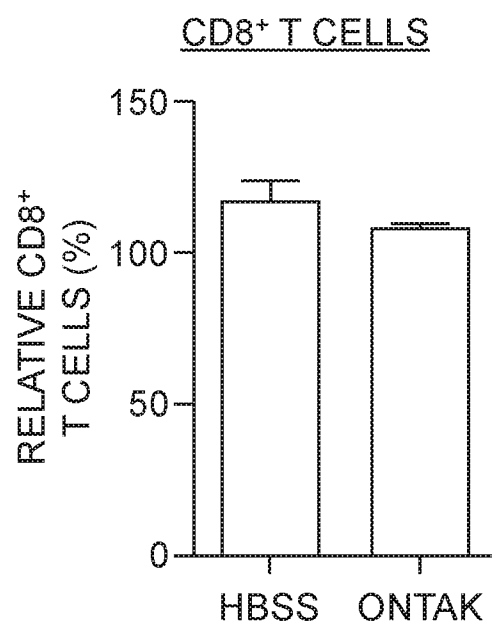
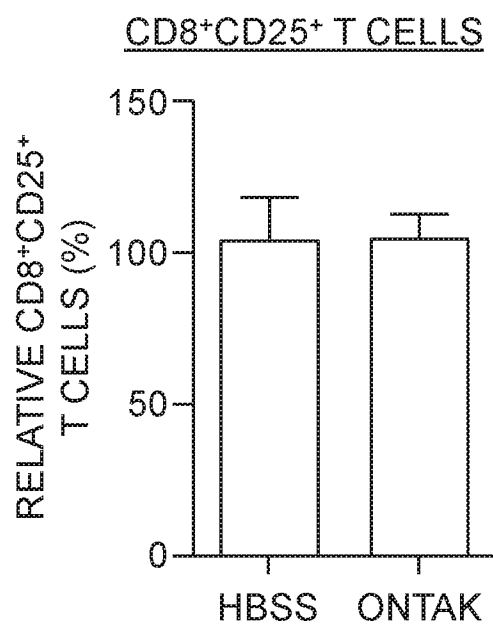


FIG. 2F

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*FIG. 3A**FIG. 3B**FIG. 3C*

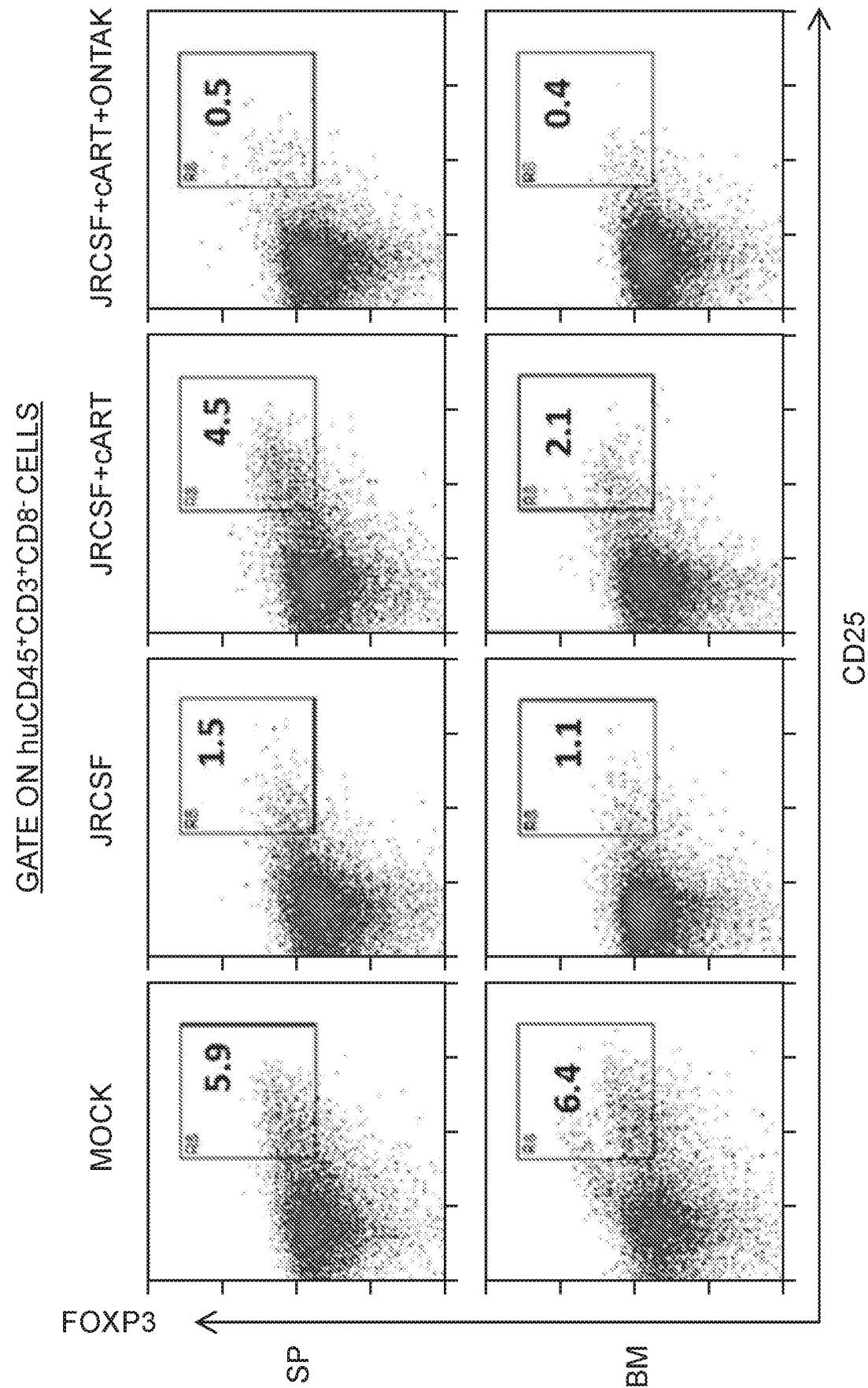
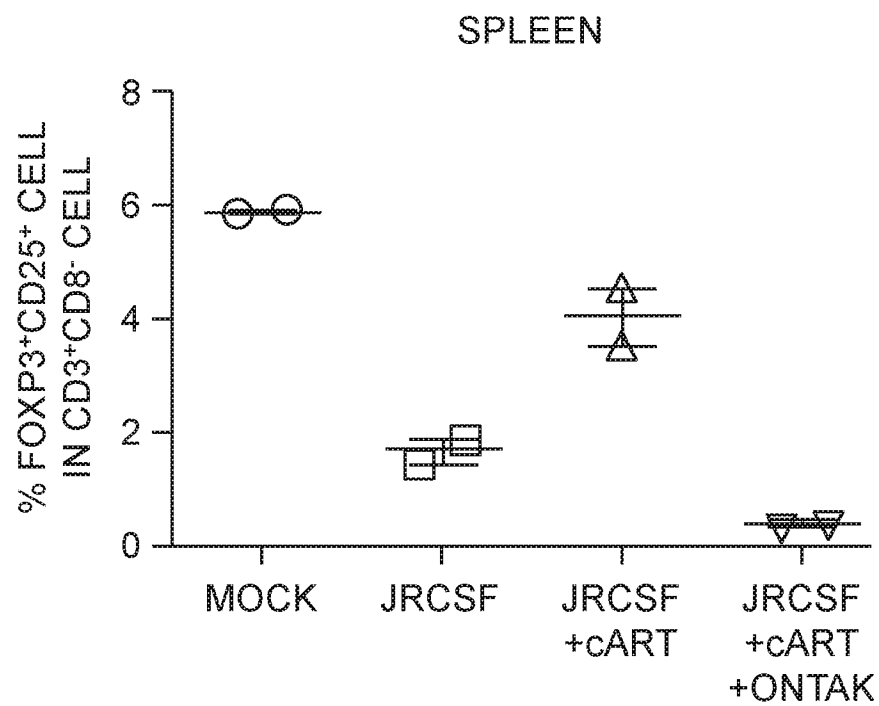
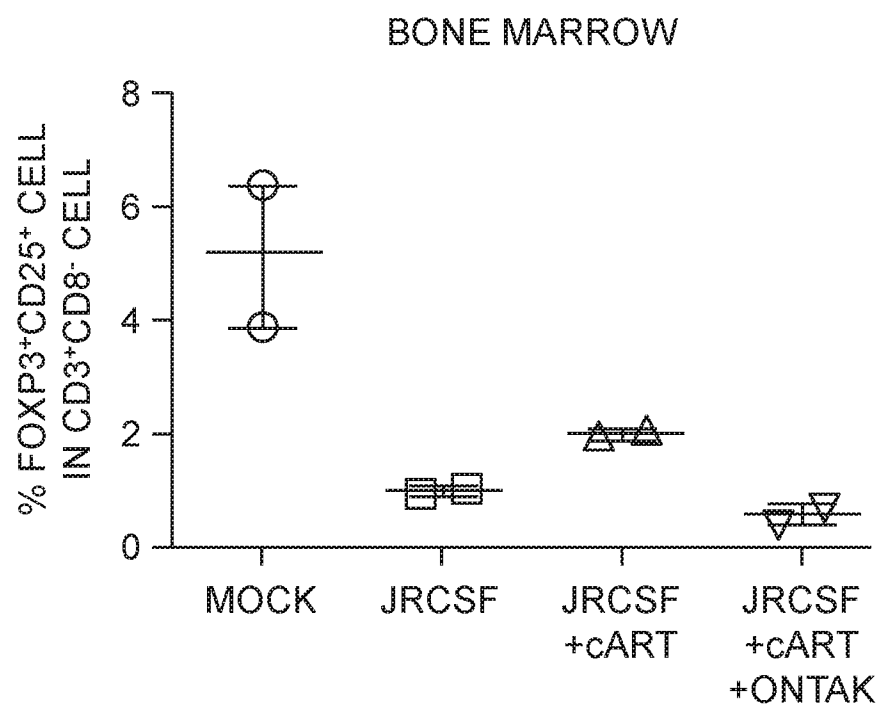


FIG. 4A

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*FIG. 4B**FIG. 4C*

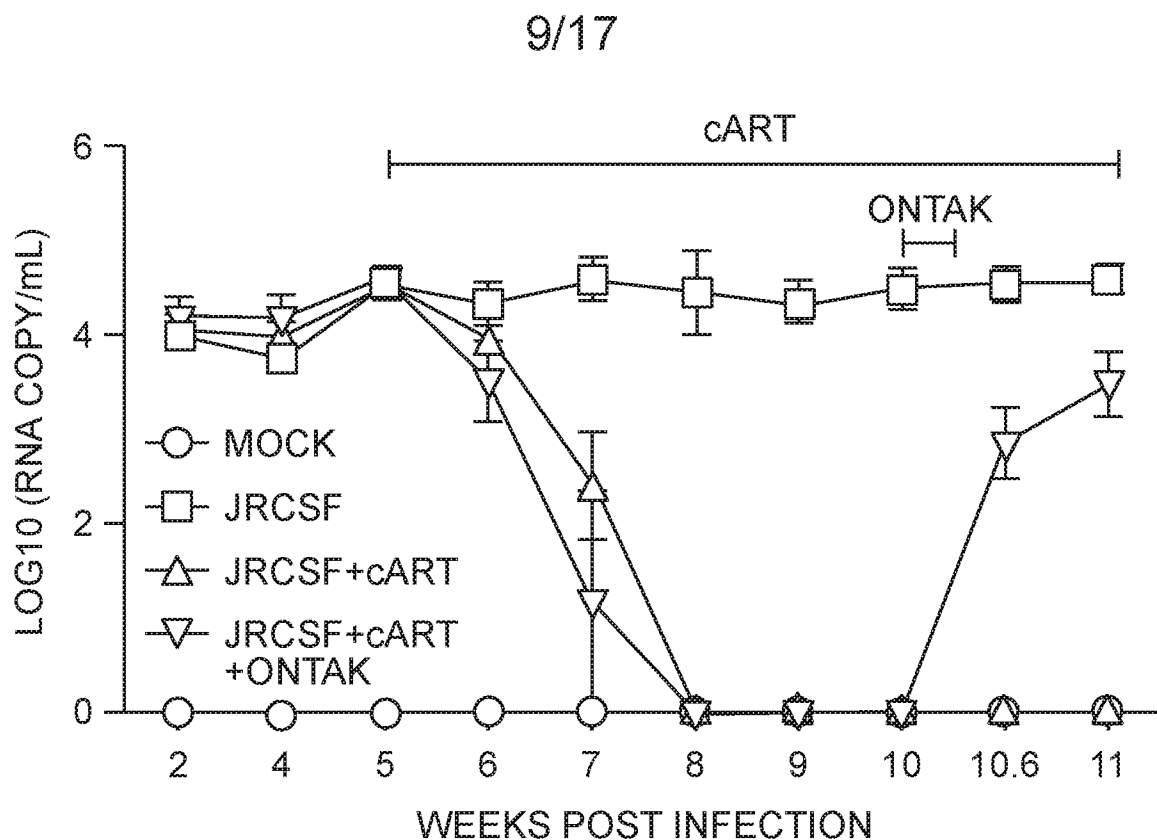


FIG. 5A

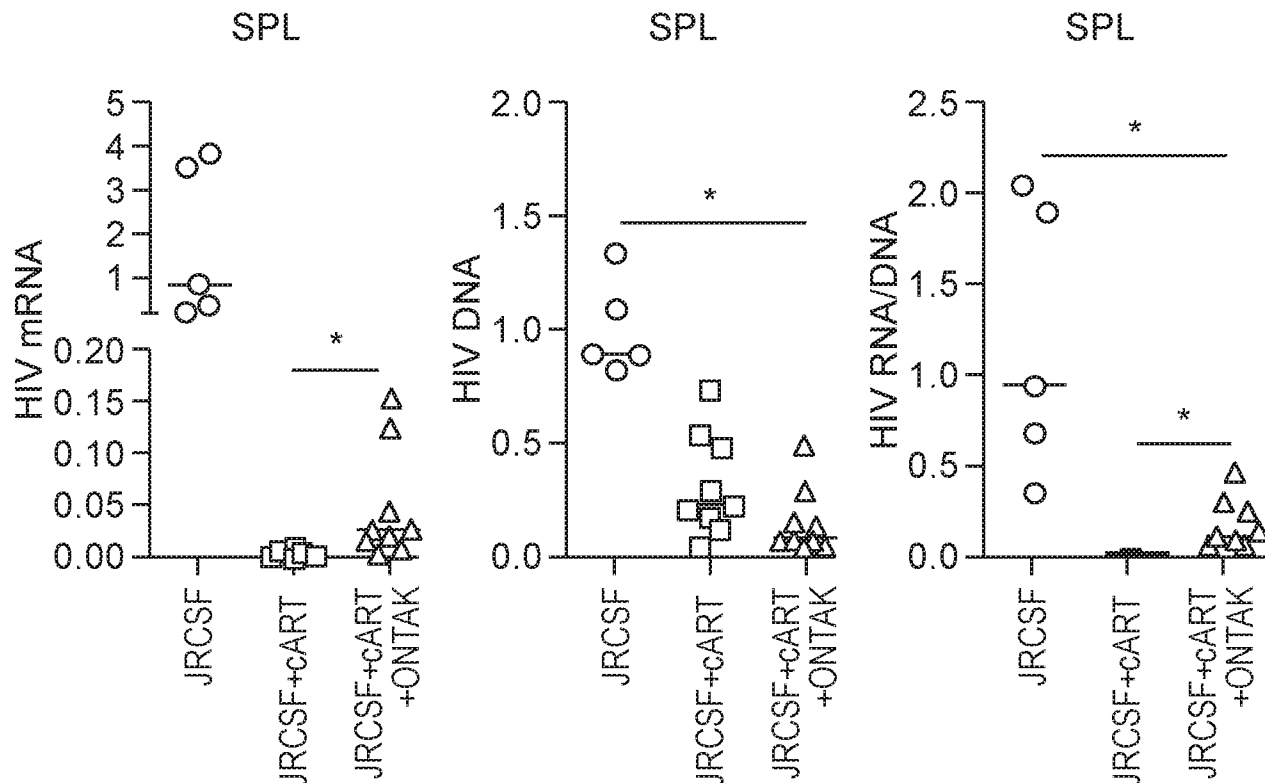


FIG. 5B

FIG. 5C

FIG. 5D

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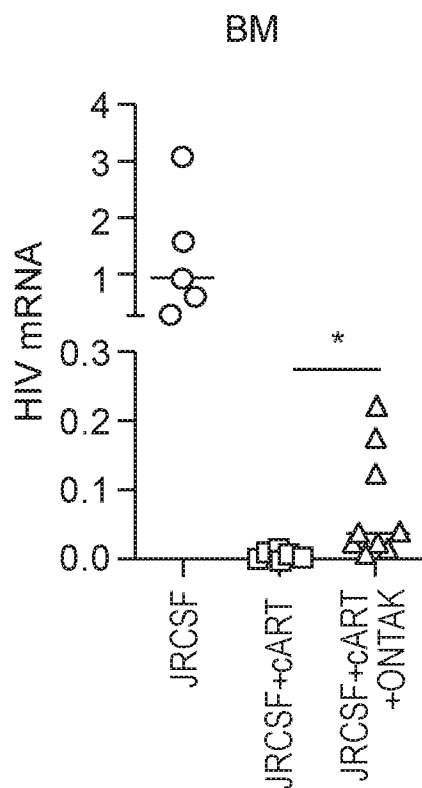


FIG. 6A

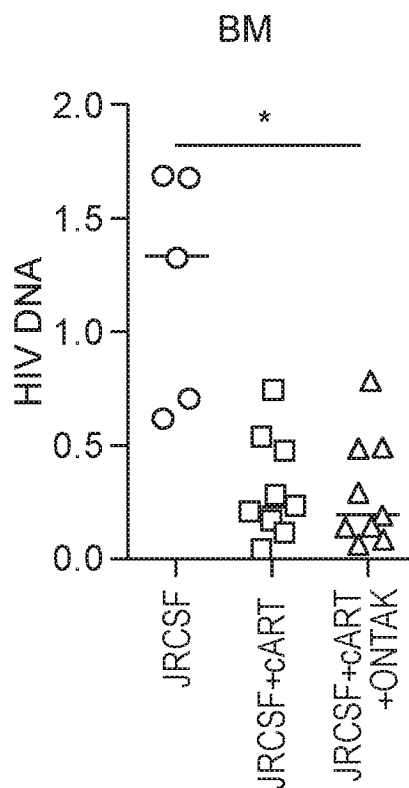


FIG. 6B

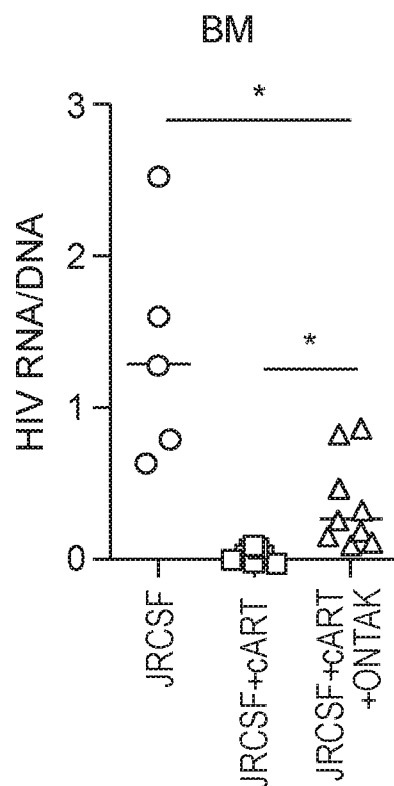


FIG. 6C

JRCSE	1	PISPIETVP	10	20	30	40	50
MOUSE #1		VKLKPGMDGP	KVKQWPLTEE	KIKALVEICT	EMEKEGKISK	IGPENPYNTP	
MOUSE #2							
MOUSE #3							
MOUSE #4							
JRCSE	60	VFAIKKKDST	70	80	90	100	110
MOUSE #1							
MOUSE #2							
MOUSE #3							
MOUSE #4							
JRCSE	120	LDKDFRKYTA	130	140	150	160	170
MOUSE #1		FTIPSINNET	PGIRYQYNVL	PQGWKGPAL	FQSSMTKILE	PFRKQNPDI	
MOUSE #2							
MOUSE #3							
MOUSE #4							
JRCSE	180	IYQYMDLDLV	190	200	210	220	
MOUSE #1		GSDLEIGQHR	TKIEELRQHL	IKWGHITFPDK	KHQKEPPFL		
MOUSE #2							
MOUSE #3							
MOUSE #4							

FIG. 7

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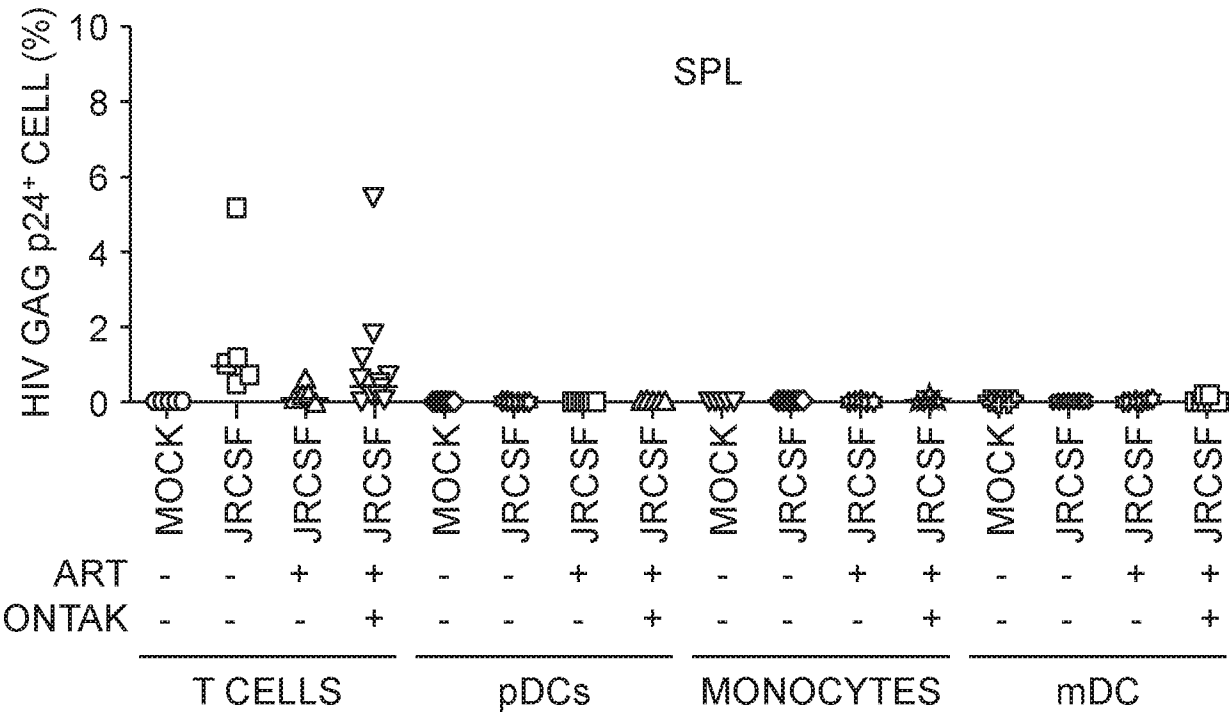


FIG. 8A

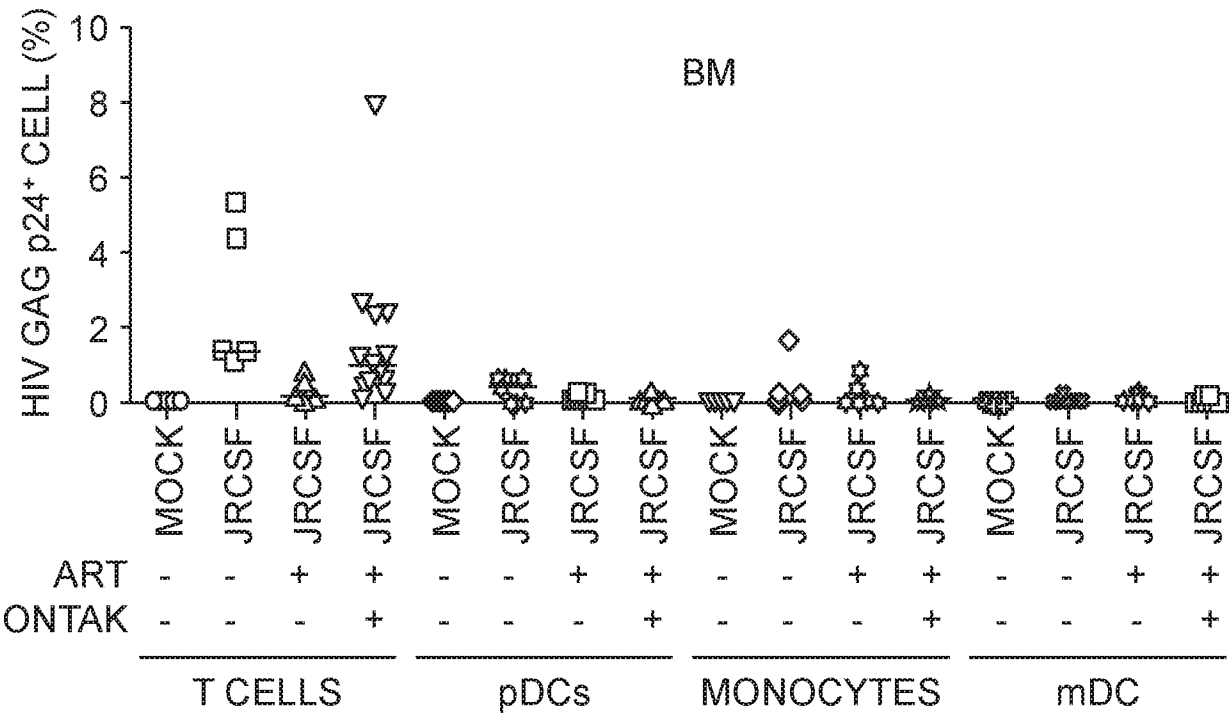


FIG. 8B

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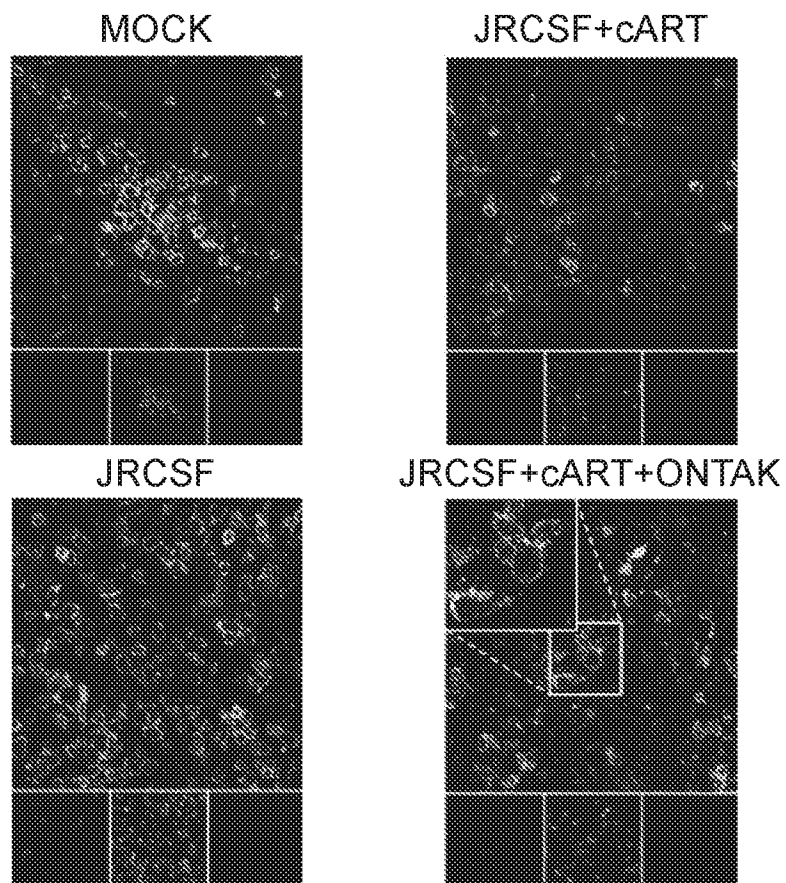


FIG. 8C

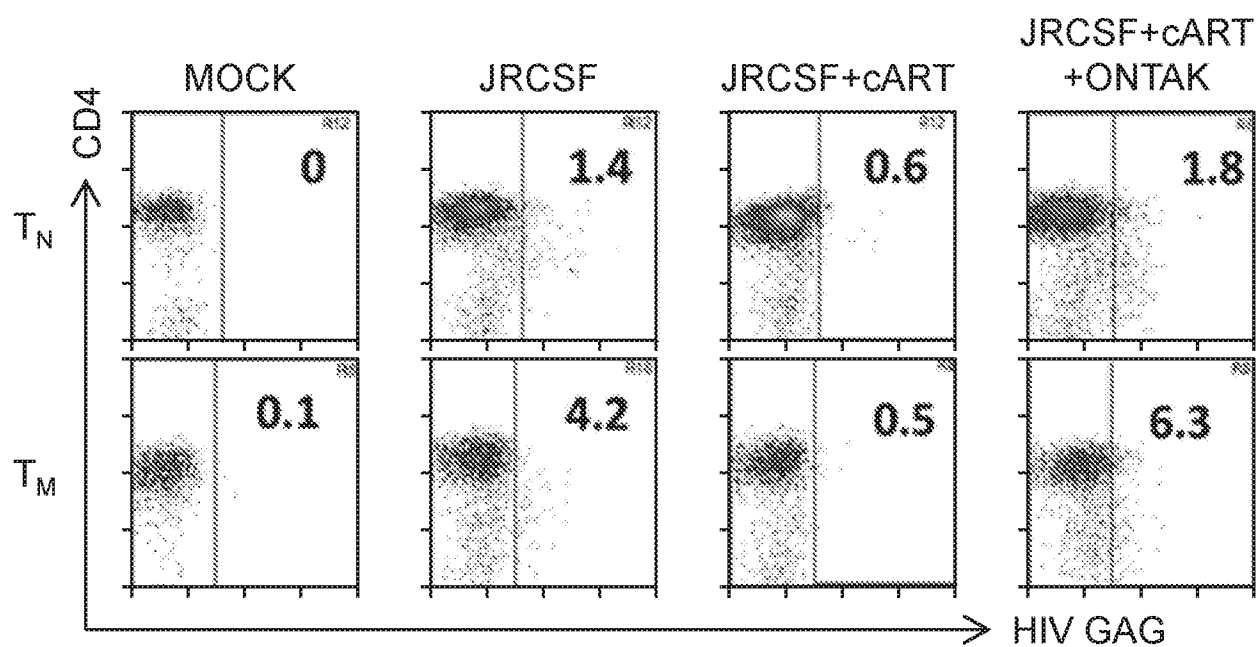


FIG. 8D

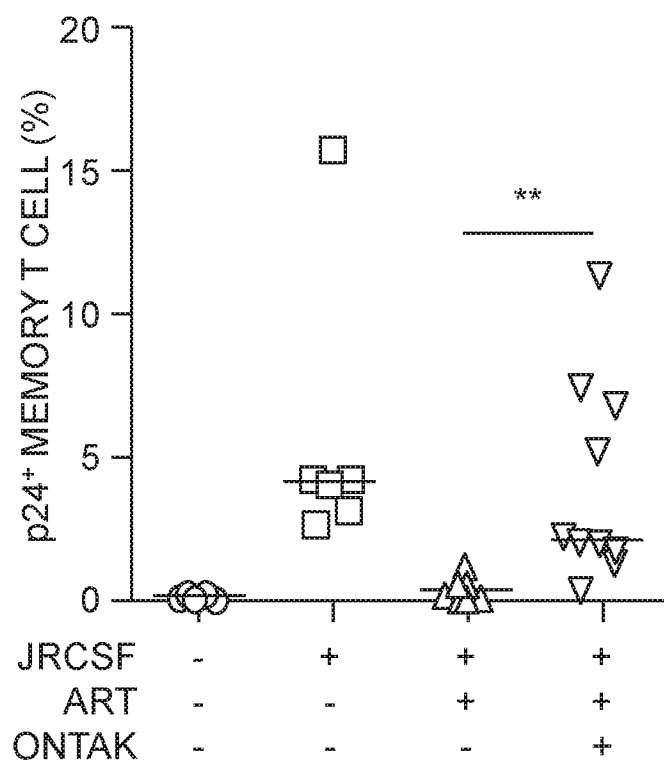
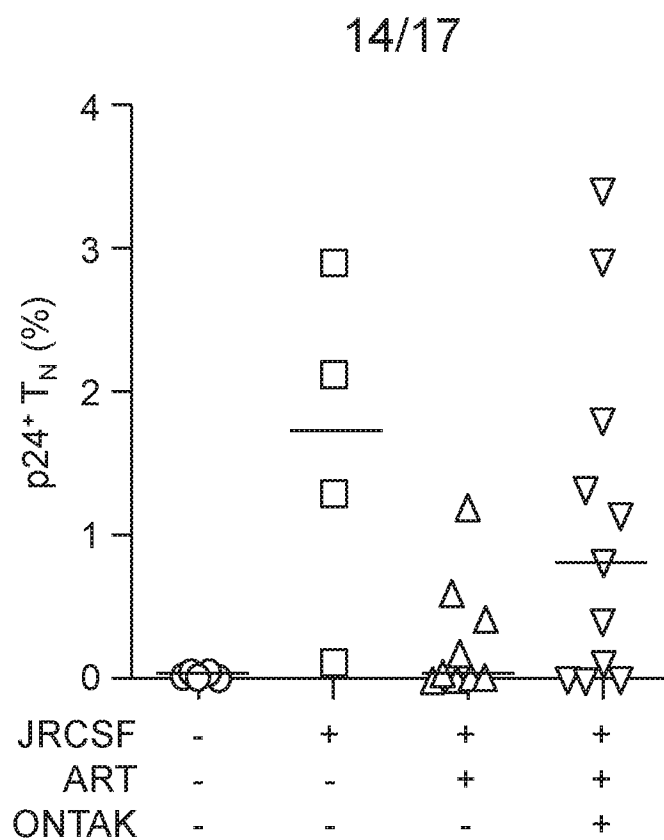


FIG. 8E

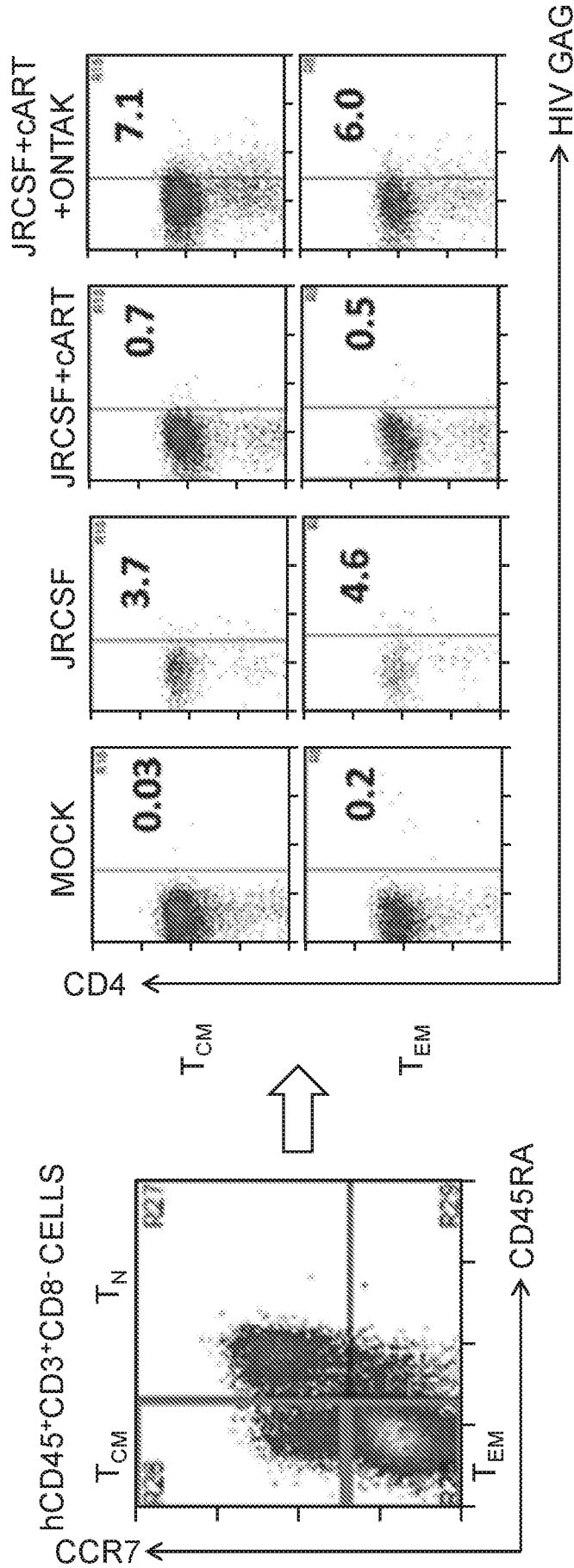


FIG. 9A

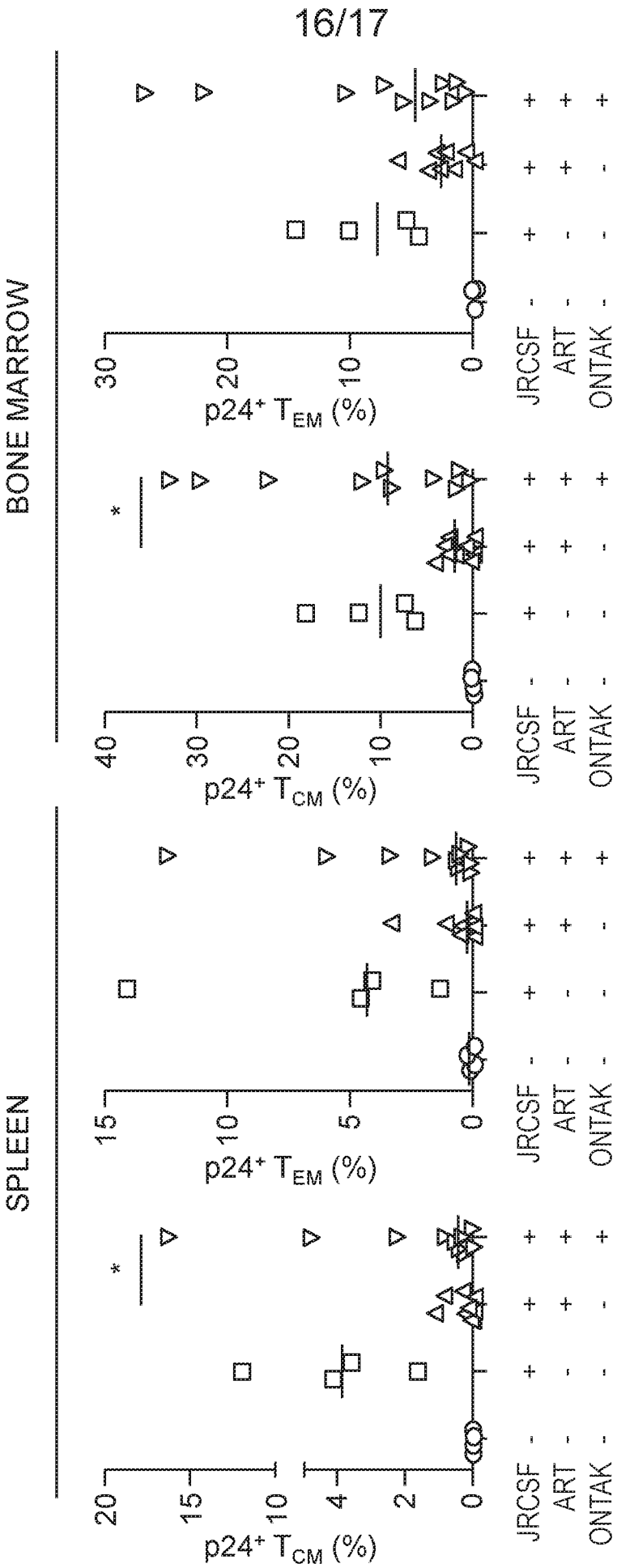


FIG. 9B

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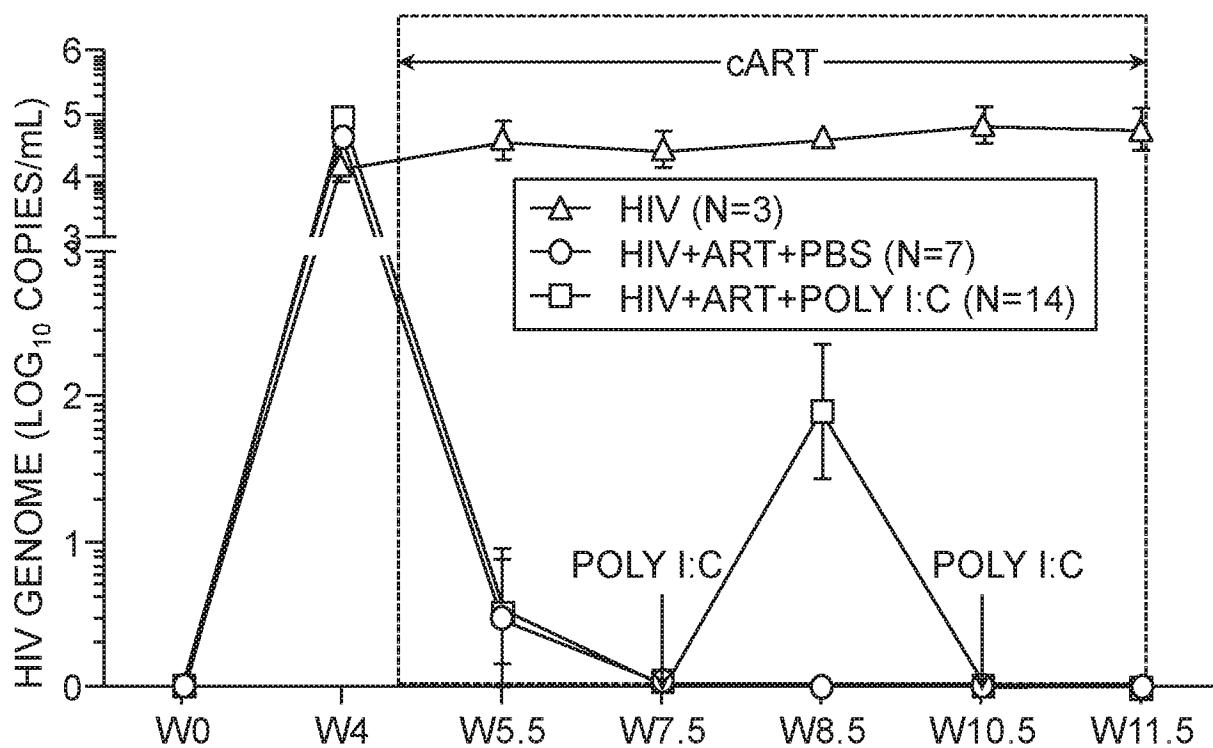


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/031991

A. CLASSIFICATION OF SUBJECT MATTER (see extra sheet) According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K 39/395, 38/20, A61P 37/00 - 37/06, C12Q 1/70, G01N 33/569 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatSearch, EMBL, NCBI, PAJ, Espacenet, DWPI, PCT Online, USPTO DP, CIPO (Canada PO), SIPO DB, Freepatentsonline														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X Y</td> <td>ZHANG L. et al. HIV-1 immunopathogenesis in humanized mouse model, Cellular & Molecular Immunology, 2012, 9, pp.237-244, especially abstract, p.241.</td> <td>1-4, 14-15, 17 5, 16</td> </tr> <tr> <td>Y</td> <td>ZOU W. Regulatory T cells, tumour immunity and immunotherapy, NATURE REVIEWS, IMMUNOLOGY, 2006, Vol.6, pp.295-307, table 2</td> <td>5, 16</td> </tr> <tr> <td>A</td> <td>ZENG H. et al. Dilazep synergistically reactivates latent HIV-1 in latently infected cells, Molecular Biology Rep., 2014, Vol. 41, pp.7697-7704, especially p.7698</td> <td>1-2</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X Y	ZHANG L. et al. HIV-1 immunopathogenesis in humanized mouse model, Cellular & Molecular Immunology, 2012, 9, pp.237-244, especially abstract, p.241.	1-4, 14-15, 17 5, 16	Y	ZOU W. Regulatory T cells, tumour immunity and immunotherapy, NATURE REVIEWS, IMMUNOLOGY, 2006, Vol.6, pp.295-307, table 2	5, 16	A	ZENG H. et al. Dilazep synergistically reactivates latent HIV-1 in latently infected cells, Molecular Biology Rep., 2014, Vol. 41, pp.7697-7704, especially p.7698	1-2
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X Y	ZHANG L. et al. HIV-1 immunopathogenesis in humanized mouse model, Cellular & Molecular Immunology, 2012, 9, pp.237-244, especially abstract, p.241.	1-4, 14-15, 17 5, 16												
Y	ZOU W. Regulatory T cells, tumour immunity and immunotherapy, NATURE REVIEWS, IMMUNOLOGY, 2006, Vol.6, pp.295-307, table 2	5, 16												
A	ZENG H. et al. Dilazep synergistically reactivates latent HIV-1 in latently infected cells, Molecular Biology Rep., 2014, Vol. 41, pp.7697-7704, especially p.7698	1-2												
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.														
<table border="0"> <tr> <td style="vertical-align: top;"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family										
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Date of the actual completion of the international search 22 July 2016 (22.07.2016)		Date of mailing of the international search report 18 August 2016 (18.08.2016)												
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhevskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37		Authorized officer U.Berezhnaya Telephone No. 495 531 65 15												

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2016/031991

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 6-12, 18-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Classification of subject matter

International application No.

PCT/US 2016/031991

A61K 39/395 (2006.01)

A61K 38/20 (2006.01)

A61P 37/00 (2006.01)

C12Q 1/70 (2006.01)

G01N 33/569 (2006.01)