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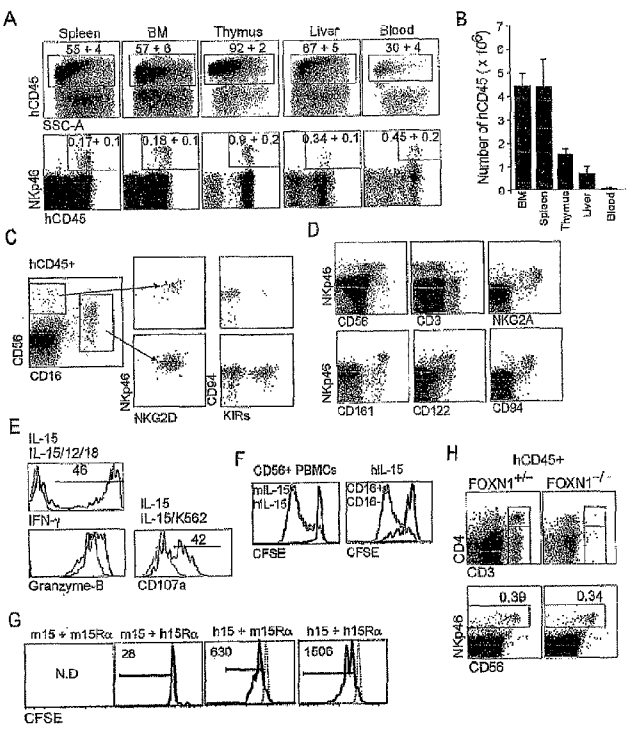

Figure 1

(57) Abstract: The present invention relates to a transgenic animal model system based on the development of transgenic mice bearing components of the human immune system, including large numbers of innate lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes. The invention further relates to the utilization of said model system to screen for, or identify, compounds that modulate (inhibit or activate) the activity or maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes. Such compounds may be used in immunotherapies for treatment of pathogenic diseases, cancer, autoimmune, infectious and inflamatory diseases, immunodeficiency and for treatment of transplant patients. The present invention is based on the discovery that NK cell differentiation and expression of killer-like inhibitory receptors (KIRs) as well as homeostasis and development of adaptive human CD4 and CD8 T cells is regulated by IL-15 mediated signal transduction.

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IL-15 MEDIATED NK AND T CELL MATURATION

1. INTRODUCTION

[001] The present invention relates to a transgenic animal model system based on the development of transgenic mice bearing components of the human immune system, including large numbers of innate lymphocytes such as mature human natural killer (NK) cells, γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes. The invention further relates to the utilization of said model system to screen for, or identify, compounds that modulate (increase or decrease) the number and/or activity or maturation of natural killer (NK) cells or other innate lymphocytes. Such compounds may be used in immunotherapies for treatment of pathogenic diseases, cancer, autoimmune, infectious and inflamatory diseases, immunideficiency and for treatment of transplant patients. The invention further relates to the use of the animal model system of the invention, wherein the immune system of the animal has been stimulated by IL-15, to generate antibodies, including monoclonal antibodies, against any antigen of interest. The present invention is based on the discovery that innate cells in general and NK cell differentiation and expression of killer-like inhibitory receptors (KIRs) in particular as well as adaptive CD4 and CD8 T cells in humans are all regulated by IL-15 mediated signal transduction that operates in a species-specific fashion where human IL-15-responsive cells require human IL-15/IL-15Rα complexes.

2. BACKGROUND OF INVENTION

2.1. NATURAL KILLER CELLS

[002] Natural Killer cells participate in host protection by eliminating cells with altered expression of self-MHC-I which can result from viral infection or transformation (1). While a role for viral and stress-induced ligands in NK cells activation is appreciated, the best described regulatory mechanism of NK cell activity is the expression of inhibitory receptors for self MHC-I ligands by mature NK cells with high cytotoxic potential. In man, killer-like inhibitory receptors (KIRs) recognizing classical MHC-I molecules HLA-A, -B or -C are expressed on the predominate peripheral NK cell ($CD56^{lo}CD16^{+}$) subset which possess abundant intracellular perforin and granzymes and display spontaneous cytotoxicity (2, 3). In contrast, $CD56^{hi}CD16^{-}$ NK cells rarely express KIRs (3) and since they are more prevalent in blood early post-bone-marrow transplant (4, 5), give rise to $CD56^{lo}CD16^{+}$ NK cells when transferred into NOD/SCID mice (6) and have longer telomeres than $CD56^{lo}CD16^{+}$ NK cells (7), it is likely that $CD56^{hi}CD16^{-}$ NK cells are (or contain within this population) precursors of $CD56^{lo}CD16^{+}$ NK cells, however the tools to definitively prove this hypothesis are lacking.

[003] While inhibitory KIRs control reactivity of mature NK cells, their expression also influences the functional maturation of developing NK cells, as NK cells expressing at least one KIR recognizing self MHC-I have a lower threshold of activation and appear more functional than NK cells expressing no KIRs or those only expressing KIRs recognizing non-self MHC-I ligands (8, 9). This phenomenon termed "licensing" or "disarming" has been well characterized in mice (10) and suggests a role for KIR-self MHC-I interactions during human NK cell development. Patients lacking the transporter associated with antigen processing (TAP) have dramatically

reduced surface expression of MHC-I molecules and consequently, NK cells that are hypo-responsive to MHC-I deficient cells (11). KIR^+ NK cells are present in these patients indicating that normal MHC-I expression itself is not required for KIR expression. Given the importance of KIR expression in regulating NK cell function, knowledge of elements influencing KIR acquisition would improve the understanding and clinical approaches to diseases where KIR and HLA haplotypes influence susceptibility, progression or outcome such as autoimmune/inflammatory disease, cancer, infections (HIV, HCV) and bone marrow transplants/graft versus leukemia effects (12, 13).

[004] Since KIRs are expressed on mature $CD56^{lo}CD16+$ NK cells, factors that influence NK cell homeostasis could potentially influence KIR acquisition *in vivo*. An elegant series of murine studies using gene targeting and bone marrow chimeras have reveled that NK cell development requires IL-15Rα expressing cells to chaperon IL-15 to the surface where it is bioactive and significantly more potent in inducing activation and proliferation of IL-15-responsive cells. This concept is called IL-15/IL-15Rα "trans-presentation" (14, 15). Both WO2007/001677 and WO2007/046006 disclose that combinations of IL-15 and IL-15Rα, or complexes of fragments thereof, are capable of modulating an immune response in a host. When not bound to IL-15Rα, IL-15 appears to have a minimal effect on NK cell homeostasis *in vivo* (16, 17). In man, mutations in IL-15Rα have not been reported however NK cells are dramatically reduced in patients carrying mutations in the common gamma chain (γ_c) cytokine receptor (used in IL-15/-7/-4/-9/-2 and -21 signal transduction), Jak3 or the shared IL-2/15Rβ, while they are present in IL-7Rα-deficient patients suggesting that IL-15 may regulate human NK cell development (18, 19).

[005] *In vivo* studies of NK cells have been largely restricted to mice and while this line of experimentation is valuable, some of this knowledge is not transferable to human NK cell biology. A clear example of this is NK cell development, where the kinetics, frequency and phenotype are clearly different between species (3). An intermediate between murine and human *in vivo* studies exists in the form of human immune system (HIS)-mice. A recently developed HIS-mouse model is the engraftment of newborn Balb/c $Rag2^{-/-}\gamma_c^{-/-}$ mice with human hematopoietic stem cells (HSC) from fetal liver, cord blood or adult bone marrow (20, 21). Balb/c $Rag2^{-/-}\gamma_c^{-/-}$ HIS mice represent a practical HIS model with high human chimerism, most lymphocyte lineages generated, adaptive immune responses occasionally evoked and unlike earlier models do not develop thymomas (20-23).

2.2. T-CELL HOMEOSTASIS

[006] The maintenance of peripheral T cell pools expressing highly diverse T cell receptors is essential for sterilizing immunity against viruses, bacteria and parasites capable of infecting humans. T cell homeostasis involves the generation of new T cells in the thymus, the survival and proliferation of T cells in the periphery and the differentiation from naïve to effector or memory [51]. Several signals have been implicated in controlling T cell homeostasis including those emanating from the T cell receptor (TCR) following interactions with self-peptide + MHC (pMHC) and those induced by growth factors including cytokines [51]. The common gamma chain (gc) family of cytokines (which comprise IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21) in particularly have been demonstrated to play a role in T cell homeostasis in mammals [52].

[007] IL-15 is a peculiar cytokine because it requires expression of the IL-15Rα chain by the same cell in order to be functional. Thus cells expressing IL-15 such as monocytes, dendritic cells and stromal cells must also express the IL-15Rα in order to trans-present IL-15 to IL-15 responsive cells (expressing the IL-2Rβ/γ_c complex), and both IL-15 and IL-15Rα are up-regulated on myeloid cells following inflammation, thereby increasing IL-15 bioavalability [63-65]. While humans carrying mutations in IL-15 or IL-15Rα have not been reported, mice lacking these proteins have reduced numbers of memory CD8 T cells (most notably the $CD122^{hi}$ subset), NK T cells and γ/δ T cells in the periphery [66, 67]. Furthermore, memory phenotype CD8 T cells fail to proliferate and survive when adoptively transferred into IL-15 deficient mice demonstrating a direct role for IL-15 in their homeostasis [60, 68].

[008] *In vivo* studies of T cell development and homeostasis have been largely restricted to mice and while this line of experimentation is valuable, 60 million years of evolution has resulted in many differences between murine and human immune systems, thus some of this knowledge may not be directly applicable to human T cell biology. An intermediate between murine and human *in vivo* studies exists in the form of human immune system (HIS)-mice. A recently developed HIS-mouse model is the engraftment of newborn Balb/c $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice with human hematopoietic stem cells (HSC) from fetal liver or cord blood [69-71]. In this model, the mouse thymus is seeded with human T cell progenitors that are able to be positively selected and differentiate into mature CD4 and CD8 T cells capable of migrating to secondary lymphoid organs indicating that a level of cross-reactivity between murine-derived IL-7 and pMHC exist [69-71]. However, the number of peripheral T cells observed in Balb/c $Rag2^{-/-}$ $\gamma_c^{-/-}$ HIS mice is limiting and despite exhibiting an abnormally high

turnover rate, T cells fail to accumulate with time suggesting T cell survival and homeostasis is not optimal [69-73].

[009] As described herein, using HIS Balb/c $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice engrafted with fetal liver HSCs, a new *in vivo* role for human IL-15 trans-presentation in human T cell development and homeostasis has been identified. Human IL-15 trans-presentation in HIS mice increases naïve CD4 and CD8 T cells, accelerates thymopoiesis providing increased peripheral T cells after hematopoeitic stem cell transplantation and results in higher immunoglobulin production against specific antigens after immunization. This discovery provides a HIS model system for use in identification or generation of novel drugs or antibodies for use in immunotherapies designed to treat a variety of different diseases and disorders.

3. SUMMARY OF THE INVENTION

[010] The present invention relates to a transgenic animal model system based on the devlopment of mice bearing components of the human immune system, including innate lymphocytes such as mature human natural killer (NK) cells, γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes. Specifically, the invention relates to immunodeficient mice which are genetically deprived of T, B lymphocytes and NK cells and which have been engrafted with human hematopoeitic stem cells. In a non-limiting embodiment of the invention, the transgenic mice may further be transgenic for expression of human HLA class I and/or HLA class II molecules. In such transgenic mice, the maturation of innate lymphocytes in general and NK cells in particular, as well as adaptive CD4 and CD8 T lymphocytes, is induced through administration of IL-15/IL-15Rα agonists, thereby providing humanized mice having large numbers of innate human lymphocytes in general and

human NK cells in particular and adaptive CD4 and CD8 T lymphocytes. Such mice provide a humanized immune system model to study activity of innate human lymphocytes in general and human NK cells in particular and adaptive CD4 and CD8 T lymphocytes *in vivo*.

[011] The transgenic animal model system of the invention provides a system for identification of novel drugs for use in immunotherapies designed to treat a variety of different diseases and disorders including but not limited to infectious disorders, cancers, autoimmune and inflammatory diseases, immunodeficiency and for treatment of transplant patients. The animal model system of the invention provides a means for assaying the efficacy, toxicity, or side effects of newly developed immunotherapies including, for example, that of vaccines. The animal model system of the invention may also be used to determine the mechanism of action of such immunotherapies.

[012] The transgenic animal model system of the invention also provides a system for generating new therapeutics. In an embodiment of the invention, IL-15 stimulated HIS mice of the invention may be used to generate, for example, new monoclonal antibodies against any antigen of interest following immunization.

[013] The present invention is based on the discovery that IL-15/IL-15Rα trans-presentation promotes NK cell maturation, innate lymphocyte development as well as CD4 and CD8 T cell homeostasis. Accordingly, the transgenic animals of the invention may be used for screening for compounds that modulate the activity, or level of expression, of IL-15 and/or IL-15Rα, thereby regulating the maturation of NK cells, CD4 T cells and CD8 T cells. Such compounds may be used in immunotherapies for treatment of pathogenic diseases, cancer, autoimmune,

infectious and inflamatory diseases and for treatment of transplant patients. In a preferred embodiment of the invention, complexes of IL-15/IL-15Rα that act as agonist on IL-15-responsive human cells are used. The composition can be administered as a therapeutic to treat an existing condition or as a prophylactic in advance of developing a condition.

4. BRIEF DESCRIPTION OF THE FIGURES

[014] Figure 1. NK cells develop and populate to various lymphoid tissue in HIS-mice. (A) 8 weeks following $CD34^+CD38^-$ HSC engraftment, various organs from HIS-mice were analyzed for human NK cell reconstitution by flow cytometry (human CD45; hCD45). FACS plots are representative. Values represent mean percentage ± s.e.m of 8 mice. (B) Cellularity of the indicated organs was enumerated and the number of $hCD45^+$ cells determined based of flow cytometry data. Numbers of cells in bone marrow (BM) are per femur and blood are per ml. Values represent mean ± s.e.m. of 8 mice. Human NK cells were analyzed in the thymus (C) and (D) spleen or bone marrow by flow cytometry using antibodies against the indicated human antigens. Events shown were pre-gated on $hCD45^+$. FACS plots are representative of 8 HIS-mice, with a total of 3 different donor HSCs represented. (E) 5×10^4 $CD56^+$ NK cells were purified from spleen and bone marrow of HIS-mice and stimulated in vitro with IL-12 and IL-18 or 5×10^5 K562 AML cells in the presence of IL-15 for 18h. Cells were stained with anti-CD107a or fixed, permeabilized and stained with anti-IFN-γ or anti-granzyme-B and analyzed by flow cytometry. (F) 2×10^5 $CD56^+$ NK cells purified from human peripheral blood were labeled with CFSE and cultured in human IL-15 (grey line) or murine IL-15 (black line) for 120 hours.

CFSE histograms of $CD56^{lo}CD16^{+}$ (black line) and $CD56^{hi}CD16^{-}$ (grey line) cultured in human IL-15 are also shown. (F) 1 x 10^4 $CD56^{+}$ NK cells purified from human peripheral blood were labeled with CFSE and cultured with the indicated combinations of human (h) and murine (m) IL-15 (15) and IL-15Rα-Fc (15Rα) for 72 hours. Cells were the analyzed flow cytometry. Numbers indicate live cells (PI) per well. N.D (not determined - due to no live cells). Histograms are representative of 2 individual experiments using different donor blood. (G) 8 weeks following $CD34^{+}CD38^{-}$ HSC engraftment, $FOXN1^{+/-}$and $FOXN1^{+/-}$ (Nude) HIS-mice were analyzed for human T and NK cell reconstitution by flow cytometry. FACS plots are gated on $hCD45^{+}$ cells in spleen (top) and BM (bottom) and are representative of 3 mice of each genotype.

[015] Figure 2. Trans-presentation by human IL-15/IL-15Rα promotes human NK cell homeostasis *in vivo*. (A) HIS mice were injected intra-peritoneally (every 5 days for 15 days) with human IL-15, IL-15/IL-15Rα-Fc or PBS commencing 6 weeks after reconstitution. 3 days after the last injection, bone marrow was analyzed for human NK cells reconstitution by flow cytometry using antibodies against the indicated human antigens. (B) HIS-mice treated as in (A) were injected intra-peritoneally with 1 mg BrdU daily on the last 2 days before being sacrificed. $hCD45^{+}NKp46^{+}$ cells from bone marrow were analyzed for intracellular proteins and incorporation of BrdU by flow cytometry. Facs plots are representative of 2 individual experiments using mice engrafted with and 2 different $CD34^{+}$ HSC sources. (C) HIS mice were injected intra-peritoneally (once per week for 4 weeks) with human IL-15/IL-15Rα-Fc, RLI or PBS commencing 6 weeks after reconstitution. 7 days after the last injection, mice were sacrificed and thymus, spleen, liver and bone marrow were analyzed for human NK cells by flow cytometry. Organ cellularities were

counted and $hCD45^+NKp46^+$ cells were enumerated based on positive surface expression determined by flow cytometry. Data is mean ± s.e.m of 5 mice in each group generated from 2 different $CD34^+$ HSC sources. P values are given when statistically significant. (D) NK cell subsets and BrdU incorporation in the bone marrow of HIS-mice treated as in (C) were analyzed by flow cytometry. (E) $hCD45^+NKp46^+$ cells from bone marrow were analyzed for intracellular proteins and incorporation of BrdU by flow cytometry. For (D) and (E) HIS-mice treated as in (C), however mice were sacrificed 3 days after final treatment and were injected intra-peritoneally with 1 mg BrdU daily on the last 2 days before being sacrificed. Facs plots are representative of 2 individual experiments of 3-5 mice per treatment group engrafted with 2 different $CD34^+$ HSC sources. (F) CD8 T cells in the spleen of RLI-treated HIS-mice were enumerated and analyzed for BrdU incorporation by flow cytometry. Facs plots are representative of 4 mice and gated on $hCD45^+$ cells. Histograms are further gated on $CD3^+CD8^+$ cells. Cellularities are mean ± s.e.m of 4 mice.

[016] Figure 3. Ectopic expression of Bcl-xL mildly augments human NK cell reconstitution *in vivo*. Human fetal liver HSCs were infected with retrovirus encoding hBcl-xL and GFP or GFP alone and used to generate HIS mice. (A) 8 weeks after engraftment thymus, spleen and bone marrow cellularities were counted and $CD45^+$ cells were enumerated based on surface expression determined by flow cytometry. (B) GFP expression within $hCD45^+$ thymocytes of HIS-mice 8 weeks after engraftment was detected by flow cytometry. (C) The percentages of GFP+ cells within the human graft were enumerated by flow cytometry. (D) NK cells were enumerated based on (A) and surface expression hCD45 and NKp46 determined by flow cytometry. (D) NK cell subsets were analyzed by flow cytometry to detect

surface expression of CD56 and CD16. FACS plots are pre-gated on $hCD45^+$ cells and are representative of 3-4 mice in each group. Data in (A) and (D) are mean ± s.e.m of 3-6 mice in each group. P values are given when statistically significant.

[017] Figure 4. Human IL-15/IL-15Rα agonists promote NK cells differentiation *in vivo*. NK cell maturation and KIR expression was analyzed in thymus, bone marrow and spleen of HIS-mice treated as in (2C) by flow cytometry for surface expression of CD56, CD16 and KIRs (KIR-2DL2/3/1/-2DS1/2/4/-3DL1/S1). HIS-mice were sacrificed 7 days after last treatment. Histograms are gated on $hCD45^+CD56^{lo}CD16^+$ cells and are representative of 5 mice and 2 different $CD34^+$ HSC sources. $^{**}P < 0.01$, $^{*}P < 0.05$. (B) The ratio of $CD16^+$:$CD16^-$ NK cells ($hCD45^+NKp46^+$) in HIS-mice treated with RLI and PBS as in (2C) were determined. Data are mean ± s.e.m of 4 mice in each group. P values are given when statistically significant. (C) Expression of KIRs (KIR-2DL2/3/1/-2DS1/2/4/-3DL1/S1) on $CD56^{lo}CD16^+$ in HIS-mice treated as in (2A) or engrafted with modified HSCs as in Fig. 3. Facs plots pre-gated on $hCD45^+$ and are representative of at least 3 mice in each group. (D) Expression of KIR2DL1/2/3 was analyzed on KIR3DL1/DS1$^+$ or KIR3DL1/DS1$^+$ NK cells in the bone marrow of HIS-mice treated with RLI as in (2C). Facs plots are gated on $hCD45^+CD56^+CD3^-$ and are representative of 3 mice.

[018] Figure 5. IL-15/IL-15Rα-dependent development of $CD16^+KIR^+$ NK cells from $CD16^-KIR^-$ precursors *in vivo*. (A) NK cell subsets in the bone marrow of RLI treated HIS-mice from (2D) were analyzed for BrdU up-take using flow cytometry. Facs plots are representative of 2 individual experiments of 3-5 mice per treatment group engrafted with 2 different $CD34^+$ HSC sources. (B) $CD56^{hi}CD16^-KIRs^-$ or $CD56^{lo}CD16^+KIRs^-$ NK cells were FACS sorted from fetal spleen ($CD45^+CD3^-$) and

transferred i.h into 1 week old sub-lethally irradiated $Rag2^{-/-}\gamma c^{-/-}$ recipients for 7 days. Mice were treated i.p with 2.5μg RLI or PBS on day 0 and day 4 post-transfer. Sorted $KIRs^{-}$ NK cells lacked KIR-2DL2/3/1/-2DS1/2/4/-3DL1/S1 expression. Data is representative of 4 mice per NK subset transferred and 2 mice per treatment group.

[019] Figure 6. Lymphocyte reconstitution in HIS-mice. 8 weeks after engraftment with human fetal liver HSCs, the indicated organs from HIS-mice were harvested and analyzed for the indicated surface antigen expression by flow cytometry. FACS plots are gated on $hCD45^{+}$ cells and are representative of at least 8 HIS-mice.

[020] Figure 7. *In vitro* NK cell cultures and *in vivo* IL-15 neutralization. (A) Splenic $DX5^{+}$ murine NK cells were labeled with CFSE and cultured in 30ng/ml murine IL-15 for 72 hours. Cells were the analyzed for surface expression of NK1.1 and DX5 by flow cytometry. (B) 5 x 104 $CD56^{+}$ NK cells purified from human peripheral blood were labeled with CFSE and cultured for 3 days in media alone or with 2 x 104 pre-activated human (same donor peripheral blood derived) or murine myeloid cells(derived from $Rag2^{-/-}\gamma c^{-/-}$ bone marrow). Myeloid cells were purified by anti-PE magnetic beads against anti-CD11b/CD11c/F480-PE for mouse and anti-CD14/CD83/CD116-PE for human and cultured over night 5mg/ml LPS and 10ng/ml IL-4. (C) 16 week old HIS-mice were treated with anti-mouse IL-15 or anti-human IL-15 neutralizing antibodies for 7 days and analyzed for $NKp46^{+}$ cells in the spleen. NK cellularity following treatment is expressed as a mean percentage + s.e.m of PBS treated age and donor HSCs matched HIS-mice. *P = 0.035.

[021] Figure 8. Trans-presented IL-15 enhances human $CD4^{+}$ and $CD8^{+}$ T cell development and reconstitution of lymphoid organs in HIS mice. (A) Experimental

scheme. Newborn $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice were irradiated with 3.3 Gy injected intra-hepatic (i.h.) with $5x10^4$ $CD34^+CD38^-$ human fetal liver cells. At 8,9,10 and 11 weeks of age, HIS mice were injected intra-peritoneally (i.p) with 2.5μg IL-15-IL-15Rα fusion protein (RLI) or PBS. Mice were sacrificed and analysed at 12 weeks. (B) Lymphoid organs from HIS mice were analysed for total human hematopoietic (human CD45; $hCD45^+$), human T cells ($hCD45^+CD3^+$ and $CD4^+$ or $CD8^+$) and (C) human B cells ($hCD45^+CD19^+$) reconstitution by flow cytometry and cellularity of the indicated organs was enumerated. $hCD45^+$ (%) is the percentage of total cells, whereas $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ (%) are the percentage within the $hCD45^+$ population. Values represent mean ± s.e.m of 9 mice per group. P values are indicated when significant. (D) HBV immunization results in marked increases in IgG levels in HIS mice that had received trans-presented human IL-15.

[022] Figure 9. Trans-presented IL-15 treatment in vivo results in minimal phenotypic alteration to expanded T cell pool in primary and secondary lymphoid organs. (A) Thymus, spleen and mesenteric lymph nodes (mLN) from HIS mice treated as in Fig 8A were analysed by flow cytometry for T cell reconstitution by CD27 and CD3. (B) Human T cells ($hCD45^+CD3^+$) were further analysed for surface antigens associated with activation (CD69, CD45RO, CD127, CD45RA, CD62L), maturation (CD122) or regulatory function (CD25). FACS plots are representative of 5 mice per group. (C) A significant increase was observed in the percentage and total number of KIR^+ T cells, which included an expansion of T cells expressing either one or both of the KIR2DL2/3 and KIR3DL1 receptors. Other NK-associated cell surface markers such as CD16 and CD161, but not CD56 were also induced or up-regulated on T cells from all organs analyzed from IL-15 treated mice.

[023] Figure 10. Trans-presented IL-15 enhances the proliferation of both naïve and activated phenotype CD8 and CD4 T cells in HIS mice. HIS-mice treated as in Fig 8A were injected intra-peritoneally with 1 mg BrdU daily on the last 2 days before being sacrificed. CD4+ and CD8+ human T cells ($hCD45^+CD3^+$) from (A) spleen and (B) mLN were analysed by flow cytometry for surface antigen corresponding to activation status (CD45RA) and incorporation of BrdU. FACS plots are representative of 3 individual experiments. * $P < 0.05$. BrdU uptake by human NK cells in the same organs are shown as a positive control for IL-15 responsiveness. (C) Serum from mice at time of sacrifice was tested by Luminex® assay for the concentration of the indicated cytokines. Values represent mean ± s.e.m of 3 mice per group except non-reconstituted mouse who served as a negative control.

[024] **Figure 11. Trans-presented IL-15 augments thymopoiesis by targeting T cell progenitors in the thymus.** The frequency of (A) DP, iSP4 and DN thymocytes from HIS mice treated as in Fig 8A were determined by flow cytometry according to the cell surface phenotype shown in (B) and cellularity enumerated. Values represent mean ± s.e.m of 9 mice per group. ** $P < 0.02$. (B) HIS-mice treated as in Fig 1A were injected intra-peritoneally with 1 mg BrdU daily on the last 2 days before being sacrificed. BrdU incorporation by the 6 thymocyte populations (DN, iSP4, DP, SP4, SP8 and NK) was determined by flow cytometry. Control mice were injected with an identical volume of PBS. * $P < 0.05$. (C) Thymic T cells and their progenitors were analysed for intracellular Bcl-xL expression by flow cytometry. Facs plots are pre-gated on $hCD45^+$ cells and are representative of 3 mice for each group. (D) The indicated thymocyte subsets were electronically purified by flow sorting and cultured in media alone or media with IL-7 or IL-15. The frequency of viable cells after 30 h was determined by PI exclusion using flow cytometry. Data is representative of 2

experiments. (E) The indicated thymocyte subsets were electronically purified by flow sorting from PBS or IL-15 treated HIS mice and cultured in media alone or media with IL-7 or IL-15. The frequency of viable cells was determined by PI exclusion using flow cytometry. Data is representative of 2 experiments.

[025] Figure 12. Diverse TCR Vβ repertoire in Balb/c Rag2$^{-/-}$ $\gamma_c^{-/-}$ HIS mice is unaltered following IL-15 treatment *in vivo*. (A) Thymocytes or (B) Splenocytes from HIS mice treated as in Fig 8A were isolated and TCR CDR3 immunoscope analysis was performed for different Vβ families. Histograms are representative of results obtained from 5 mice of each group and display amino acid length of CDR3 regions (x-axis) and relative frequency (y-axis).

[026] Figure 13. Development and diversity of γ/δ TCR$^+$ T cells in Balb/c Rag2$^{-/-}$ $\gamma_c^{-/-}$ HIS mice *in vivo*. (A) Human γ/δ TCR$^+$ T cells from thymus and LN of HIS mice were analyzed by flow cytometry. Human T cells were separated into two subsets based on CD161 expression (CD3$^+$CD161$^+$ or CD3$^+$CD161$^-$) and the expression of Vγ9, Vδ2, Vδ1 and CD8. (B) Total human γ/δ TCR$^+$ T cells from thymus and LN from HIS mice treated as in Fig 8A were analyzed by flow cytometry using a Pan γ/δ TCR antibody amongst total human T cells (hCD45+CD3+) in these organs. FACS plots are representative of (A) 4 and (B) 5 mice in each group. (C) The relative proportion of T cells expressing the indicated γ or δ TCR was determined by quantitative PCR for both control PBMC and HIS splenocytes from mice treated as in Fig 8A. Percentages represent the frequency of T cells containing the indicated Vγ or Vδ PCR products out of the total Vγ or Vδ PCR products. Histograms represent the mean percentage + SEM of 3 mice in each group (PBS and IL-15-IL-15Rα) or 1 human donor (hPBMCs). (D) Splenocytes from HIS mice treated as in Fig 8A or

human PBMCs were isolated and TCR CDR3 immunoscope analysis was performed for different Vγ and Vδ families. Histograms are representative of results obtained from 3 mice of each group and display amino acid length of CDR3 regions (x-axis) and relative frequency (y-axis).

[027] Figure 14. IL-15 accelerates thymopoeisis and appearance of peripheral T cells when administered *in vivo* to Balb/c $Rag2^{-/-}$ $\gamma_c^{-/-}$ HIS mice in the early post-HSC graft period. (A) Thymus and spleen from HIS mice (5 – 6 weeks post-HSC engraftment) were analysed by flow cytometry for human T cell reconstitution by hCD45, CD56, CD3, CD4, CD8 and CD45RA. (B) HIS mice (5 – 6 weeks post-HSC engraftment) were treated every 3 days with with 2.5μg IL-15-IL-15Rα fusion protein (RLI) or PBS and sacrificed 12 days after the first injection. Human ($hCD45^+$) lymphocytes from thymus, spleen and BM were analyzed for mature T cells (CD3 and CD45RA) and double positive thymocytes (CD4 and CD8) by flow cytometry. HIS mice treated as in 7B were bled on days 0, 4, 7 and 12 after the first injection and the total number of human T cells ($hCD45^+CD3^+$) per ml of blood was enumerated by flow cytometry. Data represents the fold increase in T cell number in mice treated with IL-15-IL-15Rα compared to PBS at each time point. Data is mean percentage + s.e.m of 3 mice. (C) Almost a 10-fold increase in human T cells in the blood after the second injection (day 4) and increasing up to 20-fold more T cells at the end of the treatment regime was observed.

5. DETAILED DESCRIPTION OF THE INVENTION

[028] As will be described in detail below, the present invention relates to a transgenic animal model system based on the development of transgenic mice bearing components of the human immune system, including innate lymphocytes such as

mature human natural killer (NK) cells, γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes. The transgenic mice of the invention may be used to identify compounds that modulate the maturation or activity of innate human lymphocytes such as mature human natural killer (NK) cells, γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes and to further assess the efficacy, toxicity and side effects of any newly developed immunotherapies, including, for example, vaccine development. Such immunotherapies are designed for treatment of pathogenic diseases, cancer, autoimmune, infectious and inflamatory diseases and for treatment of transplant patients. The present invention is based on the discovery that innate human lymphocytes in general and human NK cell differentiation and expression of killer-like inhibitory receptors (KIRs) in particular as well as adaptive CD4 and CD8 T cells in humans are all regulated by IL-15 that operates in a species-specific fashion where human IL-15-responsive cells require human IL-15/IL-15Rα complexes.

5.1. TRANSGENIC ANIMAL MODEL SYSTEM

[029] The present invention relates to a transgenic animal model system based on the devlopment of mice bearing components of the human immune system, including mature NK cells, γδ T cells, NK T cells, CD 4 and CD8 T cells and B cells. In a specific embodiment of the invention, a transgenic mouse is provided, characterized in that it has (i) a phenotye comprising a deficiency for murine T lymphocytes, B lymphocytes and NK cells; (ii) is engrafted with human hematopoietic cells; and (iii) contains human NK cells in the lymphoid tissues of said engrafted mouse. In another embodiment of the invention, the transgenic mouse further comprises human CD4 and CD8 peripheral T cells and human B cells. In a specific embodiment of the invention,

the transgenic mice contain at least 1% mature human NK cells in the lymphoid tissues of said engrafted mouse. In another embodiment of the invention, the transgenic mouse further comprises a phenotype comprising a deficiency for murine MHC class I and/or MHC class II molecules and transgenic for the expression of human HLA class I and/or HLA class II molecules.

[030] The transgenic mice according to the present invention which are deficient for murine T and B lymphocytes, and NK cells (immunologically deficient mice) may comprise two genes essential in T, B and/or NK cell development that are inactivated by a spontaneous mutation or a targeted mutation. These mutations which are well-known to those of ordinary skill in the art include, for example; a first mutation which is the mouse scid mutation ($Prkdc^{scid}$; Bosma et al., Nature 183, 301, 527-530; Bosma et al., Curr. Top. Microbiol., Immunol., 1988, 137, 197-202) or the disruption of the recombination activating gene ($Rag1^{-/-}$ or $Rag2^{-/-}$; Mombaerts et al., Cell, 1992, 68, 869-877; Takeda eta l., Immunity, 1996, 5, 217-228), and a second mutation which is the beige mutation (Lystbg; MacDougall etal., Cell. Immunol., 1990, 130, 106-117) or the disruption of the β2-microglobulin gene ($\beta 2m^{-/-}$; Kollet et al., Blood, 200, 95, 3102-3105), the IL-2 receptor γ chain (or common cytokine receptor γ chain (γ_c)) gene (IL-2R $\gamma^{-/-}$ or $\gamma_c^{-/-}$; DiSanto et al., PNAS 1995, 92, 377-381), or the IL-2 receptorβ chain (IL-2R β) gene (IL-2R$\beta^{-/-}$; Suzuki et al., J. Exp. Med., 19979, 185, 499-505). In a specific embodiment of the invention, $Rag2^{-/-}\gamma c^{-/-}$ transgenic mice may be used in the practice of the invention.

[031] In a non-limiting embodiment of the invention, the transgenic mice of the invention may further comprise a phenotype having a deficiency for murine MHC class I and/or MHC class II molecules and transgenic for the expression of human

HLA class I and/or HLA class II molecules. Such mice may be engineered using the methods set forth in WO 2008/010100, which is incorporated by reference herein in its entirety.

[032] Methods for generating transgenic animals with targeted mutations and/or transgene expression via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. No. 4,736,866 by Leder et al. and U.S. Pat. No. 4,870,009 Evans et al., U.S. Pat. No. 4,873,191 by Wagner et al., and in Hogan, B., Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986), incorporated herein by reference in their entirety.

[033] The transgenic mice of the invention, which are deficient for murine T and B lymphocytes, and NK cells, and which may also express human HLA Class I and Class II molecules, are further engrafted with human hematopoietic stem cells (HSCs). Such HSCs may be derived from, for example, fetal liver, cord blood or adult bone marrow and are characterized by expression of CD34. The cells may be cultured for an appropriate time before transplantation, to improve the engraftment rate of the hematopoietic progenitors into the transgenic mouse. The number of cells that are transplanted is determined so as to obtain optimal engraftment into the transgenic mouse. For example, human $CD34^+$ cells (from 10^4 to 10^6 cells), isolated from cord blood or fetal liver are transplanted intraperitoneally, intra-hepatically, or intraveniously, for example via a facial vein, into sub-lethally irradiated newborn transgenic mice. The engraftment of cells into immunodeficient mice can be accomplished using methods well known to those of skill in the art (Traggiai et al.,

Science, 2004,304, 104-107; Ishikawa et al., Blood, 2005, 106, 1565-1573; Gimeno et al., Blood, 2004, 104, 3886-3893; Vodyanik et al., Blood, 2005, 105, 617-626).

[034] In said engrafted transgenic mice, the numbers of innate human lymphocytes in general and NK cells in particular and the maturation of NK cells as well as human adaptive CD4 and CD8 T lymphocytes can be induced through administration of IL-15 and IL-15Rα agonists, resulting in immunodeficient mice containing large quantities of innate human lymphocytes and mature NK cells and adaptive CD4 and CD8 T cells. Such agonists include, for example, IL-15/IL-15Rα complexes,including for example IL-15/IL-15Rα, a complex of IL-15/IL-15Rα fragments, hIL-15+IL-15Rα-Fc and RLI (Receptor-Linker-Interleukin). See, for example, [029]. In an embodiment of the invention, the IL-15/IL-15Rα complex could also be administered as an expression vector (lentivirus, adenovirus, retrovirus, AAV for example) or as a transgene. Further, for expression in mammalian cells a codon optimized version of the IL-15 and IL-15Ra genes, as described in US209/0082299, may be used.

[035] The engrafted transgenic mice of the invention as defined above may also be used for the production of humanized antibodies or fragments of these antibodies. Accordingly, the subject of the present invention is also a method for preparing humanized antibodies or fragments of these antibodies, comprising (i) the immunization of the engrafted transgenic mice with an antigen of interest; and (ii) the production of humanized antibodies or fragments of these antibodies, from serum or B lymphocytes of said transgenic mice. In a further embodiment of the invention, the engrafted transgenic mice are stimulated with IL-15 or an IL-15 or IL-15Rα agonist such as an IL-15/IL-15Rα complex prior to immunization.

[036] The invention encompasses the production of polyclonal or monoclonal antibodies consisting of monomeric or dimeric antibodies, and fragments thereof, in particular the Fab, Fab'2 and Fc fragments. The transgenic mice according to the invention have the advantage of allowing the production of monoclonal antibodies which are immediately humanized antibodies. The humanized antibodies and fragments thereof are prepared by conventional techniques known to persons skilled in the art, such as those described in Antibodies: A Laboratory Manual, E. Howell and D. Lane, Cold Spring Harbor Laboratory, 1988. The humanized antibodies according to the invention and fragments thereof as defined above are well tolerated in humans and have a prolonged half-life in humans, given that the constant region of the heavy chain and the entire light chain of these antibodies are of human origin.

[037] The present invention is also directed to a pharmaceutical composition comprising a humanized antibody or a fragment of this antibody in conjunction with a carrier. Such compositions, comprising antibodies or its fragment thereof, may be used for the prevention and treatment of an infectious disease or cancer.

5.3. SCREENING ASSAYS

[038] The transgenic animals of the invention provide a model system to screen for, or identify, compounds that modulate (inhibit or activate) the activity or maturation of innate human lymphocytes in general and NK cells in particular, and adaptive CD4 and CD8 T lymphocytes. Accordingly, the present invention provides for methods for identifying a compound that induces NK cell maturation, comprising (i) contacting a transgenic mouse of the invention with a test compound and (ii) measuring the level of NK cell maturation; wherein an increased in the level of NK cell maturation in the presence of the test compound indicates that the test compound induces NK cell

maturation. Further, the present invention provides for methods for identifying a compound that induces T cell maturation, comprising (i) contacting a transgenic mouse of the invention with a test compound and (ii) measuring the level of T cell maturation; wherein an increased in the level of T cell maturation in the presence of the test compound indicates that the test compound induces T cell maturation.

[039] The present invention provides for methods for identifying a compound that modulates T cell maturation, comprising (i) contacting a transgenic mouse of the invention with a test compound and (ii) measuring the level of T cell maturation; wherein an increased in the level of T cell maturation in the presence of the test compound indicates that the test compound modulates cell maturation. In specific embodiments of the invention the T cells are CD4 or CD8 T cells.

[040] The present invention also provides for methods for identifying a compound that inhibits NK cell maturation comprising (i) contacting a transgenic mouse of the invention with a test compound, in the presence of an IL-15/IL-15Rα complex and measuring the level of NK cell maturation; (ii) in a separate experiment, contacting a transgenic mouse of the invention with an IL-15/IL-15Rα complex and measuring the level of NK cell maturation, where the conditions are essentially the same as in part (i) and then (iii) comparing the level of NK cell maturation measured in part (i) with the level of NK cell maturation in part (ii), wherein a decrease level of NK cell maturation in (i) compared to (ii) indicates that the test compound is an inhibitor of NK cell maturation.

[041] The present invention also provides for methods for identifying a compound that inhibits T cell maturation comprising (i) contacting a transgenic mouse of the invention with a test compound, in the presence of an IL-15/IL-15Rα complex and

measuring the level of T cell maturation; (ii) in a separate experiment, contacting a transgenic mouse of the invention with IL-15/IL-15Rα complex and measuring the level of T cell maturation, where the conditions are essentially the same as in part (i) and then (iii) comparing the level of T cell maturation measured in part (i) with the level of T cell maturation in part (ii), wherein a decrease level of T cell maturation in (i) compared to (ii) indicates that the test compound is an inhibitor of T cell maturation. In specific embodiments of the invention the T cells are CD4 or CD8 T cells.

[042] The animal model system of the invention, bearing components of the human immune system including expression of human NK cells, CD4 T cells and CD8 T cells further provides a means for assaying the efficacy, toxicity, or side effects of newly developed immunotherapies. Newly developed cancer treatments may also be tested for their efficacy, toxicity, and/or presence of side effects.

[043] Infectious disease treatments may be assayed using the transgenic mice of the invention. Such infectious diseases include, for example, bacterial, viral, fungal or parasitic diseases. In addition, treatments designed to inhibit transplant rejection may be assayed using the transgenic mice of the invention.

[044] The transgenic animals of the invention also provide an animal model sytem for screening for compounds that modulate the activity, or level of expression, of IL-15 and/or IL-15Rα, thereby regulating the maturation of NK cells, γδ T cells, CD4 T cells, and/or CD8 T cells. Such compounds may be used in immunotherapies for treatment of pathogenic diseases, cancer, autoimmune and inflamatory diseases and for treatment of transplant patients.

[045] The present invention provides for methods for identifying a compound that increases IL-15 expression or activity, *i.e.*, an agonist, thereby inducing NK cell maturation, comprising (i) contacting a transgenic mouse of the invention with a test compound in the presence of IL-15 and (ii) measuring the level of NK cell maturation; wherein an increased in the level of NK cell maturation in the presence of the test compound indicates that the test compound increases IL-15 expression or activity.

[046] The present invention provides for methods for identifying a compound that increases IL-15 expression or activity, *i.e.*, an agonist, thereby inducing T cell maturation, comprising (i) contacting a transgenic mouse of the invention with a test compound in the presence of IL-15 and (ii) measuring the level of T cell maturation; wherein an increased in the level of T cell maturation in the presence of the test compound indicates that the test compound increases IL-15 expression or activity. In specific embodiments of the invention the T cells are CD4 or CD8 T cells.

[047] The present invention provides for methods for identifying a compound that increases IL-15Rα expression or activity, *i.e.*, an agonist, thereby inducing NK cell maturation, comprising (i) contacting a transgenic mouse of the invention with a test compound and (ii) measuring the level of NK cell maturation; wherein an increased in the level of NK cell maturation in the presence of the test compound indicates that the test compound increases IL-15Rα expression or activity.

[048] The present invention provides for methods for identifying a compound that increases IL-15Rα expression or activity, *i.e.*, an agonist, thereby inducing T cell maturation, comprising (i) contacting a transgenic mouse of the invention with a test compound and (ii) measuring the level of T cell maturation; wherein an increased in

the level of T cell maturation in the presence of the test compound indicates that the test compound increases IL-15Rα expression or activity. In specific embodiments of the invention the T cells are CD4 or CD8 T cells.

[049] The present invention also provides for methods for identifying a compound that inhibits IL-15 or IL-15Rα activity or expression, *i.e.*, an antagonist, thereby inhibiting NK cell maturation comprising (i) contacting a transgenic mouse of the invention with a test compound, in the presence of an IL-15/IL-15Rα complex and measuring the level of NK cell maturation; (ii) in a separate experiment, contacting a transgenic mouse of the invention with an IL-15/IL-15Rα complex and measuring the level of NK cell maturation, where the conditions are essentially the same as in part (i) and then (iii) comparing the level of NK cell maturation measured in part (i) with the level of NK cell maturation in part (ii), wherein a decrease level of NK cell maturation in (i) compared to (ii) indicates that the test compound is a IL-15 of IL-15R inhibitor.

[050] The present invention also provides for methods for identifying a compound that inhibits IL-15 or IL-15Rα activity or expression, *i.e.*, an antagonist, thereby inhibiting T cell maturation comprising (i) contacting a transgenic mouse of the invention with a test compound, in the presence of an IL-15/IL-15Rα complex and measuring the level of T cell maturation; (ii) in a separate experiment, contacting a transgenic mouse of the invention with an IL-15/IL-15Rα complex and measuring the level of T cell maturation, where the conditions are essentially the same as in part (i) and then (iii) comparing the level of T cell maturation measured in part (i) with the level of T cell maturation in part (ii), wherein a decrease level of T cell maturation in

(i) compared to (ii) indicates that the test compound is a IL-15 of IL-15R inhibitor. In specific embodiments of the invention the T cells are CD4 or CD8 T cells.

[051] The ability of a test molecule to modulate the maturation of innate human lymphocytes such as mature human natural killer (NK) cells, γδ T cells , NK-T cells, as well as adaptive CD4 T cells and CD8 T cells may be measured using standard biochemical and physiological techniques. In a specific embodiment of the invention, the maturation of NK cells can be measured through detection of specific cell surface markers that are expressed on the surface of NK cells as they mature and differentiate. For example, as NK cells differentiate the level of cell surface CD56 expression decreases and the levels of CD16 and KIRs increases. NK cell differentiation can also be measured by NK cell effector functions including cell cytotoxicity and cytokine production.

[052] In a specific embodiment of the invention, the maturation of T cells can be measured through detection of specific cell surface markers that are expressed on the surface of T cells as they mature and differentiate. For example, as T cells differentiate the level of cell surface CD62L expression decreases and the levels of CD44 and KLRG-1 increases. T cell differentiation can also be measured by T cell effector functions including cell cytotoxicity and cytokine production.

[053] Preferred methods for the identification of such cell surface markers in the biological sample of a test animal can involve, for example, immunoassays wherein cell surface markers are detected by their interaction with a cell surface specific antibody. Such antibodies include, but are not limited to anti-CD56, anti-CD16 and anti-KIR, anti-CD62L, anti-CD44 and anti-KLRG-1 antibodies, to name a few. Antibodies useful in the present invention can be used to quantitatively or

qualitatively detect the presence of NK or T-cell surface markers. In addition, reagents other than antibodies, such as, for example, polypeptides that bind specifically to the cell surface marker proteins can be used in assays to detect the level of protein expression. Immunoassays useful in the practice of the invention include but are not limited to assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

[054] A sample of a biological fluid or biological tissue to be assessed for levels of NK-cells, CD4 T cells or CD8 T cells, such as blood or other biological tissue, is obtained from the test animal. Immunoassays for detecting NK-cells, CD4 T cells or CD8 T cells typically comprise contacting the biological sample, such as a blood or tissue sample derived from the test animal, with an anti-cell surface marker antibody under conditions such that an immunospecific antigen-antibody binding reaction can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, for example, can be used to detect the presence of on or more cell surface marker proteins specifically expressed on differentiated NK cells, or CD4 or CD8 T cells wherein the detection of said proteins is an indication of NK cell differentiation or T cell differentiation.

[055] Detection of antibodies bound to NK or T cell surface specific markers may be accomplished using a variety of methods. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect cytidine deaminase protein

expression through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March 1986). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. The antibody may also be labeled with a fluorescent compound. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin and fluorescamine. Likewise, a bioluminescent compound may be used to label the antibody. The presence of a bioluminescence protein is determined by detecting the presence of luminescence. Important bioluminescence compounds for purposes of labeling are luciferin, luciferase and aequorin.

5.2.5. COMPOUNDS THAT CAN BE SCREENED IN ACCORDANCE WITH THE INVENTION

[056] The assays described above can identify compounds which modulate the maturation or activity of innate human lymphocytes such as mature human natural killer (NK) cells, γδ T cells, NK-T cells, as well as adaptive CD4 and CD8 T cells. For example, compounds that affect IL-15 and/or IL-15R activity include but are not limited to compounds that bind to IL-15 or the IL-15R, and either activate the ligand/receptor activity (agonists) or block the ligang/receptor activity (antagonists). Alternatively, compounds (agonists or antagonists) may be identified that do not bind directly to IL-15 or IL-15R but which are capable of altering their activity by altering the activity of a protein involved in the IL-15 mediated signal transduction pathway.

[057] The compounds which may be screened in accordance with the invention include, but are not limited to, small organic or inorganic compounds, peptides,

antibodies and fragments thereof, and other organic compounds e.g., peptidomimetics) that bind to IL-15 or IL-15R and either mimic the activity triggered by ligand/receptor binding (*i.e.*, agonists) or inhibit the activity triggered by ligand/receptor binding (*i.e.*, antagonists).

[058] Compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries (see, e.g., Lam, K.S. et al., 1991, Nature 354:82-84; Houghten, R. et al., 1991, Nature 354:84-86); and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; (see, e.g., Songyang, Z. et al., 1993, Cell 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and Fab, $F(ab')_2$ and Fab expression library fragments, and epitope binding fragments thereof), and small organic or inorganic molecules.

[059] Other compounds which maybe screened in accordance with the invention include but are not limited to small organic molecules that affect the expression of the Il-15 or IL-15R gene or some other gene involved in IL-15 mediated signal transduction.

5.3. COMPOSITIONS CONTAINING MODULATORS OF NK CELL, CD4 AND CD8 T CELL MATURATION AND THEIR USES

[060] The present invention provides for compositions comprising an effective amount of a compound capable of increasing the number of mature NK cells, and a pharmaceutically acceptable carrier. The present invention further provides for compositions comprising an effective amount of a compound capable of increasing

the number of mature T cells, including but not limited to CD4 and CD8 T cells, and a pharmaceutically acceptable carrier. Such compounds include, but are not limited to IL-15 and IL-15R agonists. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical sciences" by E.W. Martin.

[061] The present invention relates to methods and compositions for preventing the occurance or progression of infectious diseases, including but not limited to those arising from infections with pathogens such as viruses, bacteria, fungi or parasites. The method comprises administering to a mammal a compound that induces NK cell maturation, or T-cell development, in an amount effective to prevent the occurance of the infectious diesase, or to slow or halt the progression of said disease. Such compounds include those that act as agonists of IL-15 or Il-15R. The compounds can be administered as a therapeutic to treat an existing condition or as a prophylactic in advance of exposure to pathogen.

[062] The present invention relates to methods and compositions for preventing the occurance or progression of a cancer or pre-cancerous condition. The method comprises administering to a mammal a compound that induces NK cell maturation, or T-cell development, in an amount effective to prevent the occurance of the cancer, or to slow or halt the progression of said disease. Such compounds include, but are not limited to, agonists of IL-15 or IL-15R. The compounds can be administered as a

therapeutic to treat an existing condition or as a prophylactic in advance of exposure to a carcinogenic compound or event.

[063] The present invention also relates to methods and compositions for preventing transplant rejection in transplant patients. The method comprises administering to a mammal a compound that induces NK cell maturation, or T-cell development, in an amount effective to prevent transplant rejection. Such compounds include, but are not limited to, agonists of IL-15 or Il-15R .

[064] The present invention relates to methods and compositions for preventing the occurance or progression of an autoimmune disorder. The method comprises administering to a mammal a compound that inhibits NK cell maturation, or T-cell development, in an amount effective to prevent the occurance of the autoimmue disorder, or to slow or halt the progression of said disease. Such compounds include, but are not limited to, compounds that act as antagonists of IL-15 or Il-15R .

[065] The present invention also relates to methods and compositions for preventing the occurance or progression of an inflammatory disorder. The method comprises administering to a mammal a compound that inhibits NK cell maturation, or T-cell development, in an amount effective to prevent the occurance of the autoimmue disorder, or to slow or halt the progression of said disease. Such compounds include, but are not limited to, antagonist of IL-15 or Il-15R. Such anti-inflamatory conditions include, but are not limited to arthritis, asthma and allergies.

[066] The present invention also relates to methods and compositions for preventing the occurance or progression of an immunodeficiency disorder. The method comprises administering to a mammal a compound that modulates NK cell maturation, or T-cell development, in an amount effective to prevent the occurance of

the immunodeficiency disorder, or to slow or halt the progression of said disease. Such compounds include, but are not limited to, agonist of IL-15 or Il-15R.

[067] Various delivery systems are known and can be used to administer a compound capable of modulating NK cell maturation or T-cell development, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432). Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

[068] In a specific embodiment, it may be desirable to administer the compositions of the invention locally to a specific area of the body; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

[069] The amount of the compound of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the

disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses maybe extrapolated from dose response curves derived from *in vitro* or animal model test systems. Additionally, the administration of the compound could be combined with other known efficacious drugs if the *in vitro* and *in vivo* studies indicate a synergistic or additive therapeutic effect when administered in combination.

[070] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention, optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

EXAMPLES

[071] The invention is illustrated herein by the experiments described above and by the following examples, which should not be construed as limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference. Those skilled in the art will understand that this invention may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein. Many modifications and other embodiments of the invention will come to mind in one

skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing description. Although specific terms are employed, they are used as in the art unless otherwise indicated.

6. EXAMPLE

[072] The following example demonstrates that IL-15 trans-presentation regulates human NK cell homestatis *in vivo*.

6.1 MATERIALS AND METHODS

[073] **Mice.** C57BL/6 mice were purchased from Harlan (France). $Rag2^{-/-}\gamma c^{-/-}$ mice (47) were backcrossed onto the Balb/c background and $Rag2^{-/-}\gamma c^{-/-}$ mice on a Nude background were maintained in isolators with autoclaved food and water. Mice with a human immune system (HIS) were generated as previously described (20, 21). Briefly, newborn (3 – 5 day old) $Rag2^{-/-}\gamma c^{-/-}$ mice received sub-lethal (3.3 Gy) total body irradiation from a Cs source, and were injected intra-hepatic (i.h.) with $1x10^5$ sorted CD34+CD38- or $5x10^5$ CD34+ human fetal liver cells. All manipulations of HIS mice were performed under laminar flow.

[074] ***In vivo* treatments.** HIS-mice were injected intra-peritoneally with 100ul of either human IL-15 (2.5μg), human IL-15Rα-Fc (7.5μg) + human IL-15 (2.5μg) both from R&D Systems (Minneapolis, MN), 2.5μg RLI(29) or PBS alone commencing at a minimum of 6 weeks after reconstitution. 100μl of 10mg/ml of BrdU from BrdU flow kit (BD Bioscience, CA) was injected IP daily 2 days prior to sacrificing mice. For NK cell transfer assays, 3 x 10^4 $CD56^{hi}CD16^-KIR^-$ or 2 x 10^5 $CD56^{lo}CD16^+KIR^-s$ NK cells were sorted from human $CD45^+CD3^-$ fetal splenocytes (15 weeks gestation) and transferred i.h into 1 week old sub-lethally irradiated Balb/c $Rag2^{-/-}\gamma c^{-/-}$ mice. A cocktail of KIR antibodies recognizing KIR-2DL2/3/1/-2DS1/2/4/-3DL1/S1 was used.

Recipients were then treated i.p with PBS or 2.5μg of RLI on day 0 and day 4 post-transfer. IL-15 *in vivo* neutralization was performed by i.p administration of 50μg of goat anti-human IL-15 or 25μg of goat anti-mouse IL-15 both from R&D Systems (Minneapolis, MN) every second day for 7 days.

[075] Flow cytometry analysis for cell-surface and intracellular markers. Cell suspensions were labeled with mAb against the following human cell-surface markers: CD3 (SK7), CD4 (SK3), CD34 (581), CD8 (SK1), CD19 (HIB19), CD10 (HI10a), CD38 (HB7), NKG2D (1D11), NKp46 (9E2), CD16 (3G8), CD161 (DX12), CD56 (B159), HLA-DR (L243), HLA-A/B/C (G46-2.6), CD117 (YB5.B8), IgD (IA6-2), IgM (G20-127), CD14 (M5E2), CD122 (Mik-ß3), TCR-α/ß (T10B9.1A-31), CD127 (hIL-7R-M21), CD11c (B-ly6), CD7 (M-T701), CD107a (1D4B), CD45 (2D1), CD69 (L78), Bcl-2 (Bcl-2/100), IFN-μ (XMG1.2), BrdU (B44), Ki67 (B56) from BD Bioscience (San Jose, CA), KIR2DL2/L3 (DX27), KIR3DL1 (DX9), CD94 (DX22), NKp44 (p44-8), CD27 (O323), CD62L (DREG-56), NKp30 (P30-15) from Biolegend (San Diego, CA), KIR2DS4 (FES172), KIR2DL1/DS1 (EB6B), KIR2DL2/L3/DS2 (GL183), KIR3DL1/DS1 (Z27.3.7), Bcl-xL (7B2.5), CD159a (NKG2A; Z199) from Beckman Coulter (Fullerton, CA), Granzyme-B (GB11) from CALTAG (Invitrogen, CA) and CD11b (ICRF44), CD25 (BC96), CD116 (4H1), CD83 (HB15e), anti-mouse NK1.1, CD11b, CD11c, F480 and DX5 from eBioscience (San Diego, CA). Intracellular staining was performed after fixation and permeabilization of the cellular suspensions using BD Perm/Wash and BD Cytofix/Cytoperm reagents from BD Bioscience (San Jose, CA) according to manufacturer instruction. For BrdU detection, cells were incubated for 1 h at 37°C with 30μg DNAse from BrdU flow kits (BD Bioscience, CA). All washings and reagent dilutions were done with PBS containing 2% fetal calf serum (FCS). All

acquisitions were performed using LSRII, Canto 1 or Canto 2 cytometers, cell sorting was performed using FACS ARIA, all machines were interfaced to the FACS-Diva software (BD Bioscience).

[076] Cell preparation. Human fetal material was obtained from elective abortions, with gestational age ranging from 14 to 20 weeks. The use of this tissue was approved by the Medical Ethical Committee of the AMC-UvA and was contingent on informed consent. Single cell suspensions of human fetal organs were achieved by mechanical disruption using a Stomacher® Biomaster lab system (Seward, Hadleigh, UK).

[077] Magnetic enrichment of $CD34^+$ cells (> 98% pure) was performed by using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech, Auburn, CA), after preparation of single-cell suspension and isolation of mononuclear cells by density gradient centrifugation over Ficoll-Hypaque (Nycomed Pharma, Roskilde, Denmark). Cell suspensions were prepared in RPMI medium with 2% fetal calf serum. Single cell suspensions of murine organs were prepared as previously described (24).

[078] ***In vitro*** **assays.** Human NK cells were purified from donor blood buffy coat prepared by density gradient centrifugation over Ficoll-Hypaque (Nycomed Pharma, Roskilde, Denmark) using anti-CD56 magnetic beads (Miltenyi Biotech, Auburn, CA). C57BL/6 splenic NK cells were purified by anti-DX5 magnetic beads (Miltenyi Biotech, Auburn, CA). Purified cells were loaded with 5 μM CFSE (Molecular Probes) and cultured at 2 x 10^5 cells/ml in RPMI with 10% FCS and 10ng/ml rhIL-15 (rhIL-15; R&D Systems, Minneapolis, MN) or 30ng/ml rmIL-15 (Peprotech) for 3 or 5 days. Alternatively, 1 x 104 CD56+ NK cells purified from human peripheral blood were labeled with CFSE and cultured for 72 hours in combinations of human or murine IL-15 which had been pre-incubated with human or murine IL-15Rα-Fc for 1

hour at 4°C. The final concentration of IL-15 and IL-15Rα-Fc were 5ng/ml and 20ng/ml respectively. In vitro re-stimulation of HIS-derived NK cells was performed using $CD56^+$ NK cells purified by magnetic beads (Miltenyi Biotech, Auburn, CA) from a cell suspension of spleen and bone marrow from 7 HIS-mice pooled together. NK cells were cultured at $2.5x10^5$ cells/ml in RPMI supplemented with 10% FCS, rhIL-15 (5 ng/ml) and either rhIL-12 (5 ng/ml) + rhIL-18 (20 ng/ml, R & D Systems) or $2.5x10^6$ K562 cells/ml (ATCC) for 18 hours with brefeldin A (Sigma) added for the last 4 hours of culture.

[079] Retroviral Bcl-xL expression. Human fetal liver cells were prepared and modified by retroviral transduction as follows. The Sorted $CD34^+CD38^-$ fetal liver cells were cultured overnight in IMDM (Invitrogen) supplemented with Yssel's medium, 5% normal human serum, 20ng/mL human stem cell factor, 20ng/mL human thrombopoietin and 20ng/mL human interleukin-7 (PeproTech, Rocky Hill, NJ). The following day, cells were incubated for 6 to 8 hours with control LZRS IRES-GFP or LZRS Bcl-xL-IRES-GFP virus supernatant in fibronectin-coated plates (30 μg/mL; Takara Biomedicals, Otsu, Shiga, Japan). The cell bulk was then inoculated i.h. to the newborn recipients. The cDNA sequence encoding Bcl-xL, initially provided by the laboratory of J.G. Collard (The Netherlands Cancer Institute, Amsterdam) was inserted into the multiple cloning sites of LZRS vector upstream of an internal ribosomal entry site and enhanced green fluorescent protein (GFP) (48). Control vectors were empty LZRS IRES. Retroviral supernatants were produced as described (49) using the 293T-based Phoenix packaging cell line (50).

6.2. RESULTS

[080] Human hematopoietic chimerism and NK cell development in HIS-mice. Human HSC engrafted into newborn Balb/c $Rag2^{-/-}\gamma c^{-/-}$ mice develop into mature myeloid and lymphoid cells (20, 21, 23). This approach was made to investigate human NK cell development *in vivo*. 8 - 12 weeks after HSC engraftment, HIS-mice displayed human hematopoietic chimerism in all organs analyzed, with human thymopoiesis, B lymphopoiesis and myelopoiesis evident (Fig. 1 A, B and Fig. 6). Using a NK cell specific antibody (anti-NKp46), human NK cells were identified in all lymphoid organs of HIS-mice although at low frequencies, typically between 0.3% to 1.5% human lymphocytes (Fig. 1 A). Both CD56hiCD16- and CD56loCD16+ NK cells subsets are present, with the majority of NK cells having the latter phenotype (Fig. 1 C). Some $CD16^{+}$ cells expressed CD56 at levels indistinguishable from non-NK cells. These $CD56^{lo}$ cells are NK cells as they express NKp46, NKG2D, CD94 and like their counterparts in man, express KIRs whereas $CD56^{hi}CD16^{-}$ cells are rarely $KIRs^{+}$ (Fig. 1 C). $NKp46^{+}$ cells present a phenotype consistent with peripheral NK cells in man including expression of CD122 (IL-2Rß), NKG2A, CD161, but not CD3 (Fig. 1 D). *In vivo* generated human NK express high levels of intracellular granzyme-B and IFN-α when stimulated with IL-12 and IL-18 *ex vivo*, and de-granulate when co-cultured with K562 human leukemia cells as determined by expression of CD107a (Fig. 1 E).

[081] IL-15 is a pleiotropic cytokine essential for murine NK cell development. Given the sparse numbers of human NK cells, it was hypothesized that IL-15 availability in this HIS model might be suboptimal. HIS-mice represent a hybrid human-murine system where cytokines receptors compatibilities between species may not exist. Specifically, while human IL-15 (hIL-15) induces survival and proliferation of murine NK cells (24), it is not clear whether the reverse is true. This is a critical

question as it is expected most of the IL-15 in HIS-mice to be murine-derived given the low human myeloid (20) or absent epithelial/stromal cell chimerism, (cells known to be sources of IL-15) (15, 25). It was found that human NK cells cultured *in vitro* with hIL-15 proliferated extensively (predominately the $CD56^{hi}CD16^{-}$ subset) which was in contrast to those cultured in murine IL-15 (mIL-15) which itself sufficiently induced proliferation of murine NK cells (Fig. 1 F and Fig. 7). Given mIL-15 is trans-presented *in vivo*, the effect of culturing human NK cells with low concentrations of IL-15 alone or when trans-presentated with IL-15 to IL-15Rα-Fc of the same or alternate species was investigated. Irrespective of which species of IL-15Rα was used, mIL-15 failed to induce human NK cell proliferation, although some cells survived when mIL-15 was combined with hIL-15Rα-Fc (Fig. 1 G). In contrast, hIL-15Rα was clearly superior in inducing NK cell proliferation compared to mIL-15Rα when combined with hIL-15 (Fig. 1 G). Similarly, activated human but not murine myeloid cells were able to induce human NK cell proliferation *in vitro* (Fig. 7), which is consistent with previous studies(26). Lastly, human NK cells are observed in athymic HIS-mice ($Rag2^{-/-}\gamma c^{-/-}FOXN1^{-/-}$) ruling out a major role for T cell-derived IL-2 driving NK cell development (Fig. 1 H).

[082] Taken together, these data indicate that the few resident NK cells in HIS-mice are dependent on the available human IL-15. Indeed, the number of human NK cells in the spleen of HIS-mice could be further reduced following treatment with neutralizing antibody against human not murine IL-15 (Fig. 7). Human IL-15 transcripts are found in the bone marrow of HIS-mice (27) as are $IL\text{-}15R\alpha^{+}$ cells known to express IL-15 and support NK cell development such members of the myeloid linage (15, 20) (and our unpublished observation). Given this last point, it is possible that enhancing the chimerism of human IL-15/IL-15Rα expressing cells will

improve human NK cell development in HIS-mice. This would also be important in the context of NK cell priming/activation/survival *in vivo* by IL-15Rα^{+} myeloid cells in response to pathogen exposure (26, 28).

[083] Given the likely dependence of human NK cells on hIL-15, HIS-mice were treated with the same concentration of hIL-15 alone or hIL-15 pre-incubated with hIL-15Rα-Fc to mimic IL-15 trans-presentation *in vivo*. Consistent with murine studies (16, 17), a significant increase in NK cell frequency was observed when hIL-15 was complexed to IL-15Rα-Fc but not when administered alone (Fig. 2 A). Increased NK lymphopoeisis by hIL-15^{+}IL-15R$^{\alpha}$-Fc compared to IL-15 alone was associated with increased Bcl-xL expression and cell proliferation as demonstrated by BrdU uptake and Ki-67 expression (Fig. 2 B). This data indicates that IL-15R-mediated human NK cells responses are more readily evoked *in vivo* when IL-15 is complexed to IL-15Rα-Fc.

[084] Two different IL-15R agonists were compared, both mimicking IL-15 trans-presentation, hIL-15+IL-15Rα-Fc and RLI (hIL-15 covalently linked to an hIL-15Rα extended sushi domain, but lacking any Fc fragment) (16, 17, 29). Four injections of 2.5MLg hIL-15R agonists (one per week) resulted in a significant increase in the number of NKp46^{+} cells in all organs (Fig. 2 C). Both these agonists markedly enhanced NK cell *in vivo* uptake of BrdU, expression of Bcl-xL, Ki-67 and CD69 and resulted in delayed apoptosis of NK cells when withdrawn from cytokines (Fig, 2 D, E) suggesting both proliferation and survival result from IL-15^{+}IL-15Rα binding to NK cells *in vivo*. Human CD8 T cells are known to proliferate in response to IL-15 *in vitro* (30) and were also significantly augmented in HIS-mice and had an obvious increase in BrdU uptake following treatment with RLI (Fig. 2 F). RLI that activates

both the IL-15Rß/α (trans-presentation) and IL-15Rα/ß/γ (cis-presentation) was consistently more effective *in vivo* than non-covalent association of hIL-15+hIL-15Rα-Fc that activates IL-15Rß/γ (trans-presentation) alone. This observation is in agreement with *in vitro* studies showing RLI functions more efficiently than the non-covalent associations of IL-15 + sushi domain or other soluble forms of IL-15R (29, 31). The evident effect of RLI compared to IL-15+IL-15Rα-Fc also indicates that the Fc protein does not contribute to the augmented NK lymphopoeisis and rules out any activation of human NK cells through CD16.

[085] While IL-15 is known to heighten NK cell cytotoxicity and induce proliferation, it also protects cells from apoptosis (the latter possible at lower concentrations) by suppressing pro-apoptotic Bim and elevating Bcl-2 family members such as Bcl-xL and Mcl-1 (32-35). A clear *in vivo* effect of RLI and IL-15+IL-15Rα-Fc was the up-regulation of Bcl-xL in NK cells (Fig. 2 B and E). It was next asked if enhanced survival could improve NK cell reconstitution in the limiting hIL-15 environment of HIS-mice. To address this, HSCs were infected with a bicistronic retrovirus encoding the pro-survival protein Bcl-xL and GFP (to detect infected cells) *in vitro* prior to engrafting newborn Balb/c $Rag2^{-/-}\gamma c^{-/-}$ mice. Ectopic expression of Bcl-xL in human HSCs resulted in a significant increase in thymocyte and splenocyte cellularity eight weeks after engraftment, with Bcl-xL transduced cells (GFP+) representing a greater proportion of $hCD45^{+}$ cells compared to control transduced cells in all organs (Fig. 3 A, B and C). $NKp46^{+}$ cells were significantly increased in thymus and spleen of Bcl-xL HIS-mice compared to control HIS-mice, however this appeared to be primarily a result of increased cellularity in these organs as the percentage of NK cells largely unchanged (Fig. 3 D and E). In addition, while a greater proportion of NK cells were GFP+ in Bcl-xL infected mice compared to

controls, no accumulation of Bcl-xL expressing NK cells was observed amongst the most mature subset ($CD56^{lo}CD16^{+}$). Lastly, the number of NK cells in HIS-mice engrafted with Bcl-xL infected HSCs in terms of absolute numbers and fold-difference compared to control were substantially lower than mice treated with trans-presented IL-15. These findings indicate that IL-15/IL-15Rα effects extend beyond providing survival signals in promoting human NK cell development *in vivo*.

[086] A consistent observation in all lymphoid organs following treatment with trans-presented IL-15 was the skewing of NK cell maturation towards the more differentiated $CD56^{lo}CD16^{+}$ phenotype. In particular, significant and specific increases in the frequency of $CD16^{+}$ NK cells and the ratio of $CD16^{+}$:$CD16^{-}$NK cells 7 days after the final treatment was observed (Fig. 4 A and B). This *in vivo* accumulation is consistent with model where $CD56^{lo}CD16^{+}$ NK cells represents the terminal stage of NK cell development (36) and suggests trans-presented IL-15 promotes this differentiation. CD16 expression is associated with human NK cell differentiation and increased cytotoxicity by means of increased intracellular effector granules and the ability to perform antibody dependent cell cytotoxicity. IL-15 itself is known to enhance NK cell cytotoxicity via up-regulating effector molecules such as IFN-α, perforin and granzymes at the level of transcription and/or translation and by increased surface expression of LFA-1 and IL-12Rß1 (37-39). Strikingly, amongst the enhanced $CD16^{+}$ population following IL-15 trans-presentation treatment, the percentage of NK cells expressing KIRs was also significantly elevated resulting in a large increase in the KIR^{+} NK cell pool, an effect again more prominent with RLI (Fig. 4 A). The fraction of KIR^{+} NK cells following exogenous IL-15 trans-presentation was typically highest in the thymus and elevated compared to normal frequencies in human blood (typically 50-60%). This may result from greater IL-15

concentrations in the model compared to humans, however the frequency of KIR^+ NK cells in human thymus has not been reported using all existing commercial antibodies to KIRs. In contrast to IL-15 trans-presentation, no enhanced NK cell differentiation and induction of KIR expression was observed in HIS-mice treated with IL-15 alone, nor those engrafted with Bcl-xL expressing HSCs (Fig. 4 C). Furthermore, the increase in KIR^+ NK cells did not represent an expansion of one $CD56^{lo}CD16^+KIR^+$ NK clone as $CD56^{lo}CD16^+$ NK cells expressing a combination of 1 to 5 different KIR members were present in this population (Fig. 4 D).

[087] It is unlikely the accumulation of $CD56^{lo}CD16^+$ NK cells does results from a preferential expansion of this subset in response to hIL-15 trans-presentation as both $CD56^{lo}CD16+$ and $CD56^{hi}CD16^-$ NK cell subsets had a similar level of incorporated BrdU during treatment (Fig. 5 A). While one cannot distinguish between $CD16^+$ NK cells that have incorporated BrdU and BrdU+ cells that have up-regulated CD16, it is most likely that the latter accounts for most of the $BrdU^+CD16^+$ NK cells as $CD56^{lo}CD16^+$ NK cells are refractory to IL-15 stimulation *in vitro* (40, 41), especially when compared to $CD56^{hi}CD16^-$ NK cells (Fig. 1 E). To test this hypothesis, highly purified $CD56^{hi}CD16^-KIR^-$s or $CD56^{lo}CD16^+KIR^-$sNK cells from fetal spleen into Balb/c $Rag2^{-/-}\gamma c^{-/-}$ in the presence or absence of RLI treatment were sorted and transferred. Although unable to recover the donor cells in mice receiving PBS alone, clear populations of donor cells were recovered from RLI treated mice 7 days after transfer (Fig. 5 B). Trans-presented IL-15 induced the differentiation of $CD56^{hi}CD16^-$ NK cells with between 34 – 40% of recovered NK cells becoming $CD16^+$ (Fig. 5 B). In addition a fraction of the $CD16^+$NK cells also now expressed KIRs. Similarly, while $CD56^{lo}CD16^+$NK cells retained this phenotype in the presence of RLI following transfer, expression of KIRs were acquired by a fraction of these $CD56^{lo}CD16^+$NK

cells that lacked KIR expression prior to transfer (Fig. 5 B). The absence of NK cells in non-RLI treated recipients clearly shows the available murine IL-15 is inadequate to support human NK cell survival and highlights the importance of a source of human IL-15 in HIS-mice in promoting NK cell differentiation.

[088] Binding of IL-15 to NK cells ultimately activates signaling pathways stemming from STAT5 phosphorylation such as activation of NF-αB, induction of cyclin D, down-regulation of pro-apoptotic proteins and up-regulation of Bcl-2 family members, cytolytic granules and the transcription factors Ets-1 and T-bet (34, 37, 42, 43). While it is clear how these signaling events promote cell division, survival and effector functions it is not known how these pathways regulate acquisition of KIRs. Coordination between granzyme/perforin induction and KIR expression would be one means to limit NK cell activity during differentiation and while most NK cells express other inhibitory receptors for MHC-I (CD94/NKG2A and LILR) throughout development, it appears acquisition of KIRs is a late event and likely unique compared to other forms of MHC-I-mediated inhibition. Taken together, this data provides the first *in vivo* demonstration that trans-presented IL-15 is essential for NK cell survival and subsequent differentiation and that KIR expression on mature NK cells is dynamically regulated by IL-15 signaling.

[089] Murine studies show that IL-15 functions as a membrane bound cytokine that can only be present at the cell surface and support NK cell development when bound to IL-15Rα on the same cell (14, 15). The clear effect of hIL-15Rα to enhance hIL-15 activity *in vivo* suggests this is also likely in man. The findings using a novel HIS-mouse approach demonstrate that hIL-15 trans-presentation is necessary to promote human NK cell development and differentiation *in vivo*. The failure to observe

accumulation of $CD16^{+}KIR^{+}$ NK cells in HIS-mice with HSCs ectopically expressing Bcl-xL suggests that NK cell survival is alone not sufficient to promote differentiation and a source of human IL-15 is essential for this process.

[090] The present study provides the first evidence that trans-presented IL-15 induces KIR expression on $CD56^{lo}CD16^{+}$ NK cells *in vivo* and suggests that KIRs are expressed after CD16 and that acquisition of KIRs represents a further step in NK cell differentiation. Interestingly, expression of human self-MHC-I on a fraction of the haematopoietic cells in HIS-mice appears sufficient in generating phenotypically mature KIR^{+} NK cells. The finding that this efficiently occurs *in vivo* in the absence of non-haematopoietic self MHC-I expression indicates that treatment of patients recovering from haematopoietic cell transfer (who have reduced KIR expression compared to healthy adults) (44, 45) with trans-presented IL-15 may improve clinical outcomes. Use of hIL-15R agonists such as RLI could also benefit mis-matched haematopoietic cell transfers, transporter associated with antigen processing (TAP) deficient patients (who have a reduced population of $CD56^{lo}CD16^{+}$ NK cells) (46) and cancer immuno-therapy. The efficiency of IL-15R agonists, particular RLI in augmenting human NK cell development in HIS-mice will enable us to more readily dissect the role of IL-15 dependent lymphocytes (NK cell, memory CD8 T cell, NK T cell and α/α T cell) responses to human pathogens and disease *in vivo*. Given obvious crosstalk between innate and adaptive immune cells, having robust reconstitution of IL-15-dependent cells *in vivo* improves the accuracy and application of HIS-mice for studying human immune responses. Taken together the results described herein define an essential role for IL-15 in human NK cell development *in vivo* and demonstrate the efficacy of IL-15/IL-15Rα complexes in promoting human NK cell homeostasis

suggesting human IL-15 is likely bound and trans-presented by IL-15Rα expressing cells in man.

7. EXAMPLE

[091] The following example demonstrates that IL-15 trans-presentation regulates human T cell (CD4 AND CD8 T cell) homestasis *in vivo*.

7.1 MATERIALS AND METHODS

[092] **Mice.** $Rag2^{-/-}\gamma_c^{-/-}$ mice on the Balb/c background were maintained in isolators with autoclaved food and water. Mice with a human immune system (HIS) were generated as previously described [69-71]. Briefly, newborn (3 – 5 day old) $Rag2^{-/-}\gamma_c^{-/-}$ mice received sub-lethal (3.3 Gy) total body irradiation from a Cs source, and were injected intra-hepatic (i.h.) with $5x10^4$ sorted $CD34^+CD38^-$ human fetal liver cells. All manipulations of HIS mice were performed under laminar flow. Mouse experiments were approved by an institutional committee at the Institut Pasteur and validated by the French Ministry of Agriculture.

[093] **In vivo treatments.** HIS mice were injected intra-peritoneally with 100µl of 2.5µg RLI or PBS alone commencing at a either 5-6 weeks after reconstitution or 8 weeks after reconstitution. 100µl of 10mg/ml of BrdU from BrdU flow kit (BD Bioscience, CA) was injected IP daily 2 days prior to analysis. HIS mice were immunized by intramuscular route (biceps femoris) using a 29G needle, three times on weeks 12, 14 and 16 with 100 µl of the HBV vaccine (Engerix-B, GlaxoSmithKline). These amounts correspond to 1/10 of the normal human dose. Negative controls received the same volume of PBS buffer. Two weeks after the last immunization, HIS mice were sacrificed for analysis.

[094] Flow cytometry analysis for cell-surface and intracellular markers. Cell suspensions were labeled with mAb against the following human cell-surface markers: CD3 (SK7), CD4 (SK3), CD34 (581), Vδ2 (B6), CD8 (SK1), CD19 (HIB19), NKp46 (9E2) CD38 (HB7), CD16 (3G8), CD45RO (UCLH1), CD56 (B159), CD122 (Mik-β3), TCR-α/β (T10B9.1A-31), CD127 (hIL-7R-M21), CD45 (2D1), CD69 (L78), Bcl-2 (Bcl-2/100), IFN-γ (XMG1.2), BrdU (B44) from BD Bioscience (San Jose, CA), KIR2DL2/L3 (DX27), KIR3DL1 (DX9), CD27 (O323), CD62L (DREG-56) from Biolegend (San Diego, CA), KIR2DS4 (FES172), KIR2DL1/DS1 (EB6B), KIR2DL2/L3/DS2 (GL183), KIR3DL1/DS1 (Z27.3.7), Vγ9 (IMMU 360), Bcl-xL (7B2.5) from Beckman Coulter (Fullerton, CA), Granzyme-B (GB11) from CALTAG (Invitrogen, CA) and CD25 (BC96), γ/δTCR (B1.1) and CD45RA (HI100) from eBioscience (San Diego, CA). Intracellular staining was performed after fixation and permeabilization of the cellular suspensions using BD Perm/Wash and BD Cytofix/Cytoperm reagents from BD Bioscience (San Jose, CA) according to manufacturer instruction. For BrdU detection, cells were incubated for 1 h at 37°C with 30μg DNAse from BrdU flow kits (BD Bioscience, CA). All washings and reagent dilutions were done with PBS containing 2% fetal calf serum (FCS). All acquisitions were performed using LSRII, Canto 1 or Canto 2 cytometers, cell sorting was perform using FACS ARIA, all machines were interfaced to the FACS-Diva software (BD Bioscience).

[095] Cell preparation. Human fetal liver was obtained from elective abortions, with gestational age ranging from 14 to 20 weeks. Experiments using human fetal liver cells were approved by the Medical and Ethical Committees at the Institut Pasteur and AMC-UvA and performed in full compliance with French law. Single cell suspensions of fetal material was achieved by mechanical disruption using a

Stomacher® Biomaster lab system (Seward, Hadleigh, UK). Magnetic enrichment of $CD34^+$ cells (> 98% pure) was performed by using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech, Auburn, CA), after preparation of single-cell suspension and isolation of mononuclear cells by density gradient centrifugation over Ficoll-Hypaque (Nycomed Pharma, Roskilde, Denmark). Cell suspensions were prepared in RPMI medium with 2% fetal calf serum. Single cell suspensions of murine organs were prepared as previously described [99].

[096] In vitro assays. Thymocyte populations from HIS mice treated with PBS or RLI were electronically sorted using FACSARIA (BD Bioscience) and were cultured at 1 x 10^4 cells/ml in RPMI with 10% FCS or either 10ng/ml rhIL-15 (rhIL-15; R&D Systems, Minneapolis, MN) or 10ng/ml rhIL-7 (Peprotech) for 30 hours. Viable cells were determined by PI exclusion using flow cytometry.

[097] TCR Vβγδ and CDR3 immunoscope analysis. 12 weeks after $CD34^+CD38^-$ HSC engraftment, HIS mice were killed and single cell suspensions of splenocytes were prepared. Red cells lysis was performed in 1 ml of red cell lysis buffer (Sigma) for 10 min. Splenocytes were washed, resuspended in 600 µl of RLT lysis buffer (Qiagen) and homogenized by passing through a 21-gauge needle several times using RNase free syringes. RNA was prepared using RNeasy mini kits (Qiagen) according to manufactures instructions. TCR Vβ immunoscope was performed as previously described [100]. Briefly, cDNA was prepared and real-time PCR performed by combining primers for the different Vβγδ chains (Vβ1-24, Vγ2-9 and Vδ1-8). Fluorescent products were separated on ABI-Prism 3730 DNA analyzer to determine CDR3 lengths. Analysis of five individual HIS-mice from each group containing greater than 30% human chimerism in the spleen was performed.

[098] Luminex® cytokine detection. Sera from HIS mice were prepared from total blood at time of sacrifice by centrifugation at 13,000 rpm for 10 minutes at 4 degrees. Cytokine human 25-plex panal Luminex® assays were performed according to manufactures instructions (Invitrogen).

[099] ELISA. The plasma harvested from HIS mice was screened by ELISA for the presence of antigen-specific and total antibodies. For this purpose, 96-well plates were coated either with Engerix B (glaxoSmithKline BV, Zeist) (10x diluted in PBS) or 10μg/ml goat anti–human IgM or 10 μg/ml goat anti–human IgG (Jackson ImmunoResearch Laboratories) in PBS for 1hr at 37°C or o/n at 4°C. After coating, the plates were washed in PBS with 0.5% Tween-20. A PBS solution containing 4% of milk was used as a blocking agent, before serial dilution of HIS mouse plasma or cell culture supernatants. ELISA was revealed with horseradish peroxidase–labeled goat anti–human IgM and IgG antibodies (Jackson ImmunoResearch Laboratories) followed by TMB substrate/stop solution (Biosource, Carlsbad, CA, USA).

7.2 RESULTS

[0100] Improved development of human CD4 and CD8 T cells in HIS mice receiving trans-presented human IL-15 results in improved humoral responses following immunization. It has been reported that human fetal liver HSC ($CD34^{+}CD38^{-}$) engrafted into newborn Balb/c $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice develop into mature myeloid and lymphoid cells [69-73]. This approach was used to investigate the effect of trans-presented human IL-15 on human T cell development *in vivo*, since it had previously been shown that murine IL-15 has poor activity on human NK cells [71]. 8 weeks after HSC engraftment, HIS mice were injected intra-peritoneally weekly for 4 weeks with 2.5μg of a potent human IL-15R agonist known as RLI consisting of human IL-

15 covalently linked to an extended human IL-15Rα 'sushi' domain [66, 74, 75] and therefore capable of mimicking IL-15 trans-presentation (Fig. 8A). Administration of hIL-15+IL-15Rα (RLI) results in a significant improvement in the number of human hematopoetic cells in the thymus and the spleen of recipient mice (Fig. 8B). $CD8^+$ T cells were significantly augmented in the spleen ($P = 0.025$) and thymus ($P = 0.033$) following IL-15 trans-presentation in humanized mice whereas $CD4^+$ T cells where also augmented in spleen ($P = 0.017$) and lymph node ($P = 0.025$) following IL-15 treatment. Thus the increase in total human hematopoetic cells in the thymus ($P = 0.0005$) and spleen ($P = 0.002$) following IL-15 treatment is attributed to significant increases in total T cell numbers (Fig. 8B) in addition the increase in NK cells previously reported [71], whereas B cell numbers remain constant (Fig. 8C).

[0101] To assess the impact of improved human T cell homeostasis in IL-15 'boosted' HIS mice, cohorts of HIS mice were immunized with the commercially available HBV vaccine. While overall serum IgM levels were not appreciably modified following vaccination (Fig. 8D), a slight increase in IgG levels was found in HBV-vaccinated mice compared to non-vaccinated HIS mice. In contrast, HBV immunization resulted in marked increases in IgG levels in HIS mice that had received trans-presented human IL-15 (Fig. 8D). Thus, IL-15 'boosting' resulted in improved humoral responses that correlated with improved human T cell homeostasis.

[0102] Characterization of human $CD4^+$ and $CD8^+$ T cells in HIS mice receiving trans-presented human IL-15. Since IL-15 was effective in increasing both $CD4^+$ and $CD8^+$ T cells, it was investigated whether any phenotypic changes or signs of toxicity such as overt activation were induced on expanded T cell population **(Fig. 9A)**. No differences in classic T cell markers or any change in the proportion of naïve

($CD62L^+CD45RA^+$) $CD4^+$ and $CD8^+$ T cells in the periphery following the 4 week IL-15 treatment regime was observed **(Fig. 9B)**. With respect to T cell activation, an occasional increase in the $CD45RO^+CD45RA^-$ population was observed within the splenic $CD8^+$ T cell pool but not in other lymphoid organs nor amongst $CD4^+$ T cells. In line with this more splenic $CD8^+$ T cells expressing the activation marker CD69 were observed. The expression of receptors for γ_c cytokines were also unchanged following treatment including CD122, CD127 and CD25 as were the populations of regulatory T cells ($CD25^+CD4^+CD127^-$) **(Fig 9B)**.

[0103] It was previously shown that trans-presented IL-15 induces the expression of killer-Ig-like inhibitory receptors for MHC-I (KIRs) on NK cells *in vivo* [71]. Interesting, a similar phenomenon was observed on a subset of human T cells following staining with a pool of KIR antibodies recognizing KIR2DL2/3/1, KIR2DS1/2/4 and KIR3DL1/S1 **(Fig 9C)**. A significant increase was observed in the percentage and total number of KIR^+ T cells, which included an expansion of T cells expressing either one or both of the KIR2DL2/3 and KIR3DL1 receptors. Other NK-associated cell surface markers such as CD16 and CD161, but not CD56 were also induced or up-regulated on T cells from all organs analyzed from IL-15 treated mice **(Fig 9C)**.

[0104] HIS mice receiving trans-presented human IL-15 show increased proliferation of peripheral human CD4 and CD8 T cells. The ratio of $CD8^+$ to $CD4^+$ T cells following IL-15 treatment was only mildly increased in the spleen and unchanged in other lymphoid organs of recipient mice **(Fig 9B)**. Furthermore, the clear augmentation in T cell numbers but no diminution in the relative size of the naive T cell pool compared to control mice indicates that IL-15 trans-presentation *in*

vivo targets the expansion of both naïve and activated T cells. To test this, humanized mice treated with RLI were injected IP with 10μg of BrdU 24 and 12 hours before being sacrificed and T cell populations analysed for BrdU incorporation. Both $CD8^+$ and $CD4^+$ naïve ($CD45RA^+$) and activated ($CD45RA^-$) T cell proliferation in the spleen and mesenteric lymph node were significantly increased following IL-15 trans-presentation *in vivo* **(Fig 10A, B)**. $CD8^+$ T cells, in particularly those with a naïve phenotype showed the greatest fold increase in proliferation in both the spleen and the mesenteric lymph node after IL-15 treatment followed by naïve $CD4^+$ T cells, activated $CD8^+$ T cells and lastly activated $CD4^+$ T cells **(Fig 10 A, B)**. The efficiency of IL-15 treatment and relative responsiveness of T cell populations can be compared to that of NK cells who are highly dependent on IL-15 **(Fig 10 A, B)**. Other γ_c cytokines such as IL-2 and IL-7 could also influence the observed increase in human T cells by inducing proliferation and enhancing survival. The serum concentration of these and other cytokines that are produced by IL-15 responsive lymphocytes such as NK cells was not analysed. The serum concentration of IL-2 and IL-7 were unchanged in mice treated with trans-presented IL-15 and were only slightly above the level of detection using Luminex bead array assays **(Fig 10 C)**, with the IL-2 result being confirmed by intracellular staining of T cells from these mice showing no difference between treatment groups. Noteworthy, large increases in NK cell-derived cytokines in hIL-15+IL-15Rα treated mice were not observed, suggesting the large increase in NK cells following this treatment regime is unlikely to contribute to toxicity via hyper-production of pro-inflammatory cytokines **(Fig 10 C)**.

[0105] Effects of trans-presented human IL-15 on human thymopoiesis in HIS mice. The increase in total thymocytes far outnumbered the contribution from the increase in single positive $CD4^+$ (SP4) and $CD8^+$ (SP8) T cells **(Fig 8 B)** so the IL-15

effect on the $CD3^-$ thymocyte populations was not examined. Both double positive (DP) and double negative (DN) (with NK cells being excluded), but not immature single positive CD4 (iSP4) thymocytes were significantly increased following IL-15 treatment (**$P < 0.02$; **Fig 11 A)**. Thymocytes subsets were grouped according to the phenotype in **(Fig 11 B)**. Thymocyte turnover was analysed in the same manner as previously mentioned by injecting mice with 10μg of BrdU 24 and 12 hours before being sacrificed. Interestingly, while thymic NK cell turnover is doubled following IL-15 treatment *in vivo*, SP4 and SP8 T cell turnover is unchanged, which is in sharp contrast to what was observe in the periphery **(Fig 11 B)**. The increase in DN and DP thymocytes likely arises from the significant increase (*$P < 0.05$) in proliferation of these two thymocyte populations in IL-15 treated mice **(Fig 11 B)**. While both the number and turnover of iSP4 thymocytes were always increased in IL-15 treated mice, this increase was not statistically significant. Of all the thymocyte populations, iSP4 thymocytes presented the highest rate of proliferation with around 75% of these cells dividing within 24 hours, thus if IL-15 is capable of enhancing the division of this population it is less likely to be observed given the high baseline turnover. As this is the first report that trans-presented IL-15 is functional on human DN and DP thymocytes, other parameters were next examined such as cell survival. Intracellular staining for pro-survival proteins revealed enhanced expression of Bcl-xL in DN and SP4 thymocytes of IL-15 treated mice but not in DP, CD8 or iSP4 thymocytes, whereas Bcl-2 protein levels were unchanged in each population following IL-15 treatment **(Fig 11 C)**. Futhermore, it is clearly evident that Bcl-xL expression is up-regulated during human thymocyte maturation in HIS mice, Bcl-xL being most highly expressed in DP thymocytes before being down-regulated in positively selected SP4 and SP8 T cells.

[0106] Interestingly, IL-15 offered very little survival advantage to purified DN thymocytes compared to media or IL-7, whereas both SP4 and SP8 thymocyte populations displayed a clear increased survival when cultured in IL-15 for 30 hours **(Fig 11 D)**. DP thymocytes however only displayed improved survival when cultured in IL-7, but not IL-15 **(Fig 11D)**. In light of this *in vitro* finding, an improved ex vivo survival of thymocyte populations sorted from IL-15 treated humanized mice compared to those sorted from control mice was not observed **(Fig 11 E)** indicating that increased proliferation as opposed to improved survival is the major contributing factor for the increased number of immature thymocytes following this treatment regime.

[0107] TCR repertoires in HIS mice receiving trans-presented human IL-15. The maintenance of a highly diverse T cell receptor repertoire (TCR) is essential for efficient immune responses against the extensive number of foreign antigens encountered during our lifetime. We next examined the effect of IL-15 treatment on the human TCR repertoire by performing immunoscope profiling on the β, γ and δ variable chains of the TCR expressed by splenic and thymic T cells. This analysis is important as it is plausible that the T cell expansion we observed following IL-15 treatment arises from clonal proliferation of a relatively restricted population of IL-15 responsiveness T cells such as NKT (Vα24/Vβ11) or γ/δ TCR^+ T cells which in turn could compromise the homeostasis of α/β T cells reducing overall T cell diversity.

[0108] The peripheral T cell pool generated in our model of humanized mice appears extremely diverse especially considering the limited number of peripheral T cells that are generated in this model. Indeed, T cells bearing TCRs from all Vβ family members with numerous CDR3 lengths used amongst each family member were

detected **(Fig 12 A, B)**. This is a valuable piece of data considering that the model of humanized mice is becoming more popular for the study of T cell immune responses and indicates at least that an extensive TCR repertoire exists and could react against a large number of peptide antigens. A similar diversity was observed in the CDR3 length amongst all Vβ chains used except for the occasional over-representation of clones within the Vβ11 and Vβ7. This was not consistent observed in all mice following IL-15 treatment and was far less evident amongst thymic T cells from the same mice **(Fig 12 A, B)**.

[0109] The degree of γ/δ TCR^+ T cell development in humanized mice has not been characterized. Using commercially available antibodies a clear population of $V\gamma9^+V\delta2^+$ T cells in the thymus and LN of humanized mice was observed and these γ/δ T cells were largely restricted to the $CD161^+CD3^+$ T cell subset **(Fig 13 A)**. In the thymus, a clear $V\gamma9^-V\delta2^+$ and a minor $V\gamma9^+V\delta2^-$ T cell population was detected that appeared absent or at least diminished in LN. Following IL-15 treatment, it became obvious that the γ/δ T cell population was increased in frequency and number ($P < 0.05$; **Fig 13 B)**, however this was inline with the general augmentation in total T cells and was much less impressive than the increase we previously reported for NK cells using a identical treatment regime [71] and indicates in the HIS model, γ/δ T cells are not more responsive to IL-15 than α/β T cells.

[0110] CDR3 immunoscope analysis of γ and δ variable chain genes was next used to identify all γ and δ usage in HIS mice (since monoclonal antibodies against each unique γ and δ are not commercially available). These type of analyses on spleen-derived human T cells were used as they are more numerous than those derived from LN in the HIS model. Consistent with the flow cytometry data, most human γ/δ T

cells found in the periphery of HIS mice use Vδ2 and this is similar to human PBMCs and unaffected by IL-15 treatment **(Fig 13 C)**. Interestingly, a very small population of $V\gamma5^+$ T cells and two large populations of $V\gamma8^+$ and $V\gamma9^+$ T cells were observed which each represent between 40 – 60% of total γ/δ T cells and who obviously pair almost exclusively with Vδ2, although occasional usage of Vδ3, 5 and 8 was observed **(Fig 13 C)**. Interestingly, significantly greater use of Vγ8 TCR in humanized mice was observed compared to what was detected in healthy human PBMC, although the significance of this in terms of T cell development or potential immune response is not evident **(Fig 13 C)**. *In vivo* administration of IL-15 did not influence the ratio of the different γ and δ chain usage nor the variability in CDR3 length for any given Vγ or Vδ chains. Thus the modest increase in γ/δ TCR^+ T cells following IL-15 treatment likely results from an increase in all γ/δ TCR^+ T cells subsets generated **(Fig 13 C)**.

[0111] Trans-presented human IL-15 accelerates T cell development in HIS mice. Since trans-presented IL-15 was extremely efficient in increasing total human T cell numbers in HIS mice and displayed a clear effect in the thymus, it was next determined whether IL-15 immunotherapy could promote early thymopoeisis, before mature T cells are generated, thus accelerating mature T cell production. In order to perform this study, the thymus and spleen from HIS mice were analysed at various ages and it was found that between 5 – 6 weeks after HSC engraftment represented the ideal window to commence the IL-15 immunotherapy as the thymus was seeded with immature thymocytes including DPs, DNs and iSP4s and almost no mature T cells, the latter also being the case for the spleen **(Fig 14 A)**. These HIS mice were then treated with a more intense IL-15 treatment regime that entailed 2.5μg of IL-15R agonist, IL-15+IL-15Rα (RLI) every three days (day 0, 3, 6 and 9) and HIS mice

were then sacrificed and analyzed on day 12. Thymus, spleen and BM from IL-15 immunotherapy HIS mice and PBS controls were then analyzed for human chimerism ($hCD45^+$) and mature T cell development ($hCD45^+CD3^+$). IL-15 trans-presentation resulted in significant increase in human T cells in all organs analysed, promoting both SP4 and SP8 development in the thymus resulting in a significantly increased pool of naïve mature T cells ($hCD45^+CD45RA^+CD3^+$; **Fig 14 B)**. Furthermore, this effect could be monitored over time in the blood of HIS mice receiving IL-15+IL-15Rα where the we detected almost a 10-fold increase in human T cells in the blood after the second injection (day 4) and increasing up to 20-fold more T cells at the end of the treatment regime **(Fig 14 C)**.

[0112] The experiments described herein demonstrate that the homeostasis of both $CD4^+$ and $CD8^+$ human peripheral T cells and some immature human thymocyte populations is markedly improved in HIS mice when human IL-15 is trans-presented (IL-15/IL-15Rα). The findings differ from a similar murine study reporting increased $CD8^+$ T cell numbers following treatment with trans-presented mouse IL-15 [74, 76]. The results further underscore the fact that human and murine immune systems are not identical, and highlight the value of the HIS model to study human immunology *in vivo*. During the past 30 years HIS mice models have been continually improved and are now at a stage where immune responses can be elicited by the engrafted human lymphoid system (reviewed in [77]). Indeed both antibody and cellular immune responses against vaccines such as tetanus toxoid and viruses such as Epstein Barr virus have been detected in various HIS models [78-80]. Given that the immune system in HIS mice is heavily skewed towards B cell development (with around 90% of peripheral blood or splenic human hematopoietic cells being B cells), there is a clear need to improve T lymphopoeisis in these models [69, 70]. It has been recently

reported that a beneficial effect is observed on human thymopoeisis following human IL-7 treatment in the HIS model, however peripheral T cell numbers remained unchanged despite transient increases in thymocytes suggesting that other factors are involved [73]. The beneficial effect of human 'trans-presented' IL-15/IL-15Ra resulted in increased T cell numbers that also impacted on the ability to generate humoral immune responses with increased immunoglobulin levels following immunization. These results further enlarge the spectrum of potential applications of the HIS model for human vaccine testing.

[0113] The observation that both naïve and activated phenotype $CD4^+$ T cells proliferate in response to IL-15 receptor ligation *in vivo* differs from *in vitro* studies where only memory but not naïve $CD4^+$ T cells respond to IL-15 [81]. Moreover, this *in vitro* study observed that IL-15 promoted the generation of effector memory phenotype $CD4^+$ T cells from central memory phenotype $CD4^+$ T cells. While impact of trans-presented IL-15 on non-naïve $CD4^+$ T cells was not dissected in the study, the collective data suggests that *in vivo* IL-15 could assist in the generation or maintenance of both antigen-dependent and –independent memory CD4 T cells. *In vitro* studies also suggested that IL-15 can skew activated T cells towards a Th1 phenotype as determined by an increased IFN-γ/IL-4 protein ratio. Tehre was a failure to detect any difference in serum or intracellular IFN-γ levels in $CD4^+$ and $CD8^+$ T cells of IL-15 treated mice compared to controls. Furthermore, intracellular granzyme-B levels in $CD4^+$ and $CD8^+$ T cells were also unchanged.

[0114] Several *in vitro* reports suggest that IL-15 induces an NK-like phenotype (with CD56 expression) on thymocytes or preferentially expands the existing $CD56^+$ T cell pool [82-84]. It was shown that $CD56^-$ T cells from PBMC do not proliferate to IL-15,

whereas CD8αα and $V\alpha24^{+}V\beta11^{+}$ robustly proliferated to IL-15 *in vitro* [82]. We failed to detect CD56 expression on T cells in HIS mice following treatment with trans-presented human IL-15 treatment. In addition, γ/δ T cells were only increased to a similar level as α/β T cells, which is in contrast to data suggesting that human γ/δ T cells are more responsive on IL-15 *in vitro*. It is possible that other factors (such as the selecting ligands for human γ/δ T cells) are limiting in HIS mice. Indeed, a recent report using IL-15 supplementation in Macaques demonstrated a rare but severe preferential expansion of $CD4^{-}CD8^{-}$ γ/δ T cells that comprised 70% of the T cell compartment in the blood of one subject [85]. IL-15 treated Macaques displayed increases in CD8 T cells, NK cells and to a lesser extent CD4 T cells that was associated with increased turnover *in vivo*. Still, IL-15 toxicity in these macaques was also associated with hypocellularity, decline in hemoglobin, weight loss, low-grade fever and non-specific dermatitis, that was avoided when IL-15 was given intermittently [85]. In contrast, we failed to detect any obvious signs of IL-15 toxicity in HIS mice using our treatment regime.

[0115] It was previously reported that a likely reason for the poor human NK cell development in HIS mice was a result of the poor responsiveness of human NK cells to murine IL-15 [71]. Indeed, exogenous trans-presented human IL-15/IL-15Rα (RLI) induced a large increase in human NK cells in HIS mice [71]. It is speculated that a similar mechanism may explain the improved T cell homeostasis that is observed. It is possible that trans-presented IL-15 together with signals delivered through peptide/MHC complexes may be critically involved in maintaining human T cell homeostasis *in vivo*. An additional factor may result from the expansion and potential activation of NK cells and T cell following trans-presented IL-15 treatment that

induces production of other cytokines and growth factor which provide bystander effects. IL-2 is a likely candidate as it is produced by antigen activated T cells and promotes T cell division. There was a failure to detect any difference in the serum level of IL-2 between treated and control mice, nor in the intracellular concentration of IL-2 in T cells from both groups of mice. While human IL-15 is functional on mouse hematopoeitic cells, HIS mice lack their endogenous γ_c receptor, thus all of the observed effects involve IL-15 receptor triggering on human cells.

[0116] The ability of trans-presented IL-15 to promote human thymopoeisis offers a unique new therapeutic approach to augment human T cell numbers during immunotherapy to cancer or to reduce the period of immunodeficiency following radiotherapy/chemotherapy and HSC transplant by promoting NK and T lymphopoeisis. It has been previously shown that trans-presented IL-15 (RLI) is effective in an NK cell-dependent murine metastatic melanoma and colorectal cancer model [86], while others have reported similar effects with $CD8^+$ T cell-dependent systems [87, 88]. Furthermore, IL-15 has been shown to induce antigen independent expansion of naïve, virus specific and tumor specific cytotoxic $CD8^+$ T cells *in vitro* [74, 89-92] and rescue tolerant $CD8^+$ T cells for use in adoptive immunotherapy of established tumors [93]. IL-15 has been shown to be more potent than IL-2 in inhibiting apoptosis of memory $CD4^+$ T cells *in vitro* as well as of naïve phenotype $CD4^+$ T cells from HIV infected individuals in both examples by up-regulating Bcl-2 [94-96]. IL-15 has also been shown to enhance number, function and survival of HIV-specific $CD8^+$ T cells [97,98] indicating that trans-presented IL-15 treatment could improve the clinical condition of diverse disease states by not only boosting T cell numbers but by augmenting antigen specific T cell responses. The findings using human immune system mice demonstrates that human IL-15 trans-presentation is

effective in promoting human T cell development and homeostasis *in vivo* and in the absence of any obvious toxicity. IL-15 boosted HIS mice provide an improved humanized mouse model for evaluating pre-clinical immunotherapies and for deriving and screening novel immunomodulatory drugs.

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What is claimed:

1. A transgenic mouse, characterized in that it has (i) a phenotye comprising a deficiency for murine T lymphocytes, B lymphocytes and NK cells; (ii) is engrafted with human hematopoietic cells; and (iii) contains innate lymphocytes such as mature human natural killer (NK), cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes.

2. The transgenic mouse of claim 1, wherein the innate human lymphocytes in general and NK cells in particular and the maturation of NK cells as well as the human adaptive CD4 and CD8 T lymphocytes are induced through administration of an IL-15 or IL-15Rα agonist.

3. The transgenic mouse of claim 2, wherein said IL-15 or IL-15Rα agonist is an IL-15/IL-15Rα complex.

4. The transgenic mouse of claim 1 further comprising a phenotype comprising a deficienccy for murine MHC class I and/or MHC class II molecules and transgenic for the expression of human HLA class I and/or HLA class II molecules.

5. The transgenic mouse of claim 1, wherein the mouse is $Rag2^{-/-}\gamma c^{-/-}$ transgenic mice.

6. The transgenic mouse of claim 1 wherein the human hematopoietic cells are derived from cord blood or fetal liver.

7. A method for increasing the number of innate lymphocytes such as mature human natural killer (NK), cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in a subject, comprising the administration of an IL-15 agonist.

8. A method for increasing the number of innate lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in a subject, comprising the administration of an IL-15R agonist.

9. A method for increasing the number of innate lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in a subject, comprising the administration of an IL-15/IL-15Rα complex.

10. The method of claim 7 to 9, wherein the subject is a transplant patient.

11. The method of 7 to 9, wherein the subject is a cancer patient.

12. The method of claim 7 to 9, wherein the subject is infected with a pathogen.

13. The method of claim 7 to 9, wherein the subject is an immunodeficient patient.

14. The method of claim 7 to 9, wherein the mature NK cells are CD16+Kir+.

15. A method for identifying a compound that induces maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes, comprising

(i) contacting a transgenic mouse characterized in that it has

(a) a phenotye comprising a deficiency for murine T lymphocytes, B lymphocytes and NK cells;

(b) is engrafted with human hematopoietic cells

with a test compound and

(ii) measuring the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes; wherein an increased in the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in the presence of the test compound indicates that the test compound induces cell maturation.

16. The method of claim 15, wherein the compound is tested in the presence of an IL-15/IL-15Rα complex.

17. A method for identifying a compound that inhibits maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes comprising

(i) contacting a transgenic mouse characterized in that it has

(a) a phenotye comprising a deficiency for murine T lymphocytes, B lymphocytes and NK cells;

(b) is engrafted with human hematopoietic cells

with a test compound, in the presence of an IL-15/IL-15Rα complex and measuring the level of NK cell maturation;

(ii) in a separate experiment, contacting a transgenic mouse of the invention with an IL-15/IL-15Rα complex and measuring the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes, where the conditions are essentially the same as in part (i) and then

(iii) comparing the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes measured in part (i) with the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in part (ii),

wherein a decrease level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in (i) compared to (ii) indicates that the test compound is an inhibitor of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes.

18. A method for identifying a compound that increases IL-15 expression or activity, *i.e.*, an agonist, thereby inducing maturation of innate human lymphocytes

such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes, comprising (i) contacting a transgenic mouse of the invention with a test compound in the presence of IL-15 and (ii) measuring the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes; wherein an increased in the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in the presence of the test compound indicates that the test compound increases IL-15 expression or activity.

19. A method for identifying a compound that increases IL-15R expression or activity, *i.e.*, an agonist, thereby inducing maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes, comprising (i) contacting a transgenic mouse of the invention with a test compound and (ii) measuring the level of maturation of innate human lymphocytes such as mature human natural killer (NK cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes; wherein an increased in the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in the presence of the test compound indicates that the test compound increases IL-15R expression or activity.

20. A method for identifying a compound that inhibits IL-15 or IL-15R expression or activity, *i.e.*, an antagonist, thereby inhibiting maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes comprising (i) contacting a transgenic mouse of the invention with a test compound, in the presence of an IL-15/IL-15Rα complex and measuring the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes; (ii) in a separate experiment, contacting a transgenic mouse of the invention with an IL-15/IL-15Rα complex and measuring the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes, where the conditions are essentially the same as in part (i) and then (iii) comparing the level of

maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes measured in part (i) with the level of maturation of innate human lymphocytes such as mature human natural killer (NK) cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in part (ii), wherein a decrease level of maturation of innate human lymphocytes such as mature human natural killer (NK cells γδ T cells, and NK-T cells as well as adaptive CD4 and CD8 T lymphocytes in (i) compared to (ii) indicates that the test compound is an inhibitor of IL-15 of IL-15R expression or activity.

21. A method for preparing humanized antibodies, or fragments thereof, comprising (i) immunizing the transgenic mouse of claim 1 with an antigen of interest; and (ii) producing humanized antibodies, or fragments thereof, from the serum or B lymphocytes of said transgenic mice.

22. The method of claim 17 wherein the engrafted transgenic mouse is stimulated with IL-15 or an IL-15 agonist such as an IL-15/IL-15Rα complex prior to immunization.

23. An antibody, or fragment thereof, produced using the method of claim 21.

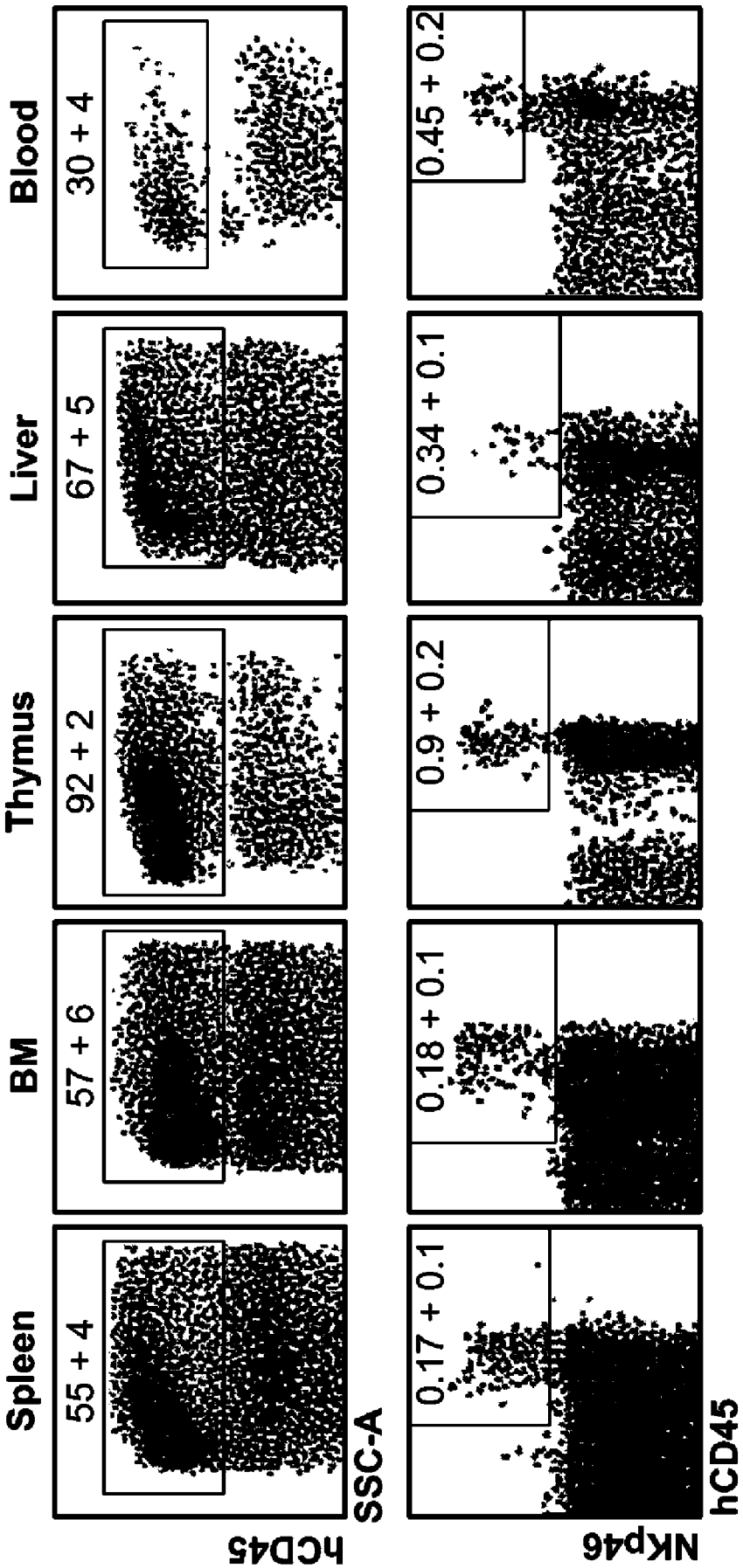

Spleen
BM
Thymus
Liver
Blood
55 + 4
57 + 6
92 + 2
67 + 5
30 + 4
0.17 + 0.1
0.18 + 0.1
0.9 + 0.2
0.34 + 0.1
0.45 + 0.2
SSC-A
hCD45
NKp46
hCD45

FIG. 1A

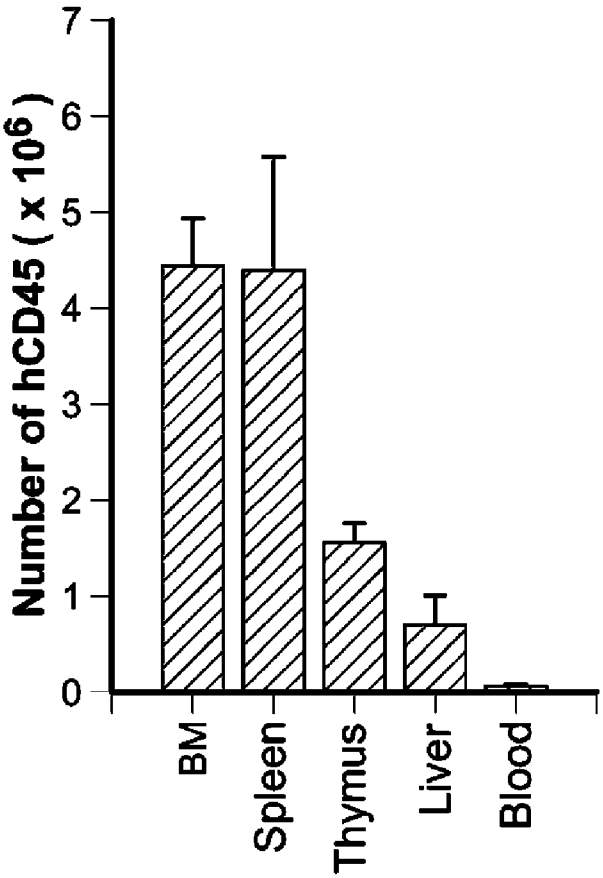

Number of hCD45 (x 10^6)
7
6
5
4
3
2
1
0
BM
Spleen
Thymus
Liver
Blood

FIG. 1B

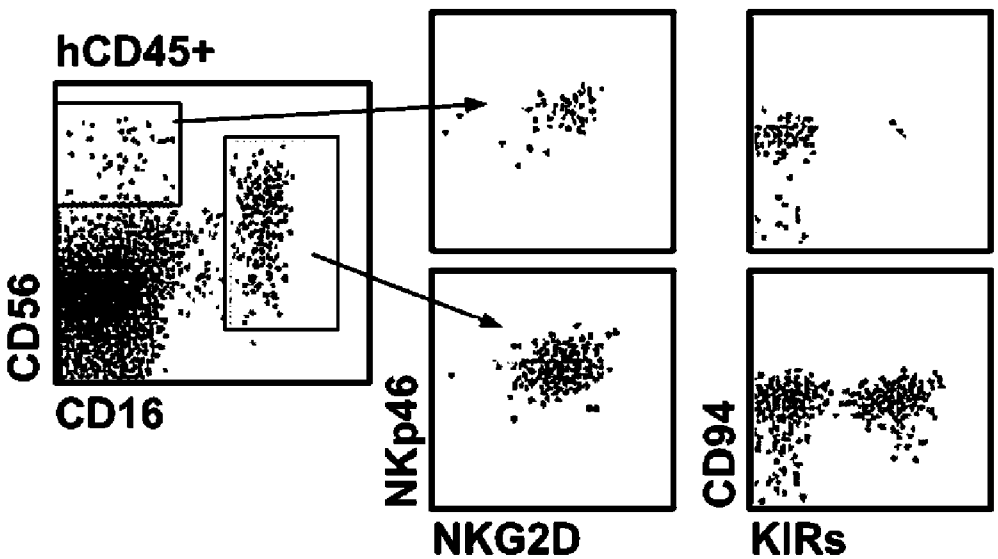

hCD45+
CD56
CD16
NKp46
NKG2D
CD94
KIRs

FIG. 1C

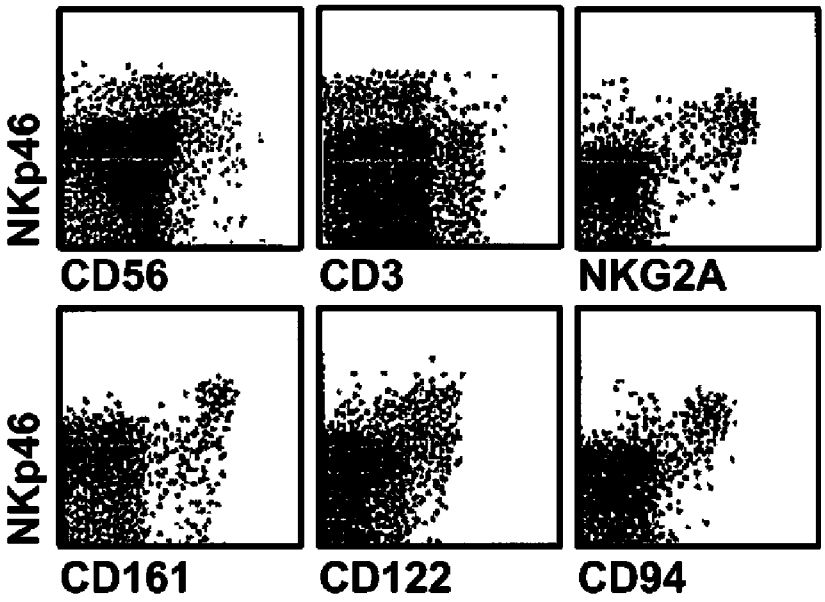


FIG. 1D

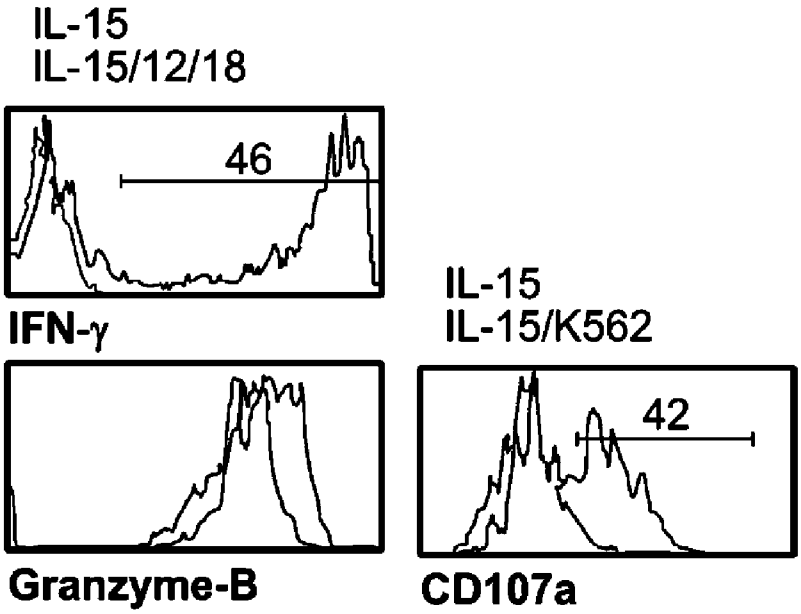


FIG. 1E

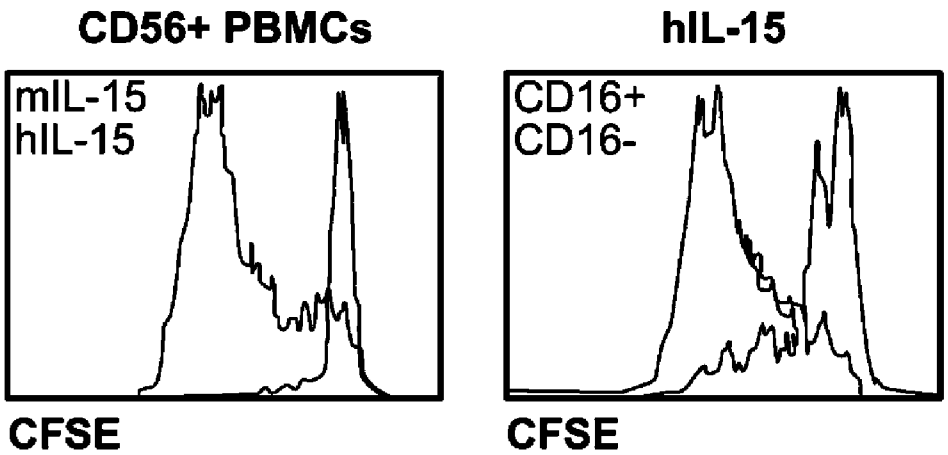


FIG. 1F

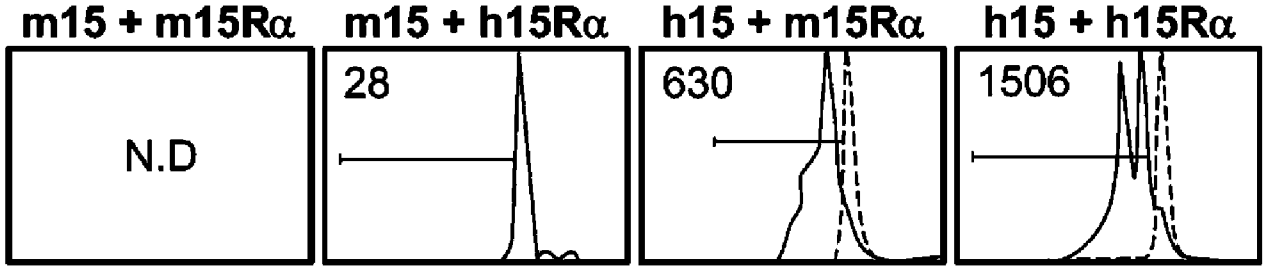


FIG. 1G

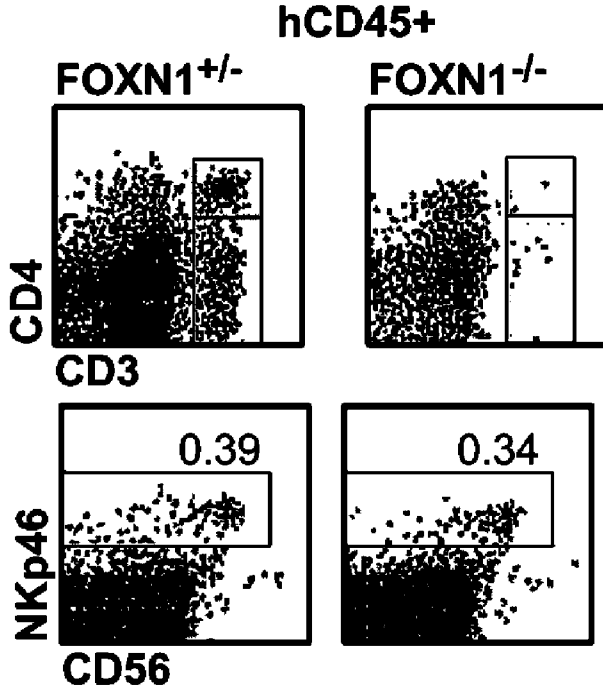


FIG. 1H

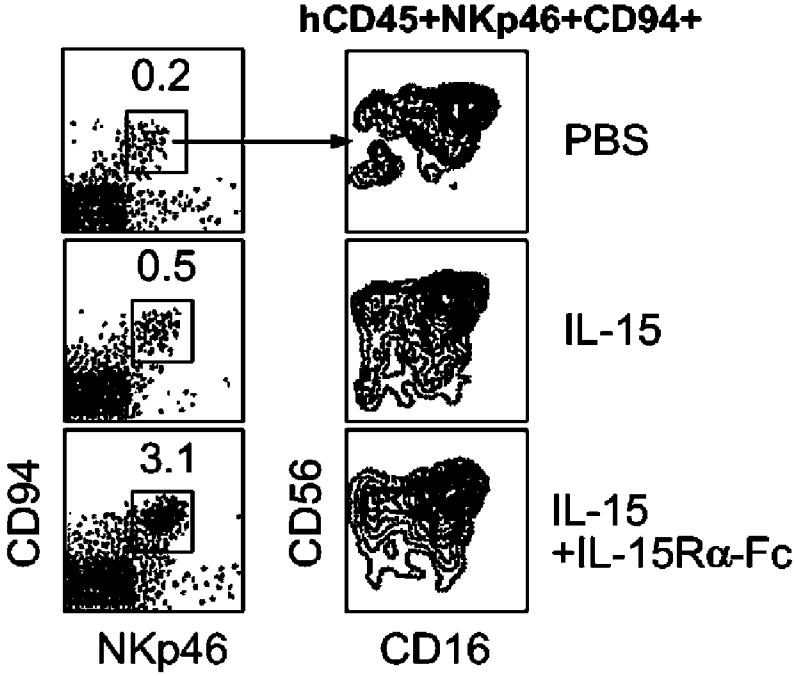


FIG. 2A

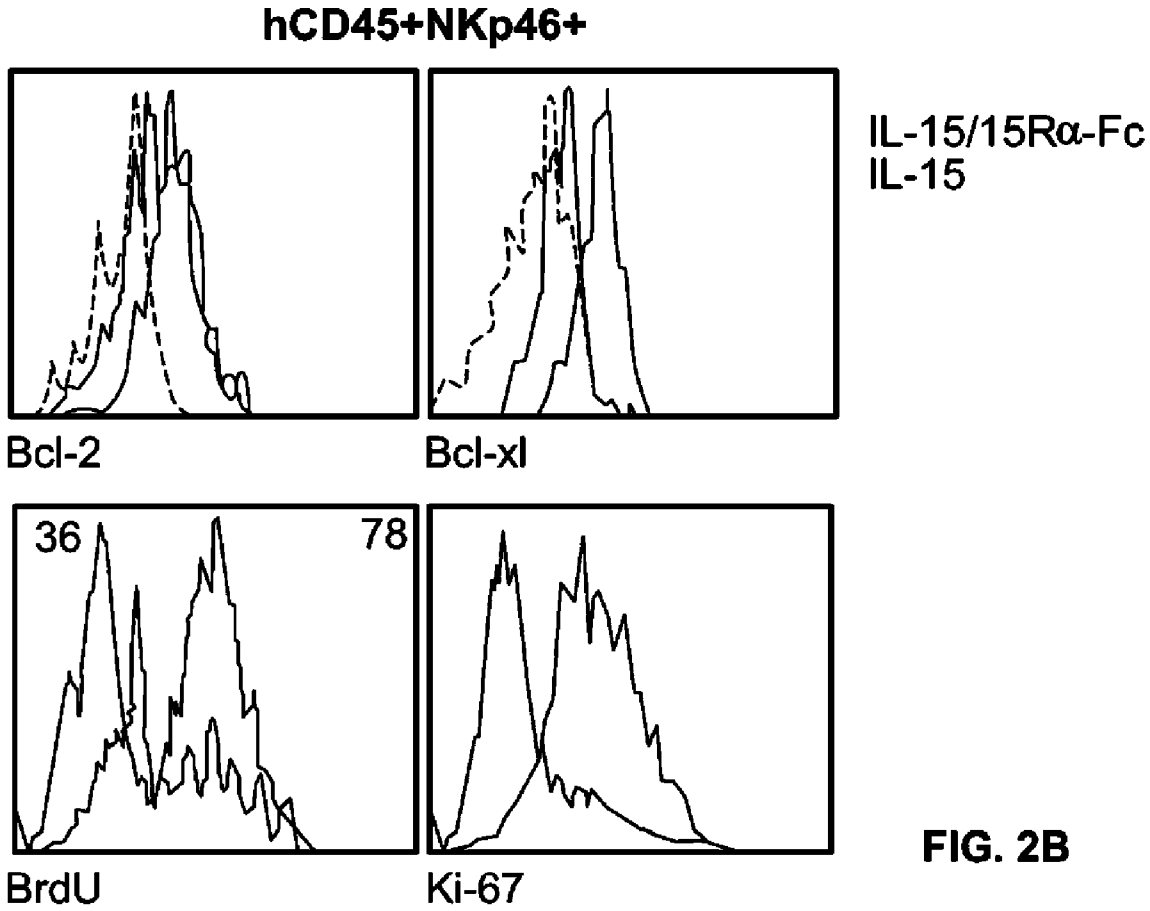


FIG. 2B

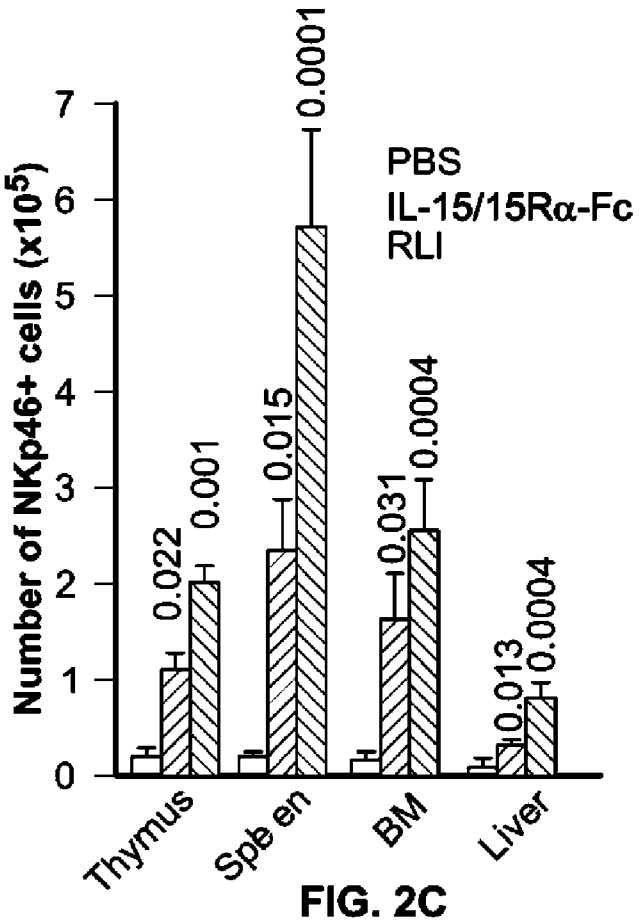


FIG. 2C

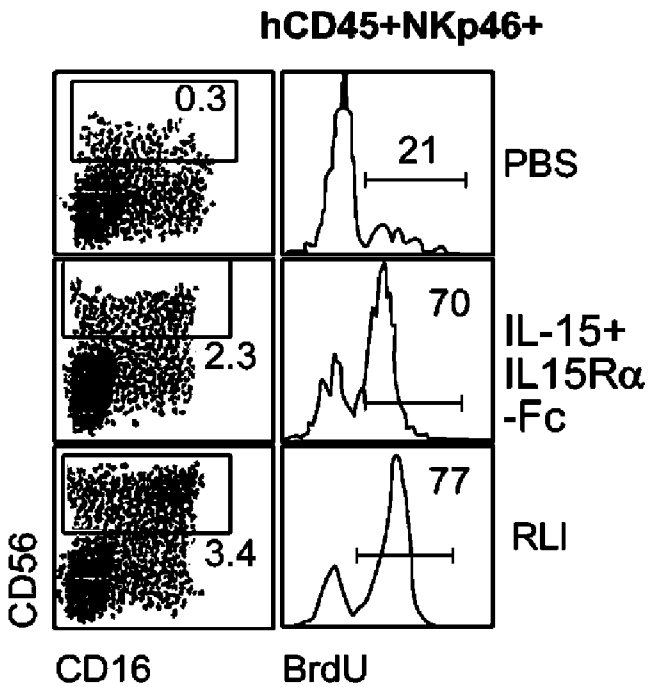


FIG. 2D

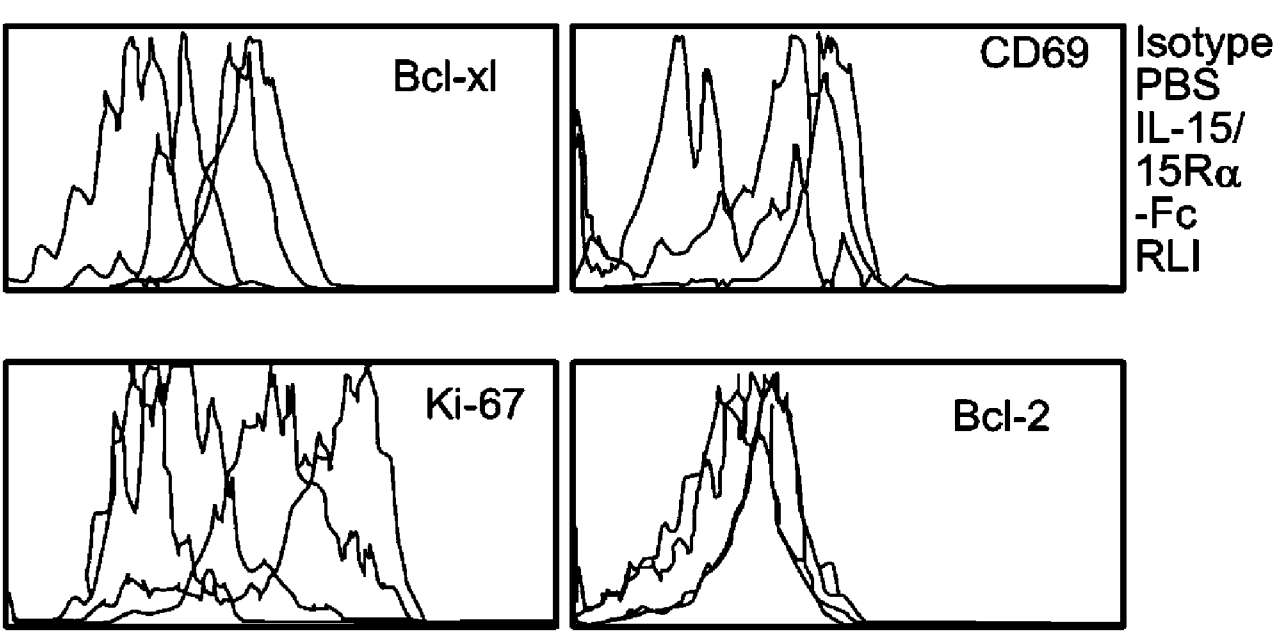

hCD45+NKp46+
Bcl-xl
CD69
Isotype
PBS
IL-15/
15Rα
-Fc
RLI
Ki-67
Bcl-2

FIG. 2E

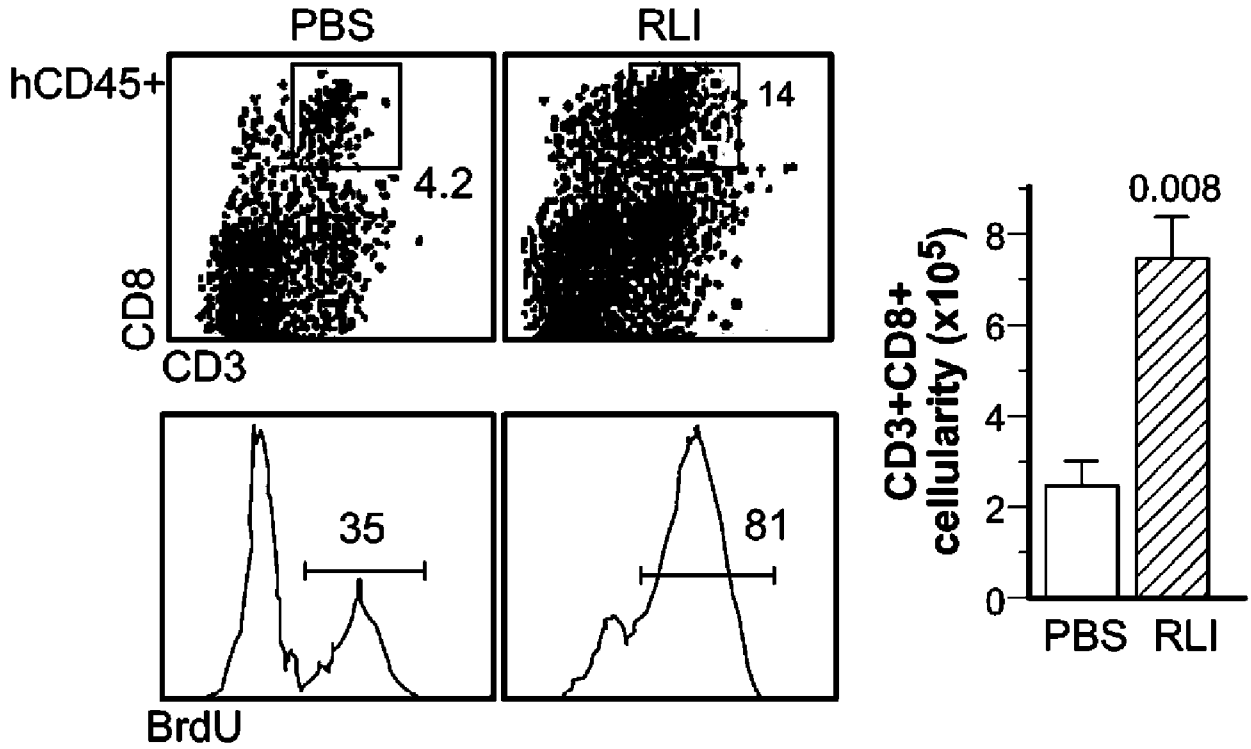

PBS
RLI
hCD45+
4.2
14
CD8
CD3
35
81
BrdU
0.008
CD3+CD8+ cellularity (x10^5)
8
6
4
2
0
PBS
RLI

FIG. 2F

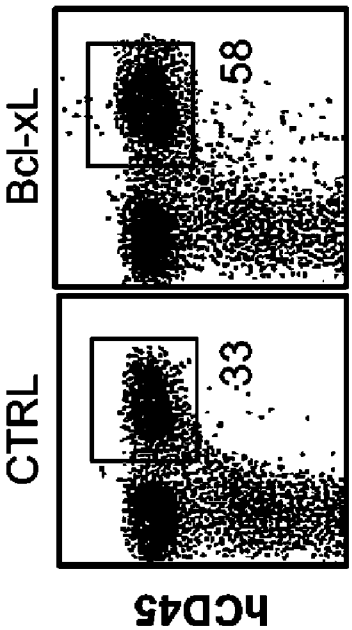


FIG. 3B

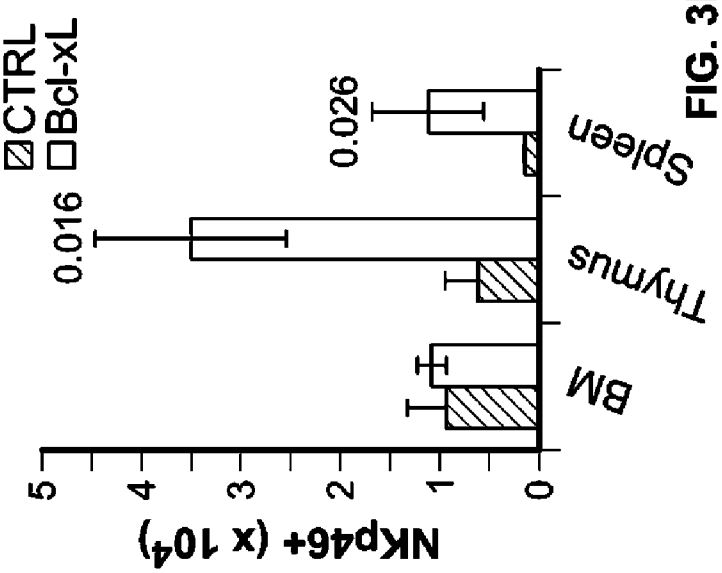


FIG. 3D

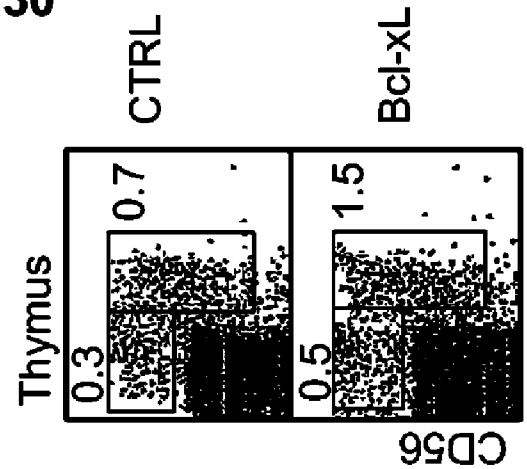


FIG. 3E

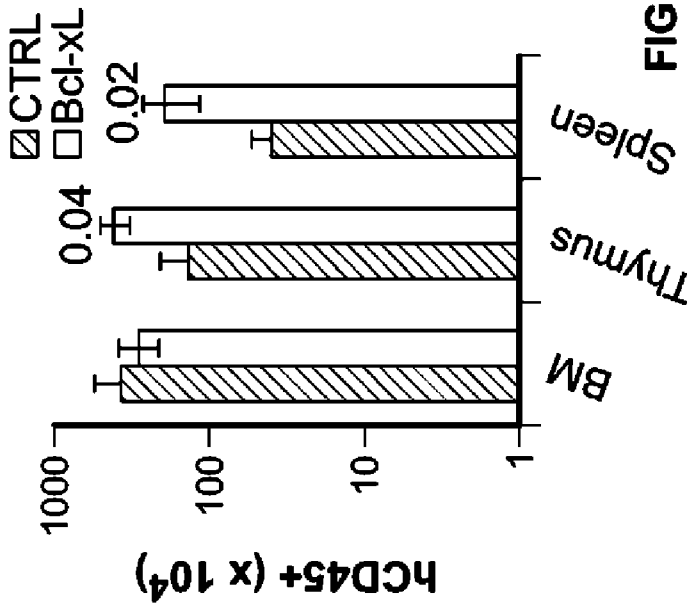


FIG. 3A

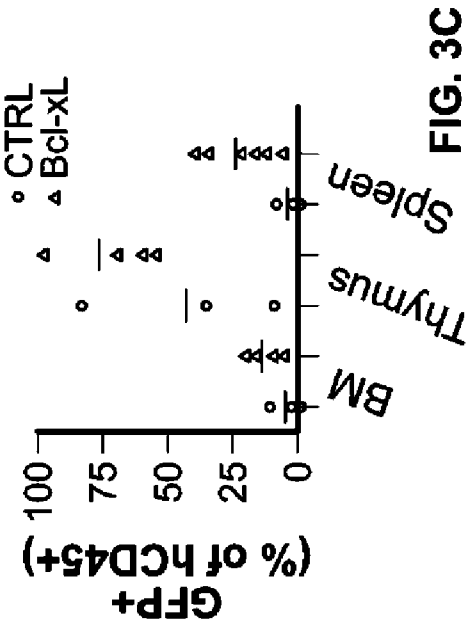


FIG. 3C

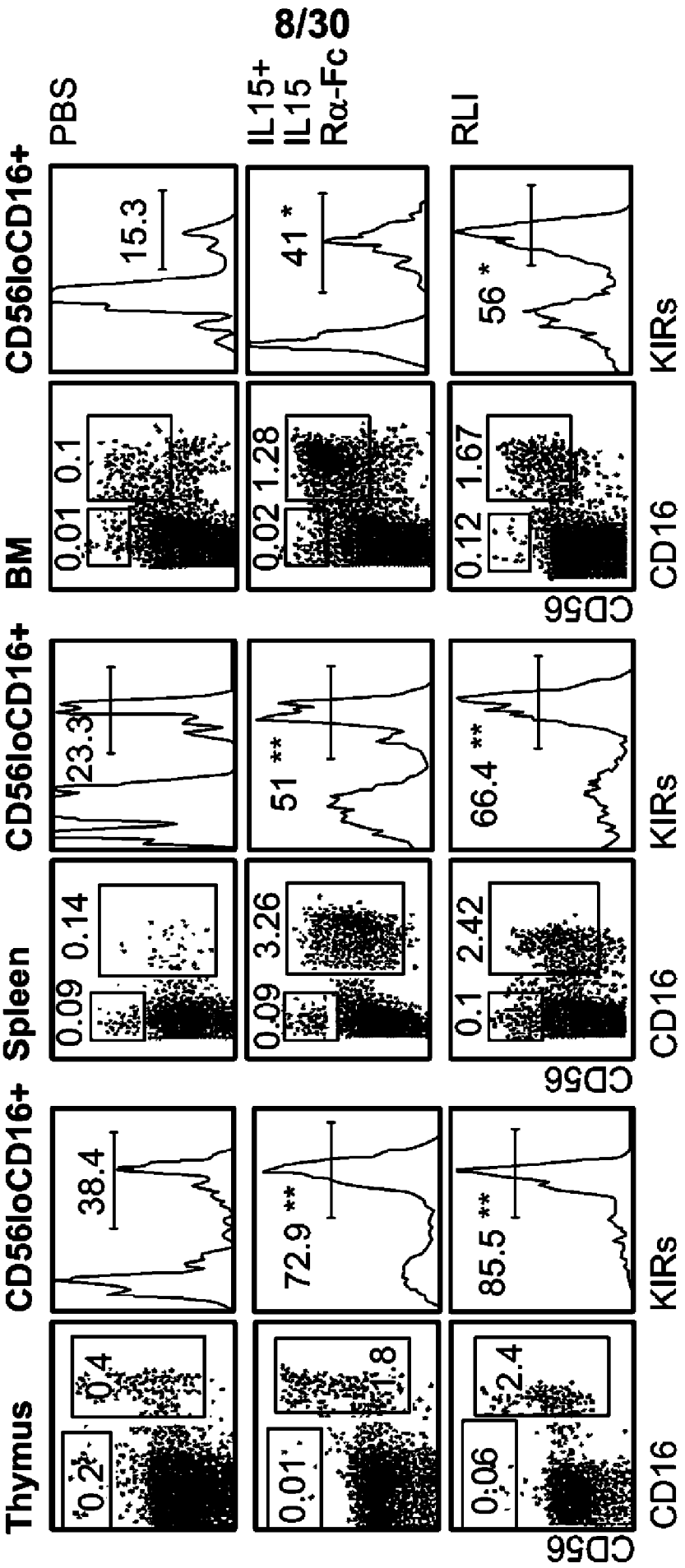

Thymus
Spleen
BM
CD56loCD16+
PBS
IL15+ IL15 Rα-Fc
RLI
CD56
CD16
KIRs
0.2
0.4
38.4
0.01
1.8
72.9 **
0.06
2.4
85.5 **
0.09
0.14
23.3
0.09
3.26
51 **
0.1
2.42
66.4 **
0.01
0.1
15.3
0.02
1.28
41 *
0.12
1.67
56 *

FIG. 4A

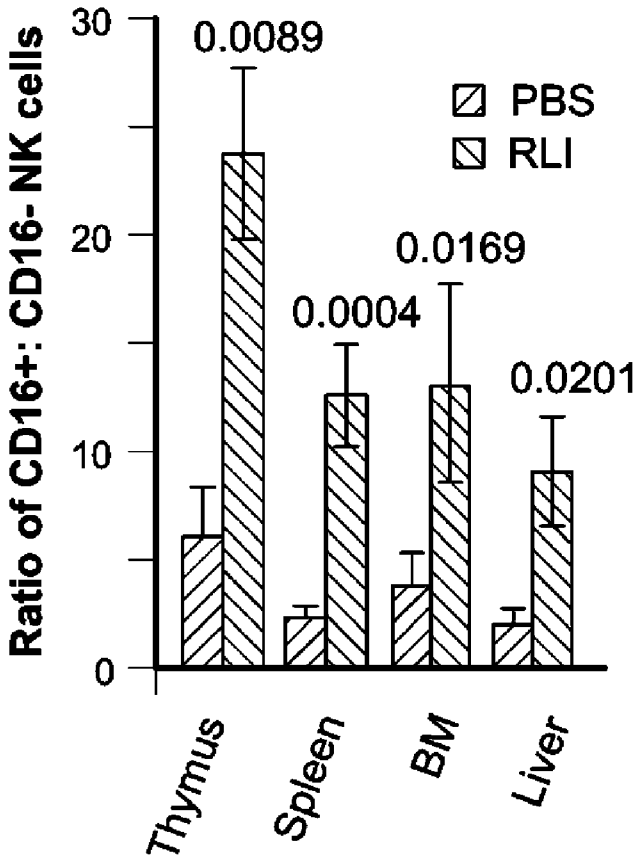


FIG. 4B

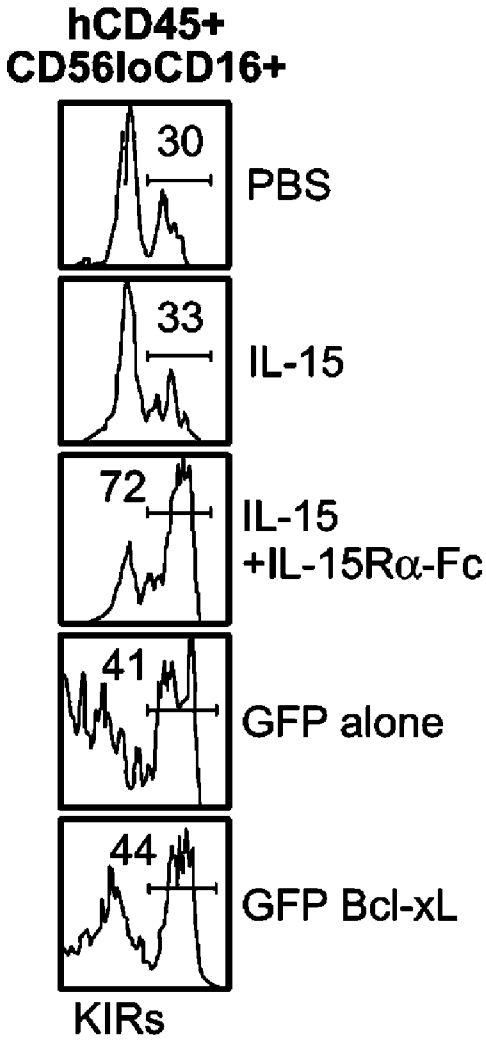


FIG. 4C

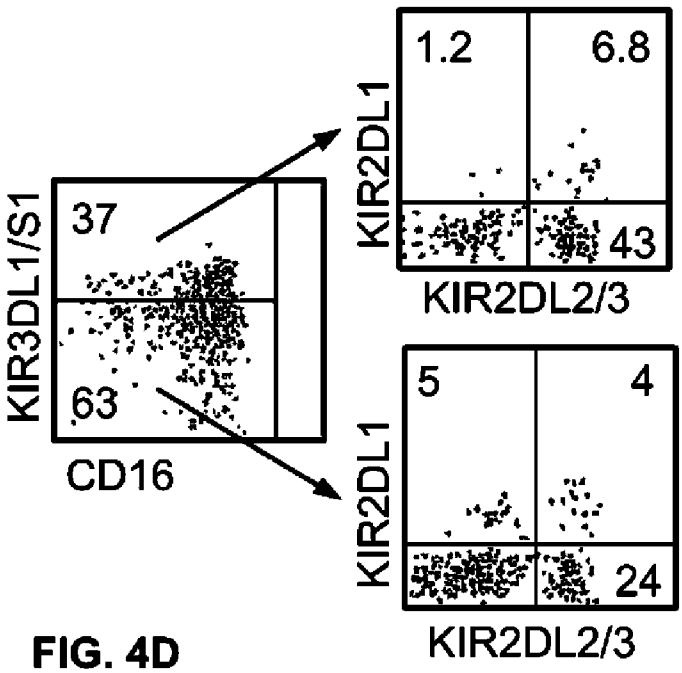


FIG. 4D

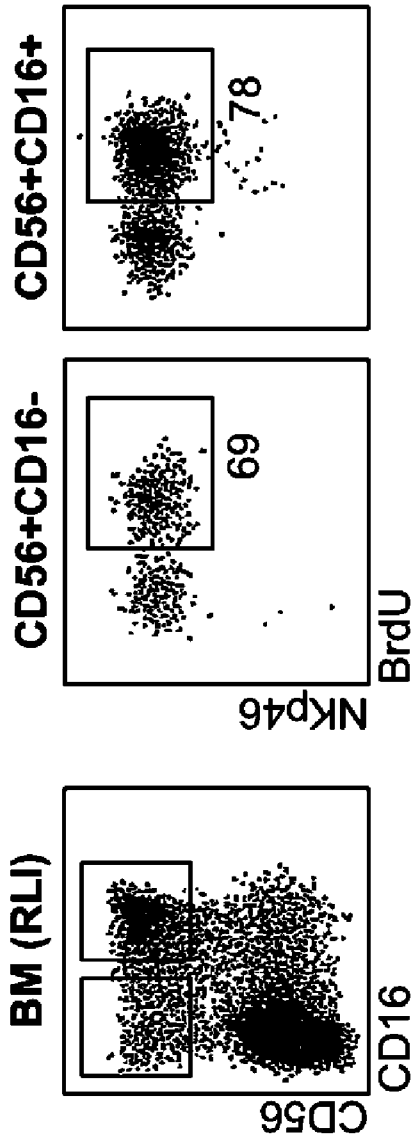

BM (RLI)
CD56
CD16
CD56+CD16-
NKp46
BrdU
69
CD56+CD16+
78

FIG. 5A

Donor cells
7 days post-transfer
PBS
RLI
CD56hi CD16- KIRs-
CD56lo CD16+ KIRs-
0.0006
0.0003
0.06
0.1
34
99
27
33
NKp46
hCD45
CD56
CD16
KIRs

FIG. 5B

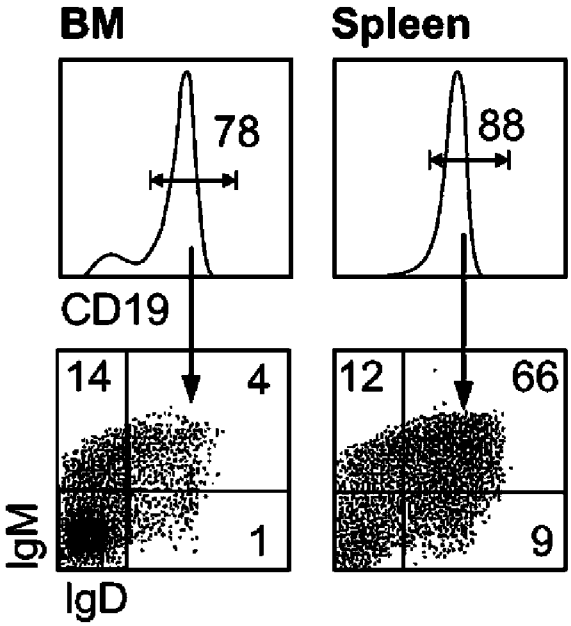


FIG. 6A

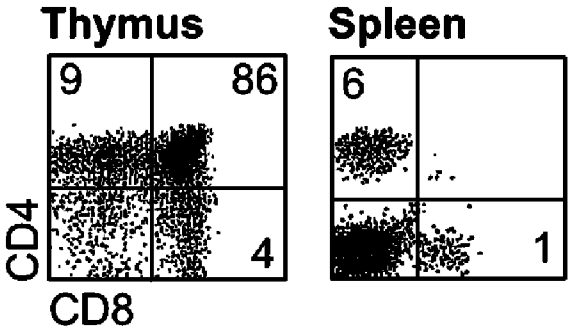


FIG. 6B

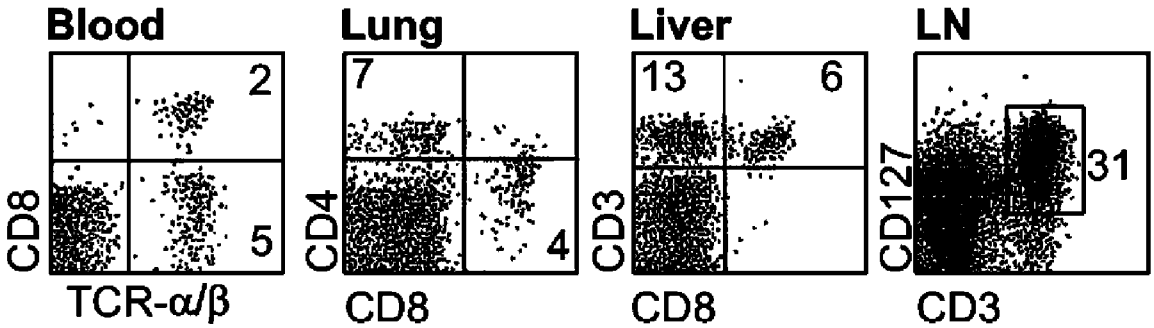


FIG. 6C

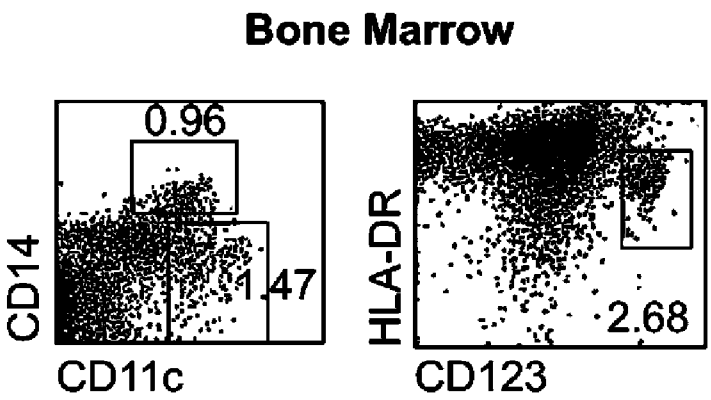


FIG. 6D

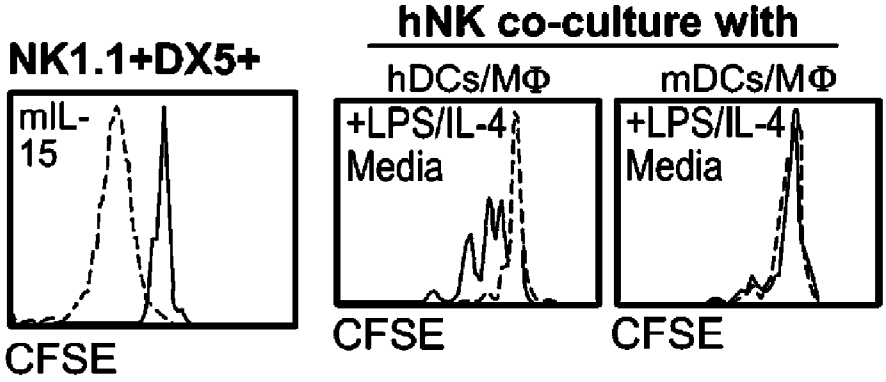


FIG. 7A

FIG. 7B

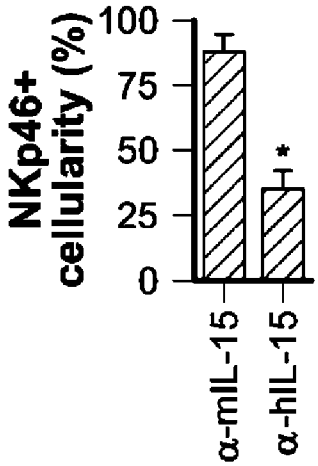


FIG. 7C

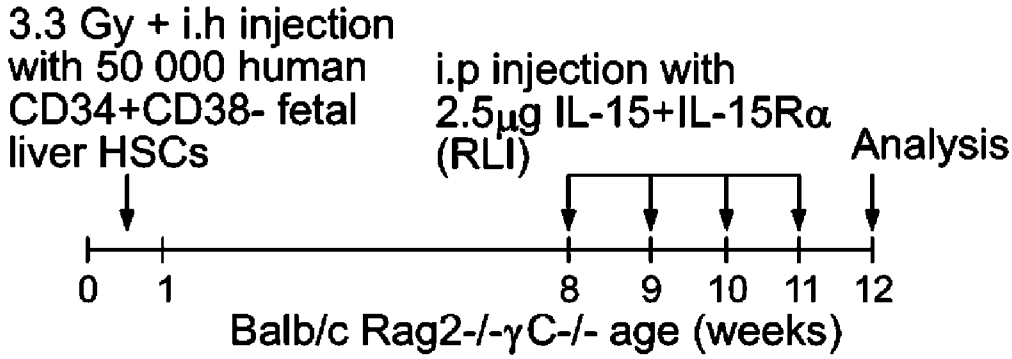


FIG. 8A

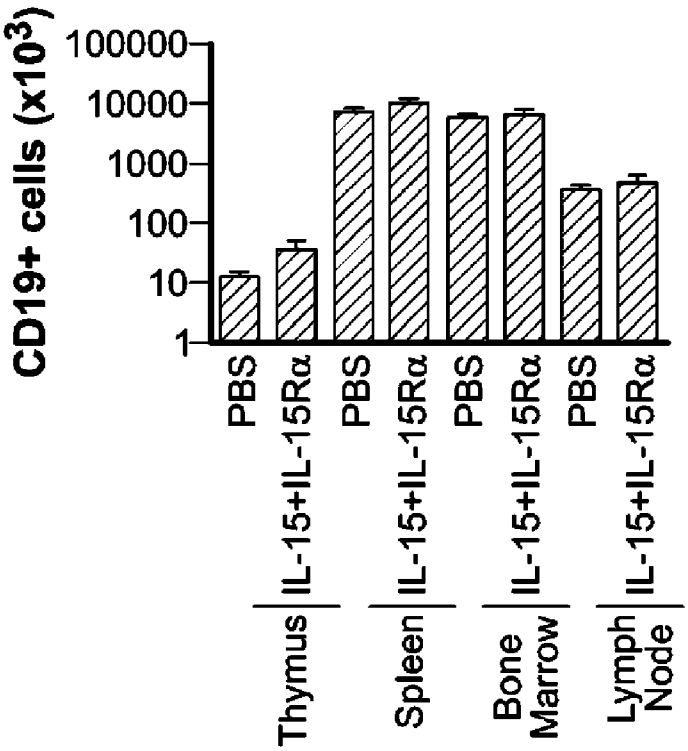


FIG. 8C

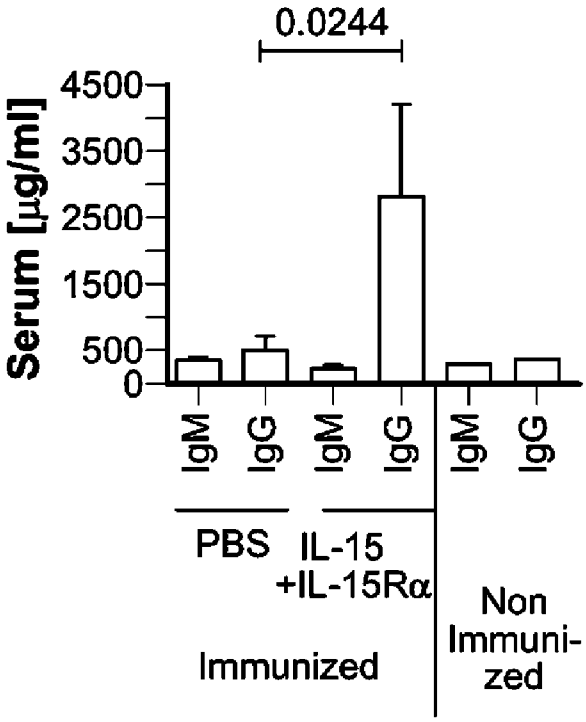


FIG. 8D

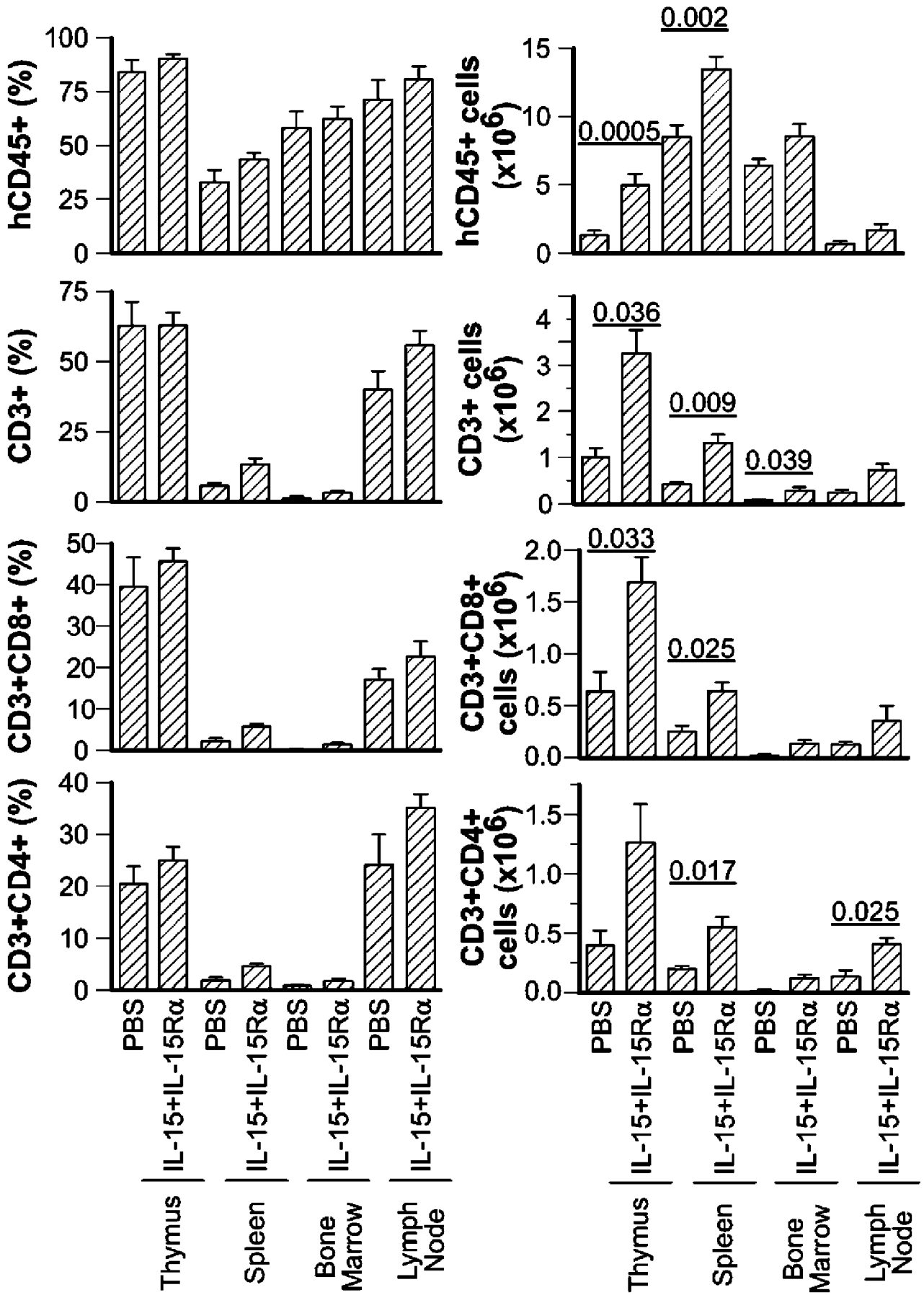

hCD45+ (%)
hCD45+ cells (x10^6)
CD3+ (%)
CD3+ cells (x10^6)
CD3+CD8+ (%)
CD3+CD8+ cells (x10^6)
CD3+CD4+ (%)
CD3+CD4+ cells (x10^6)
0.002
0.0005
0.036
0.009
0.039
0.033
0.025
0.017
0.025
PBS
IL-15+IL-15Rα
Thymus
Spleen
Bone Marrow
Lymph Node

FIG. 8B

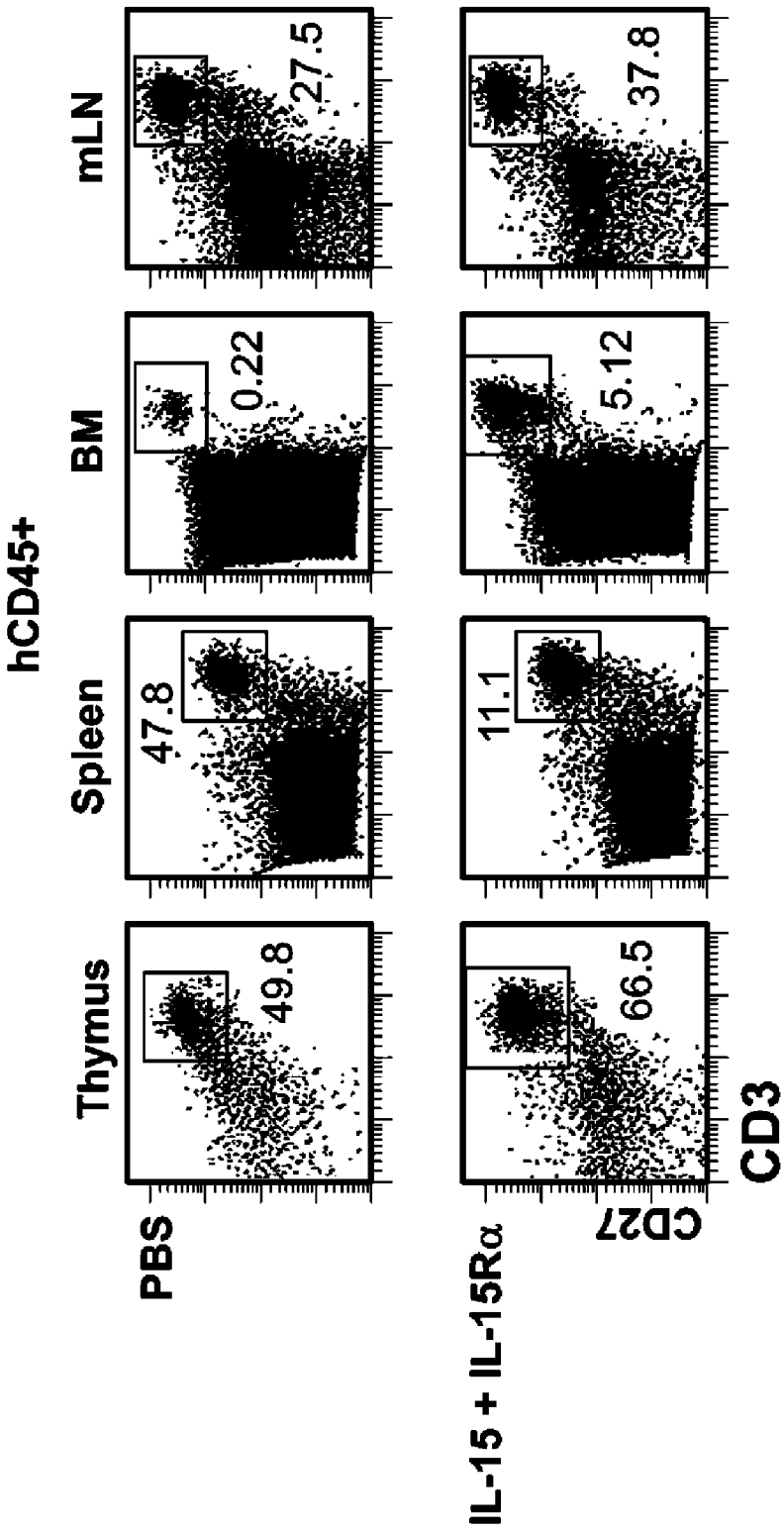

hCD45+
Thymus
Spleen
BM
mLN
PBS
IL-15 + IL-15Rα
49.8
47.8
0.22
27.5
66.5
11.1
5.12
37.8
CD27
CD3

FIG. 9A

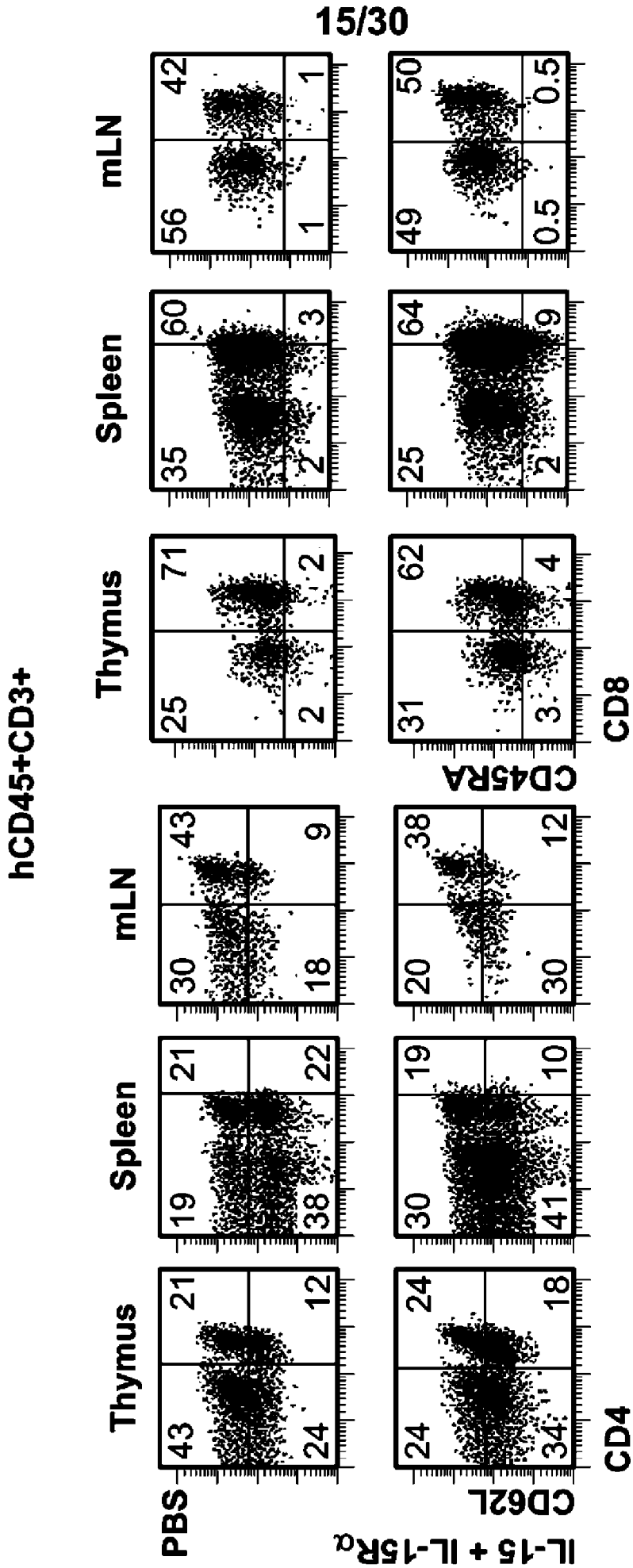
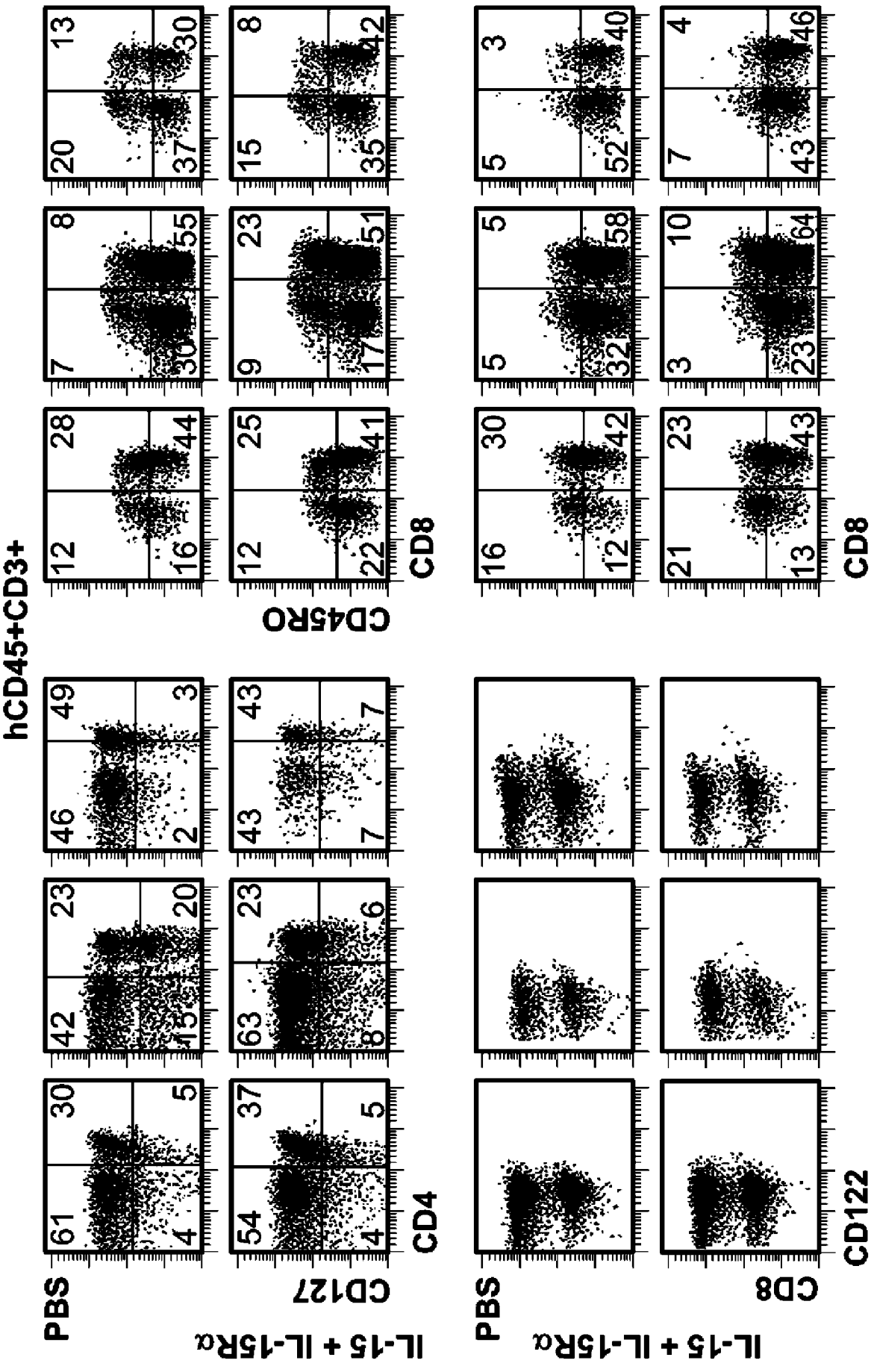

hCD45+CD3+
PBS
IL-15 + IL-15Rα
Thymus
Spleen
mLN
Thymus
Spleen
mLN
CD62L
CD4
CD45RA
CD8
43
21
24
12
19
21
38
22
30
43
18
9
24
24
34
18
30
19
41
10
20
38
30
12
25
71
2
2
35
60
2
3
56
42
1
1
31
62
3
4
25
64
2
9
49
50
0.5
0.5

FIG. 9B


hCD45+CD3+
PBS
IL-15 + IL-15Rα
CD127
CD45RO
CD4
CD8
CD8
CD122
CD8
FIG. 9B (Cont.)

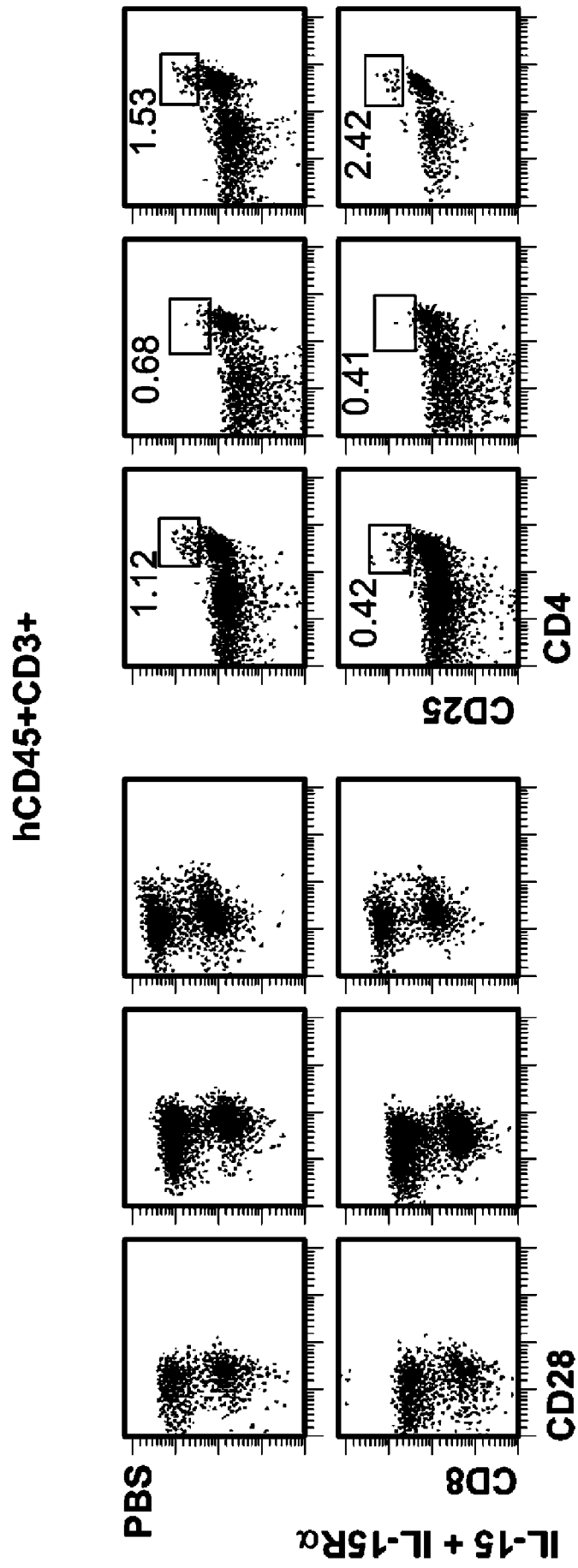

hCD45+CD3+
1.53
2.42
0.68
0.41
1.12
0.42
CD25
CD4
PBS
CD8
CD28
IL-15 + IL-15Rα

FIG. 9B (Cont.)

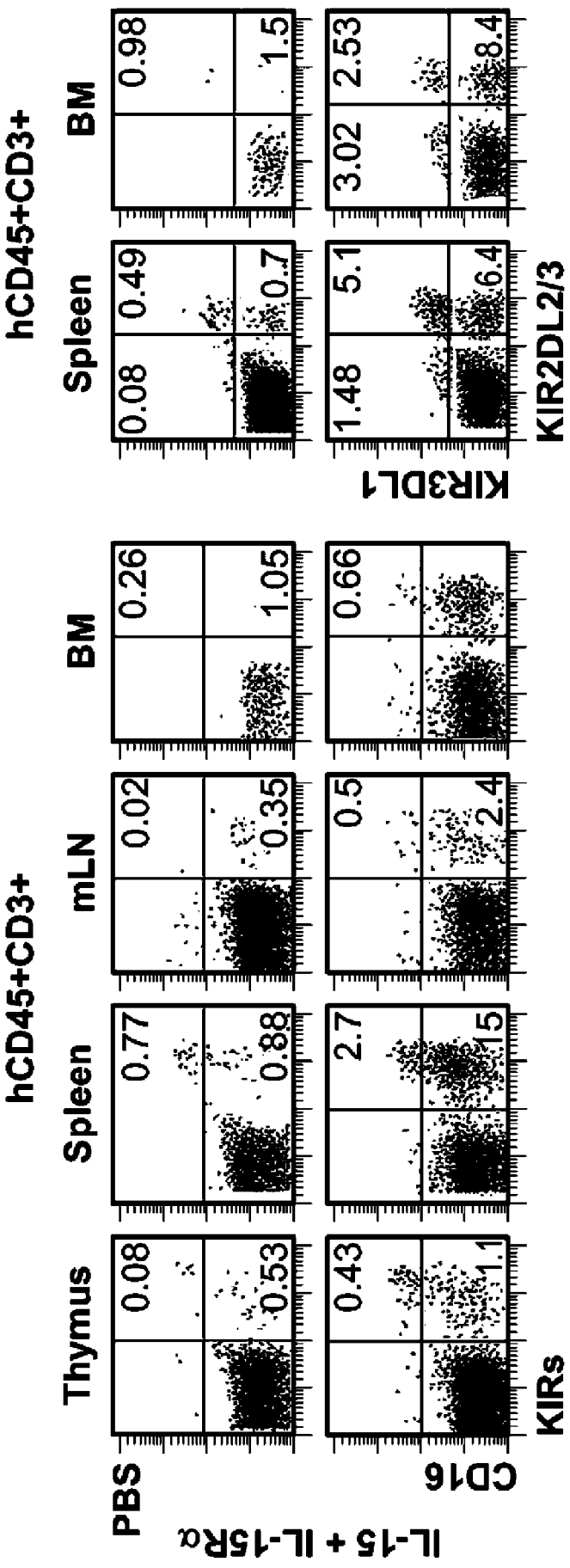

hCD45+CD3+
Thymus
Spleen
mLN
BM
PBS
IL-15 + IL-15Rα
0.08
0.53
0.77
0.88
0.02
0.35
0.26
1.05
0.43
1.1
2.7
15
0.5
2.4
0.66
CD16
KIRs
hCD45+CD3+
Spleen
BM
0.08
0.49
0.7
0.98
1.5
1.48
5.1
6.4
3.02
2.53
8.4
KIR3DL1
KIR2DL2/3

FIG. 9C

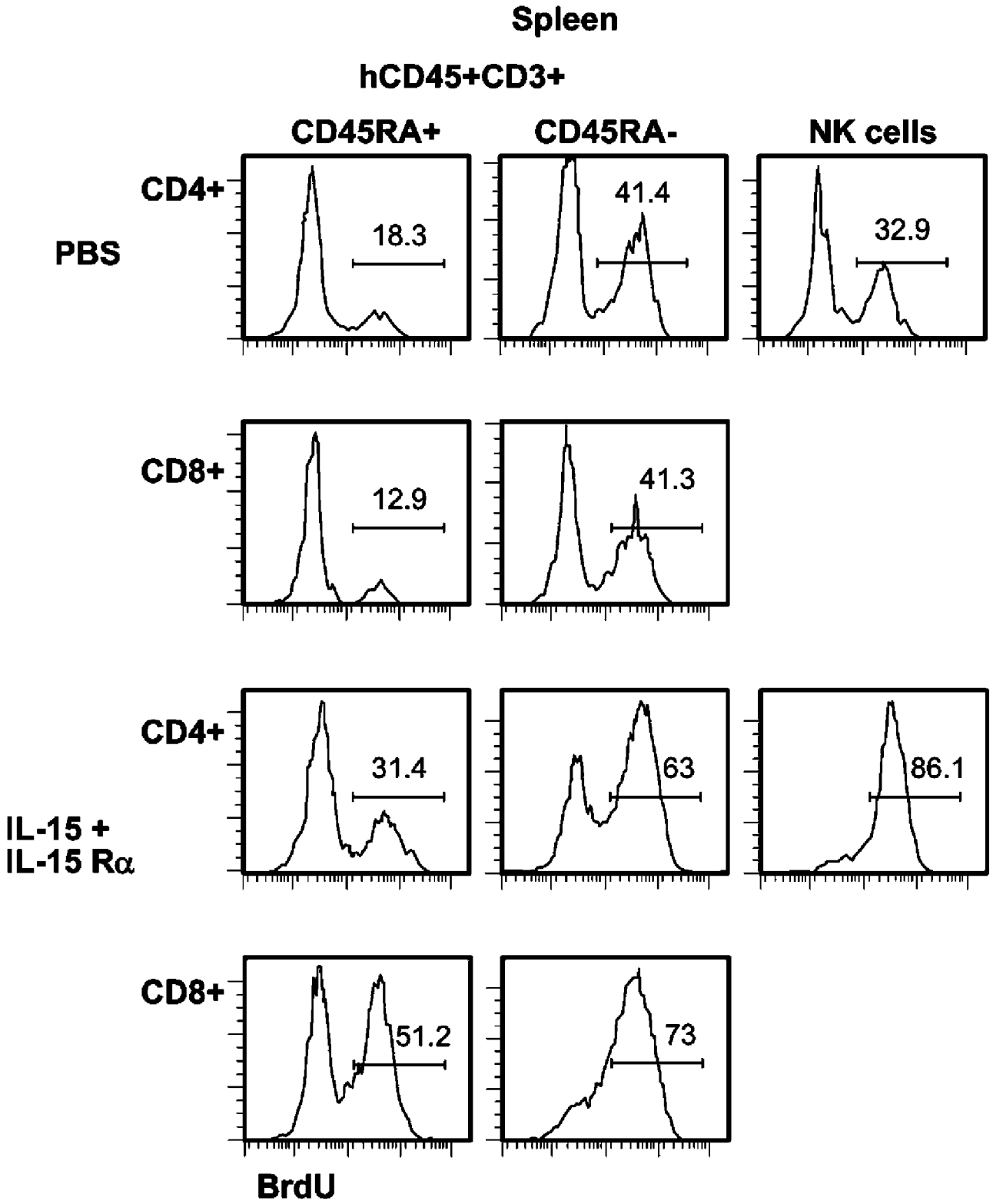

Spleen
hCD45+CD3+
CD45RA+
CD45RA-
NK cells
PBS
CD4+
18.3
41.4
32.9
CD8+
12.9
41.3
IL-15 +
IL-15 Rα
CD4+
31.4
63
86.1
CD8+
51.2
73
BrdU

FIG. 10A

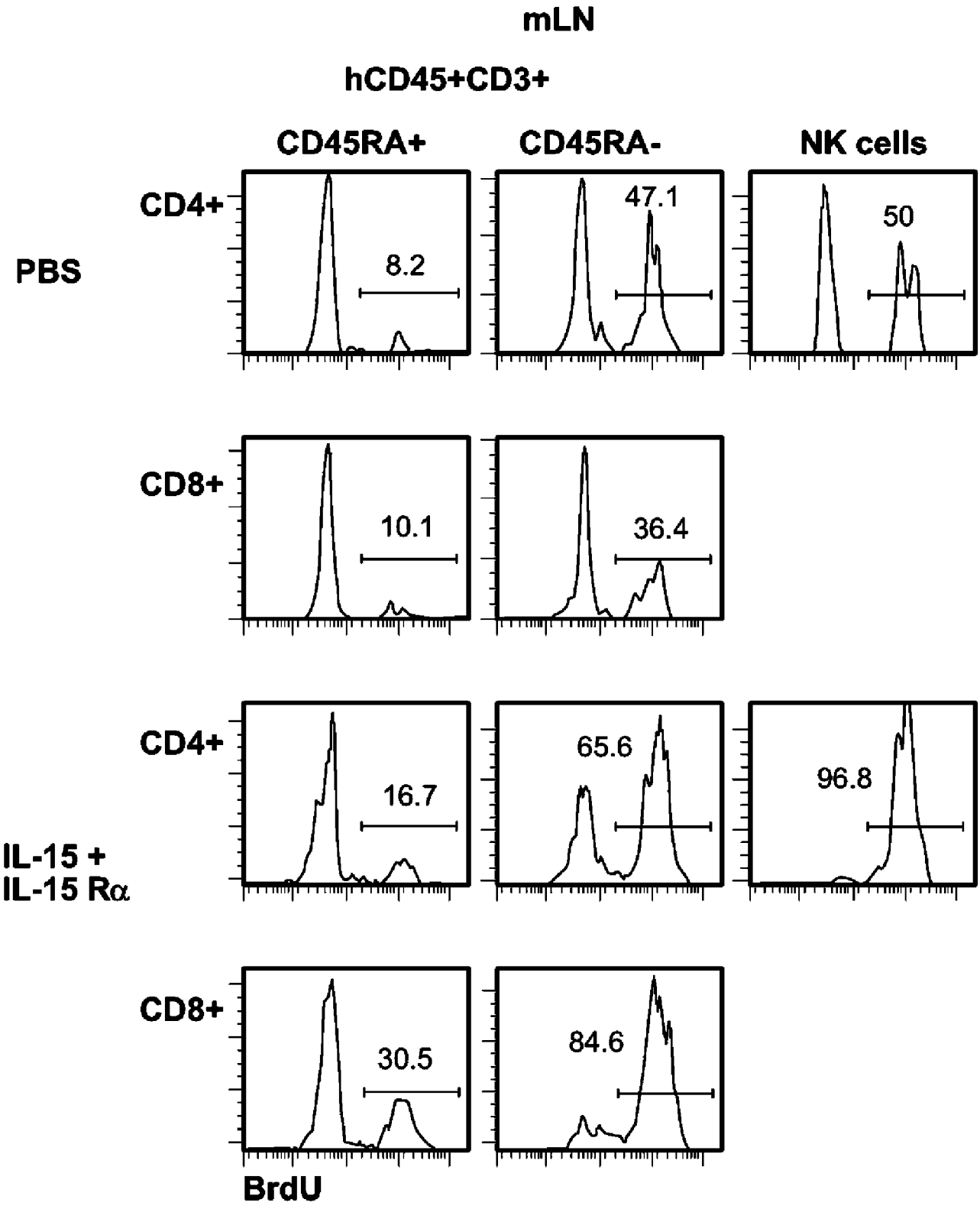

mLN
hCD45+CD3+
CD45RA+
CD45RA-
NK cells
PBS
CD4+
8.2
47.1
50
CD8+
10.1
36.4
IL-15 +
IL-15 Rα
CD4+
16.7
65.6
96.8
CD8+
30.5
84.6
BrdU

FIG. 10B

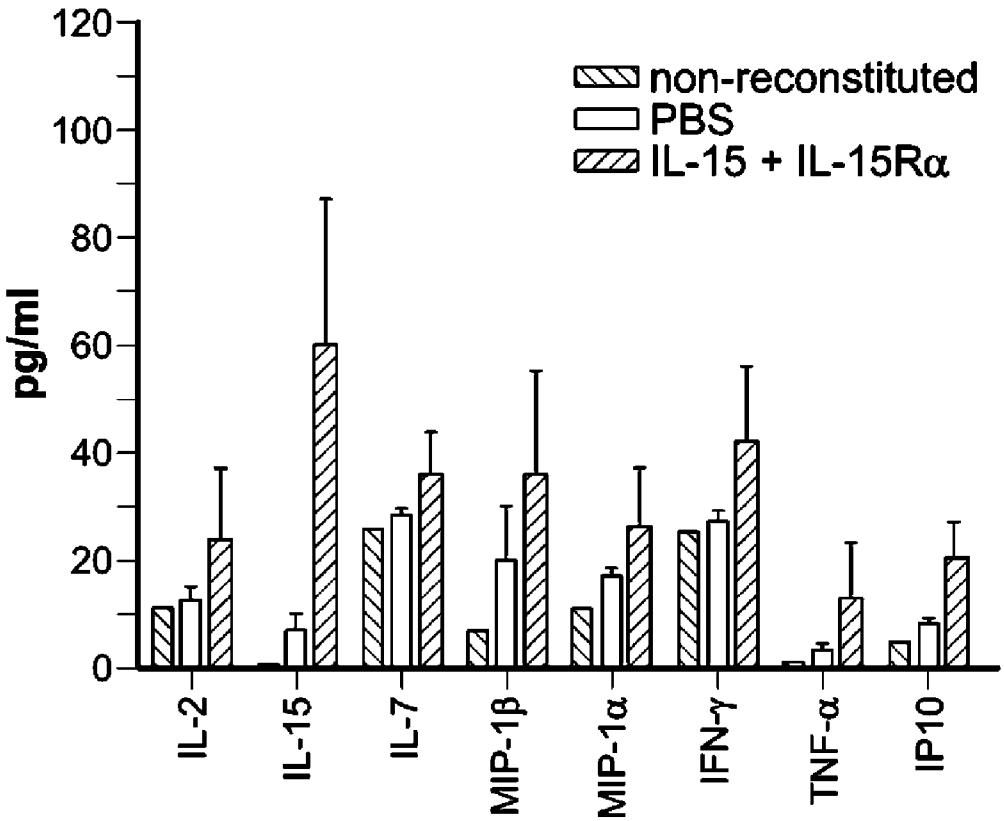

120
100
80
60
40
20
0
pg/ml
non-reconstituted
PBS
IL-15 + IL-15Rα
IL-2
IL-15
IL-7
MIP-1β
MIP-1α
IFN-γ
TNF-α
IP10

FIG. 10C

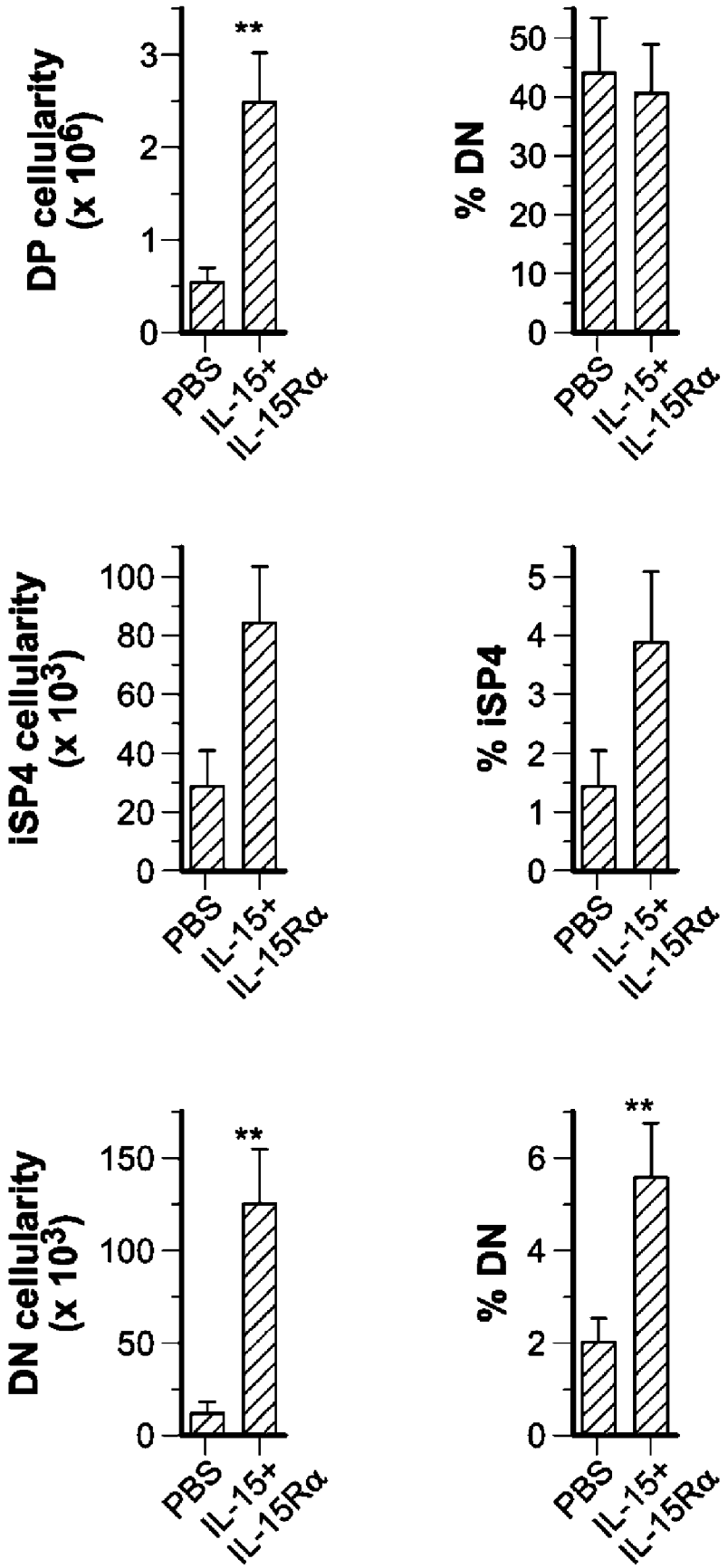

DP cellularity (x 10^6)
**
3
2
1
0
PBS
IL-15+ IL-15Rα
% DN
50
40
30
20
10
0
PBS
IL-15+ IL-15Rα
iSP4 cellularity (x 10^3)
100
80
60
40
20
0
PBS
IL-15+ IL-15Rα
% iSP4
5
4
3
2
1
0
PBS
IL-15+ IL-15Rα
DN cellularity (x 10^3)
**
150
100
50
0
PBS
IL-15+ IL-15Rα
% DN
**
6
4
2
0
PBS
IL-15+ IL-15Rα

FIG. 11A

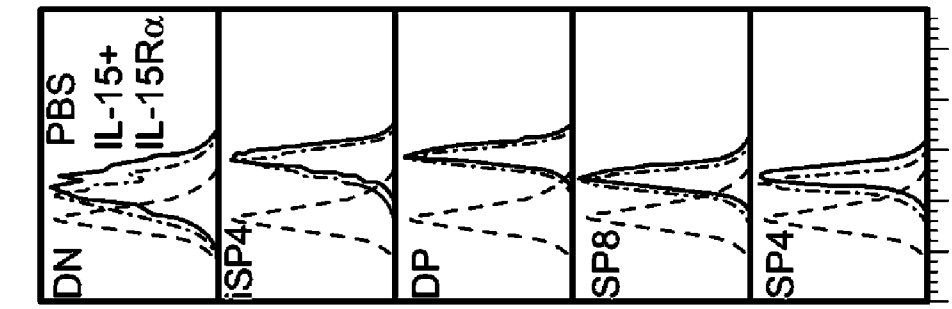


FIG. 11C

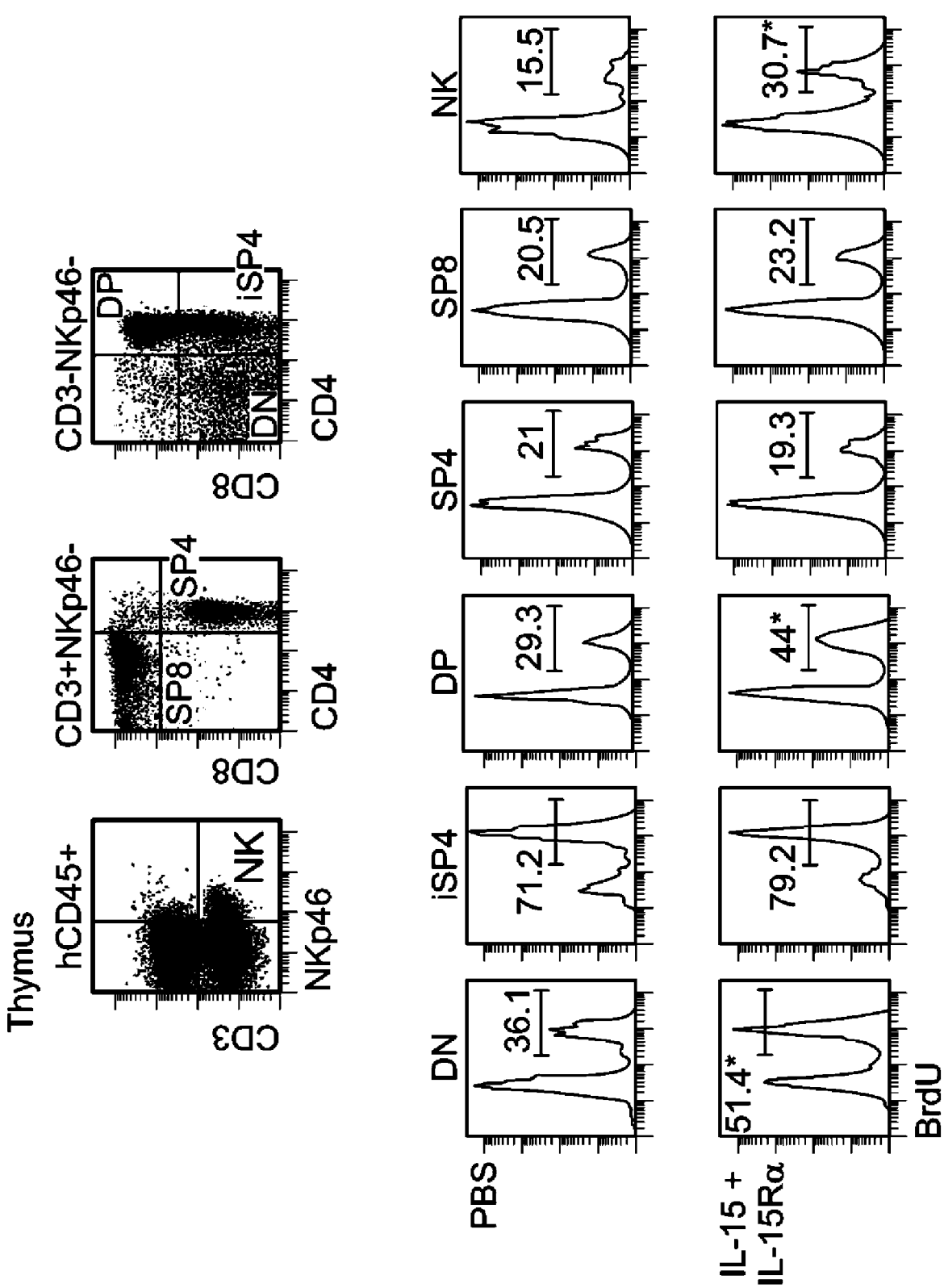


FIG. 11B

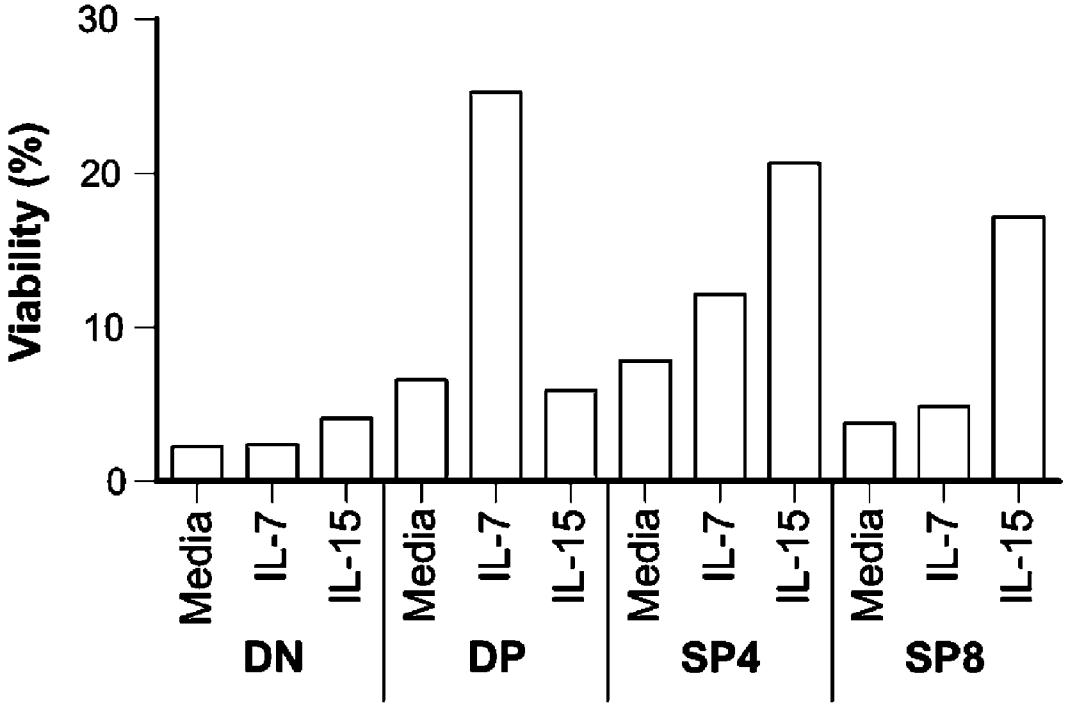


FIG. 11D

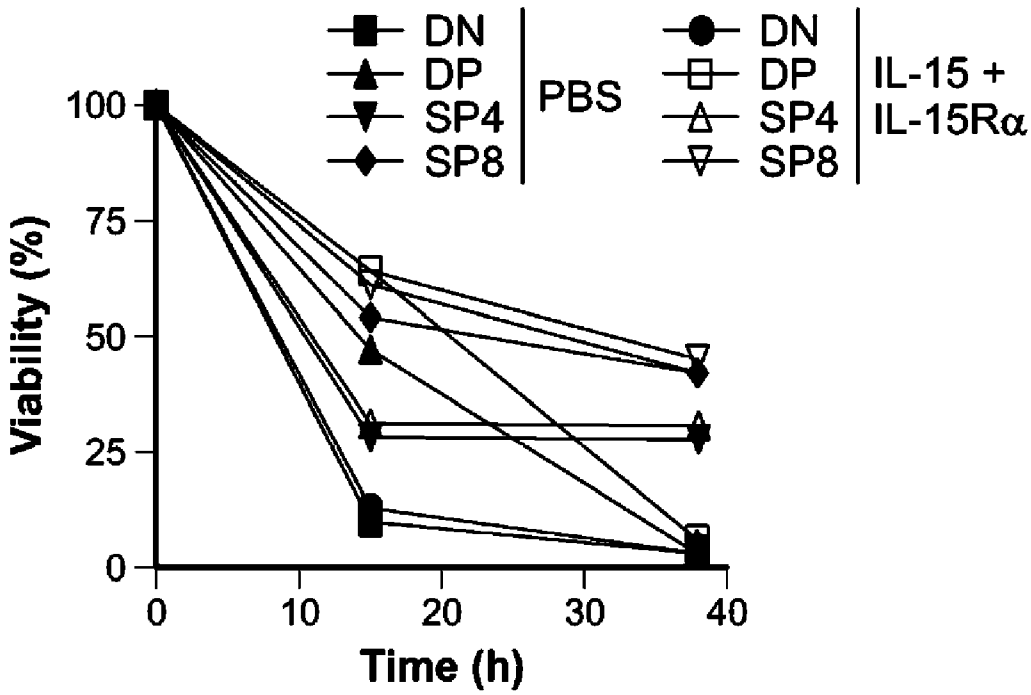


FIG. 11E

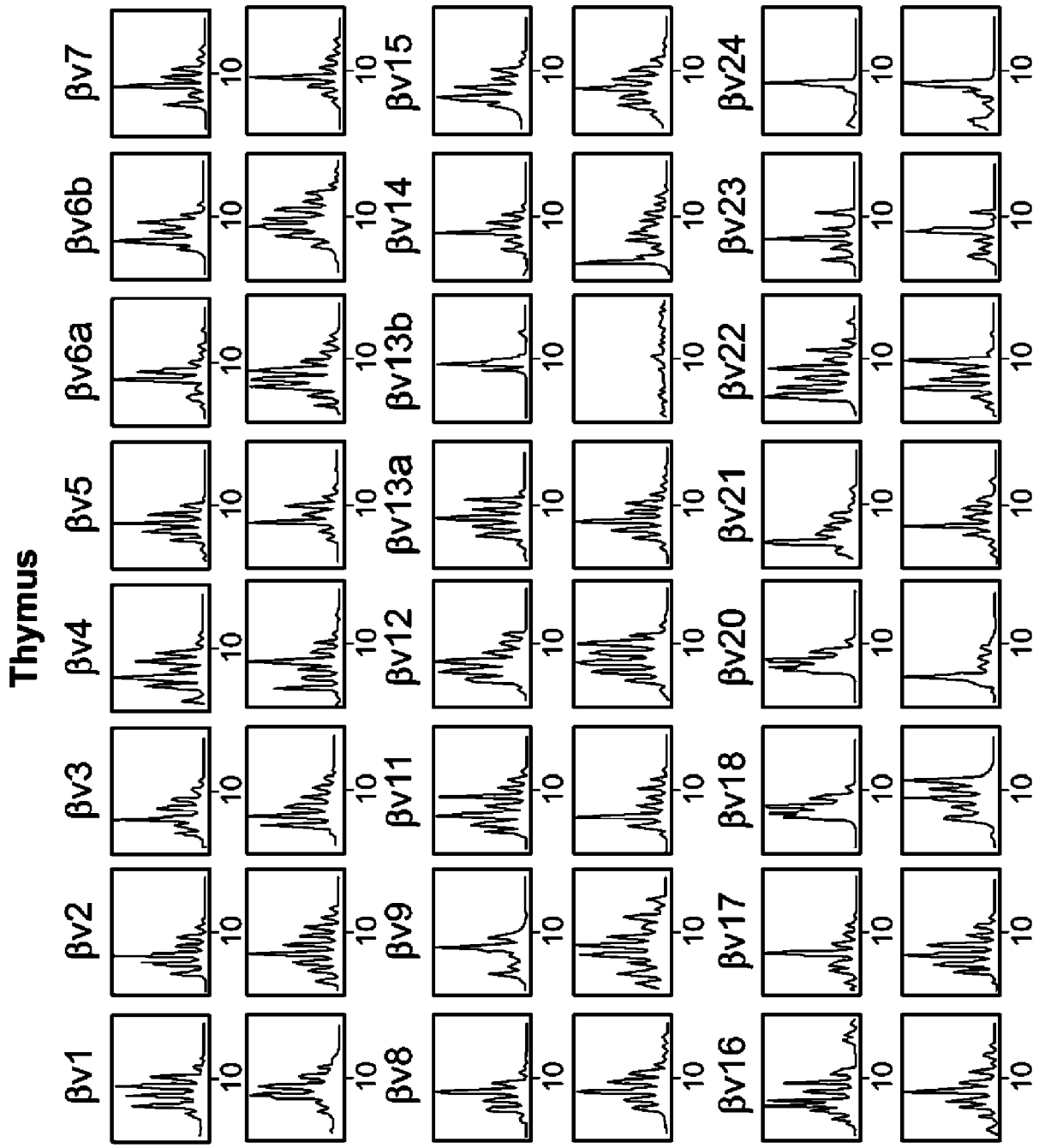

Thymus
βv1
βv2
βv3
βv4
βv5
βv6a
βv6b
βv7
βv8
βv9
βv11
βv12
βv13a
βv13b
βv14
βv15
βv16
βv17
βv18
βv20
βv21
βv22
βv23
βv24
PBS
IL-15 + IL-15Rα
PBS
IL-15 + IL-15Rα
PBS
IL-15 + IL-15Rα
10
Amino acid length

FIG. 12A

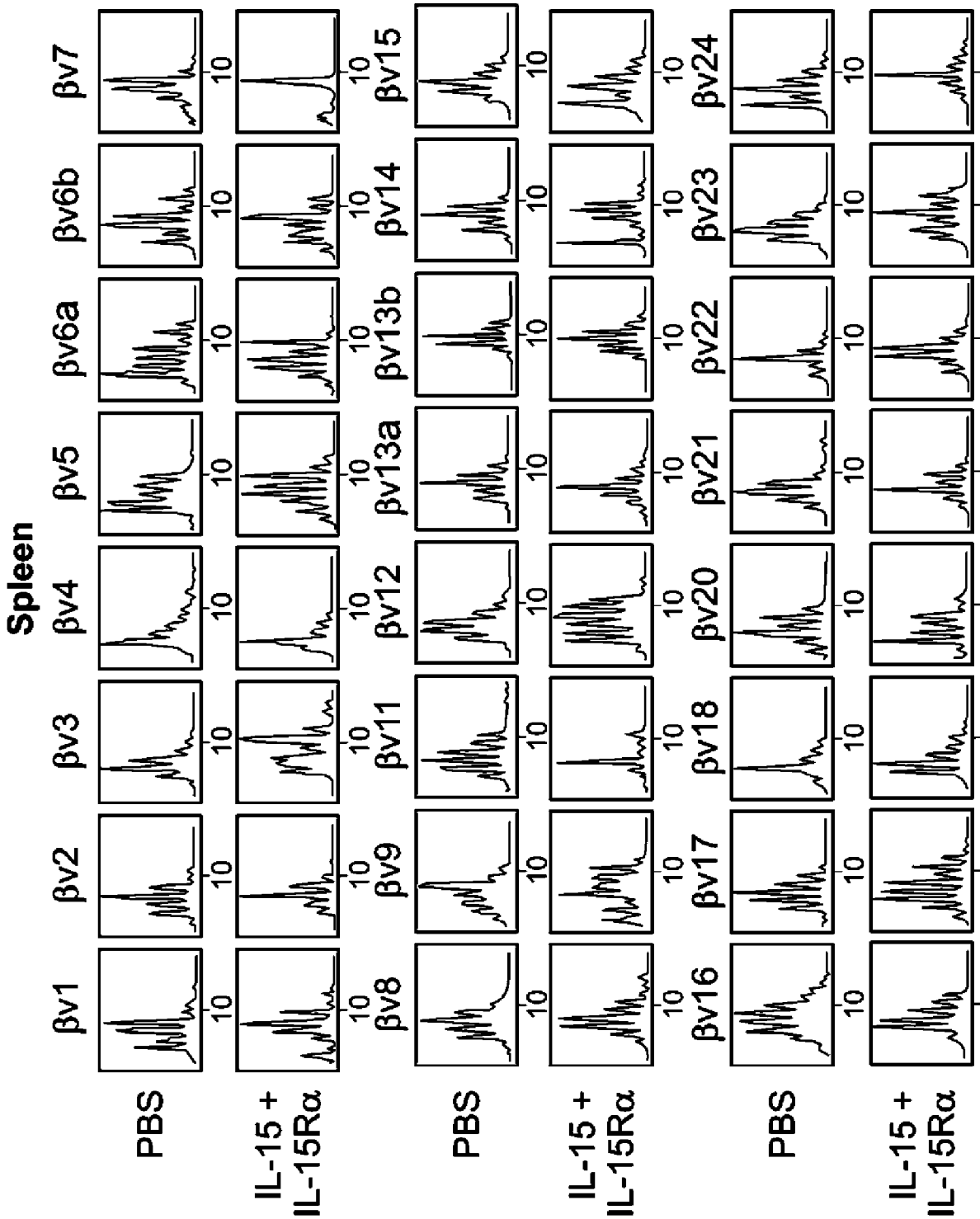

Spleen
βv1
βv2
βv3
βv4
βv5
βv6a
βv6b
βv7
βv8
βv9
βv11
βv12
βv13a
βv13b
βv14
βv15
βv16
βv17
βv18
βv20
βv21
βv22
βv23
βv24
PBS
IL-15 + IL-15Rα
PBS
IL-15 + IL-15Rα
PBS
IL-15 + IL-15Rα
10
Amino acid length

FIG. 12B

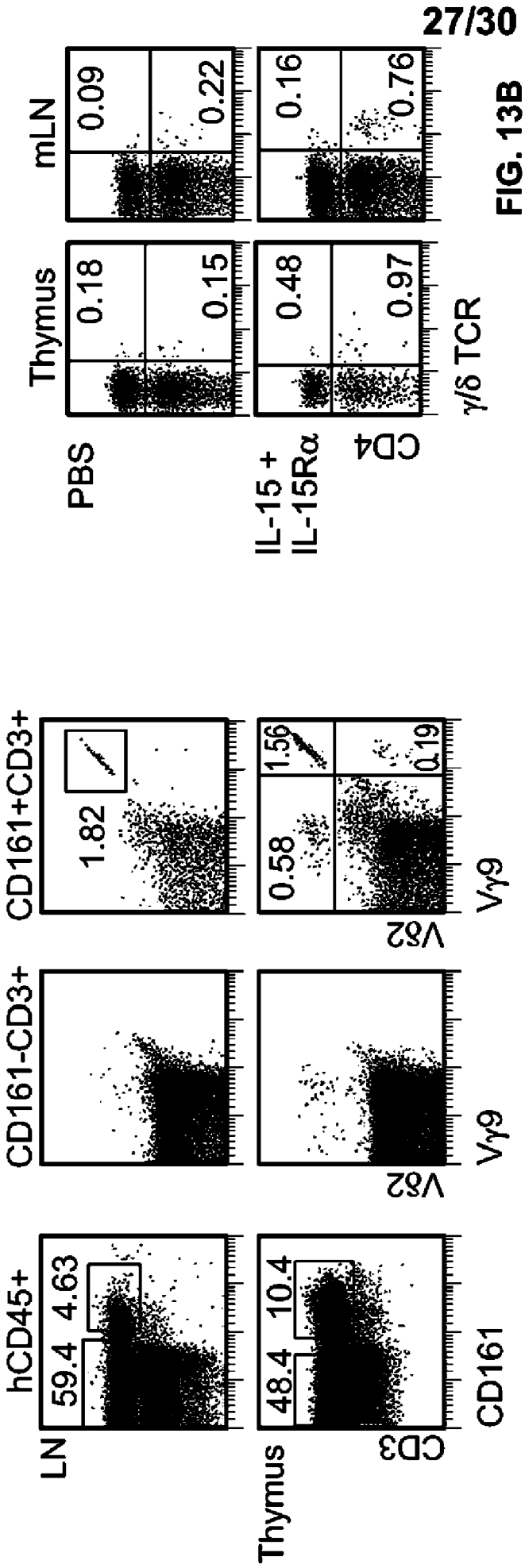


FIG. 13A

FIG. 13B

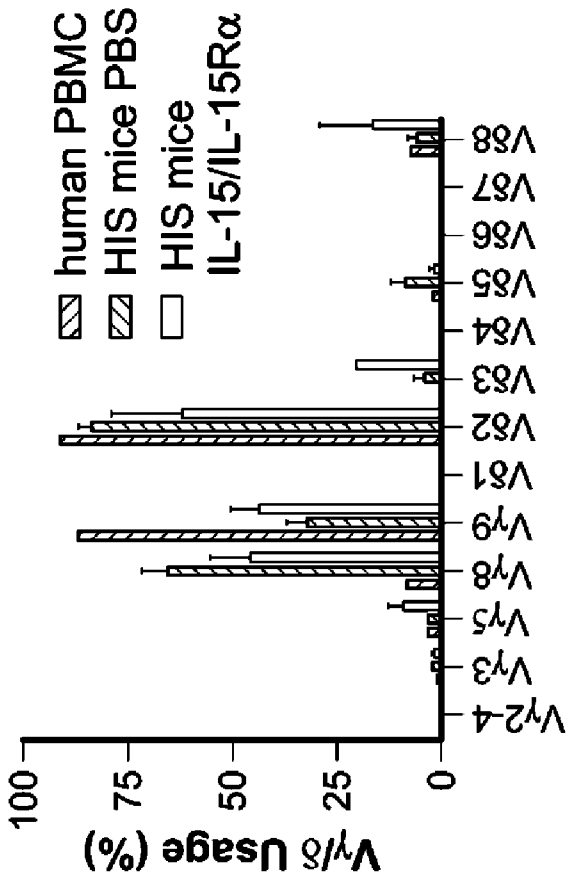


FIG. 13C

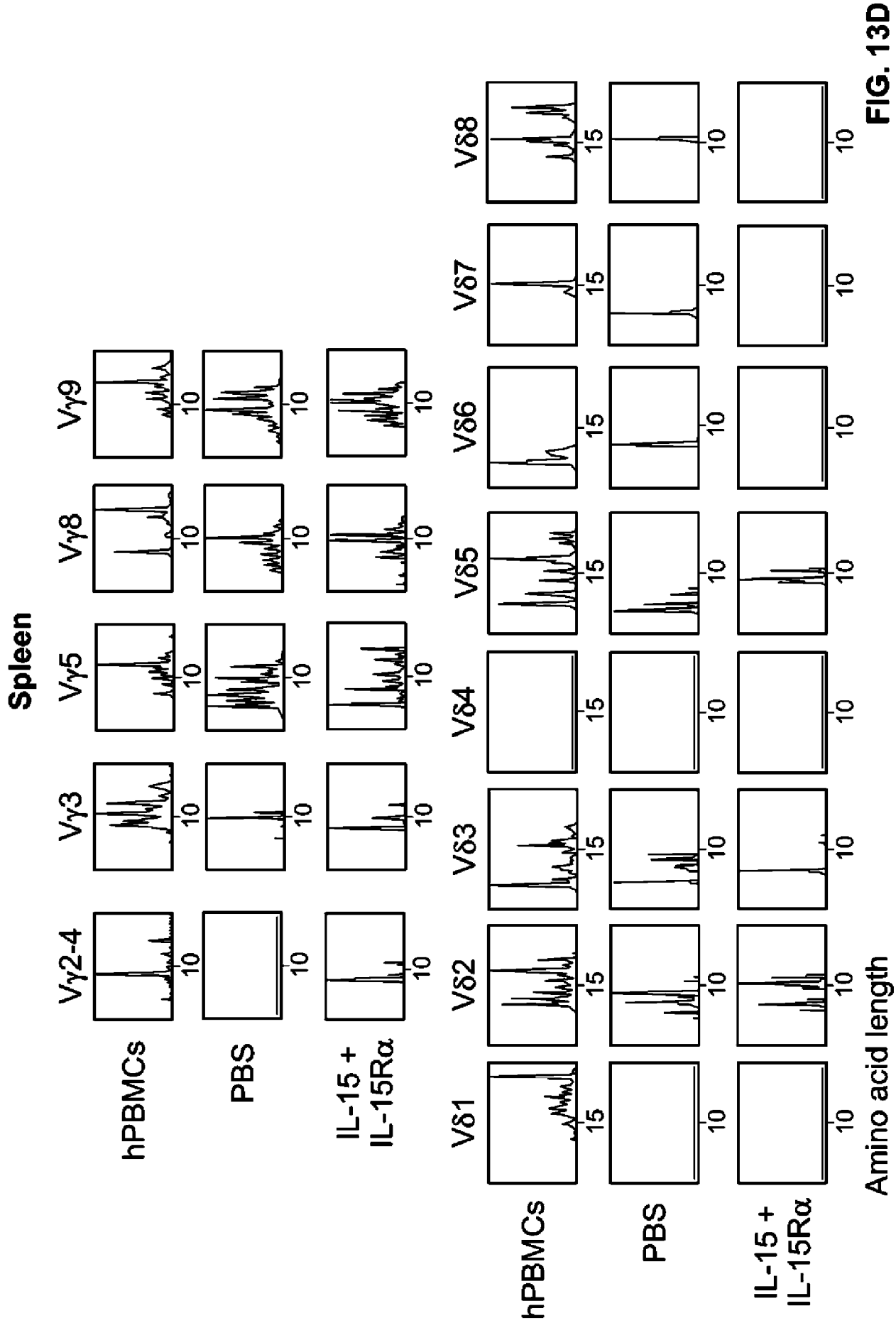

Spleen
Vγ2-4
Vγ3
Vγ5
Vγ8
Vγ9
Vδ1
Vδ2
Vδ3
Vδ4
Vδ5
Vδ6
Vδ7
Vδ8
hPBMCs
PBS
IL-15 + IL-15Rα
Amino acid length

FIG. 13D

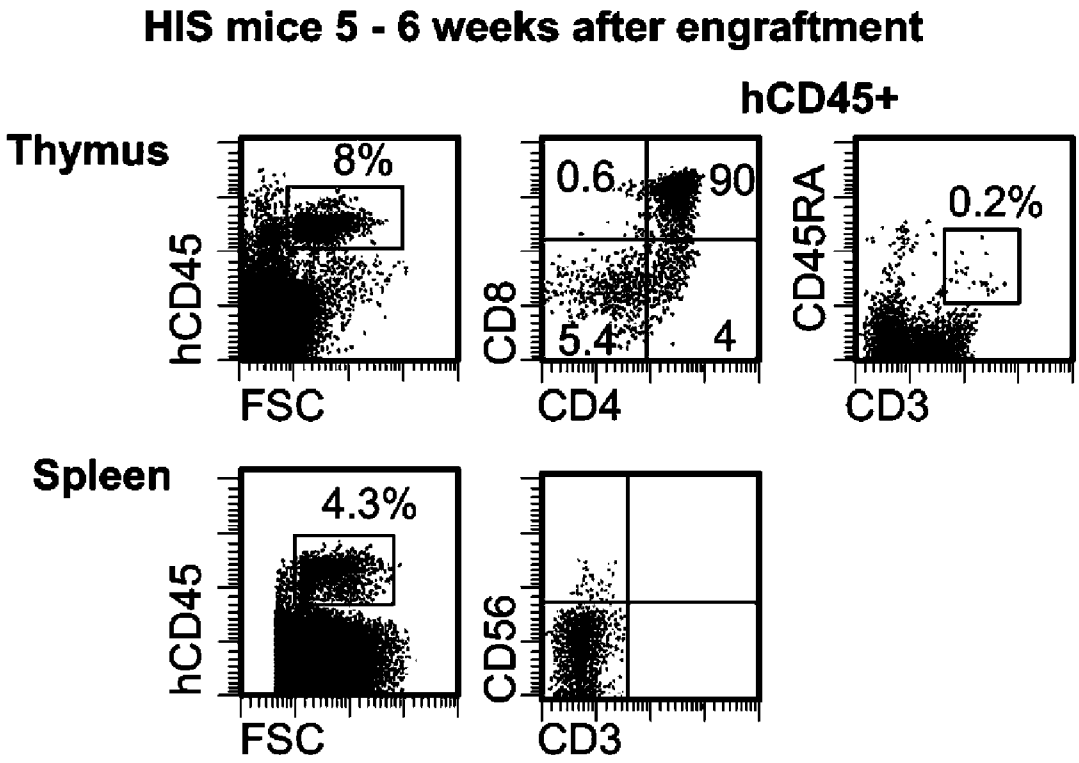


FIG. 14A

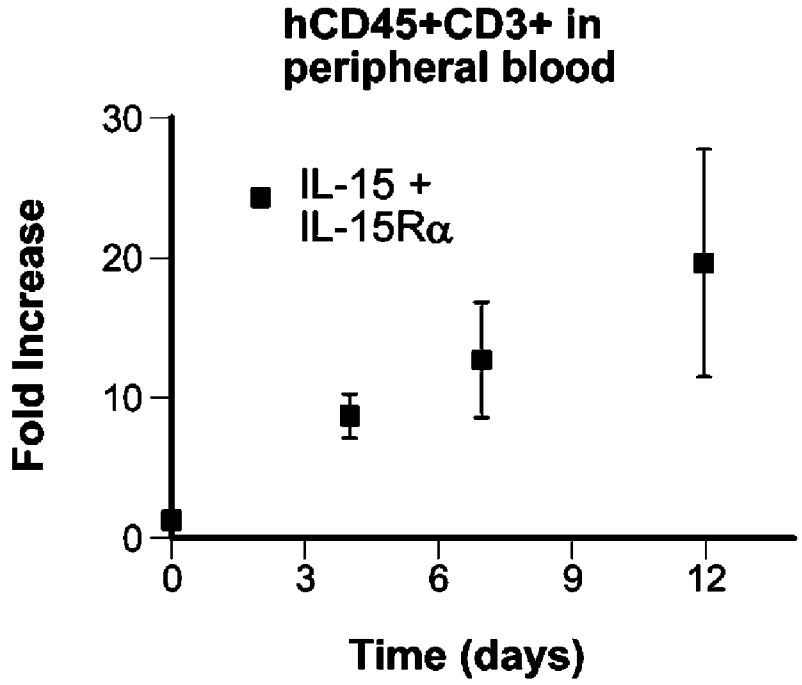


FIG. 14C

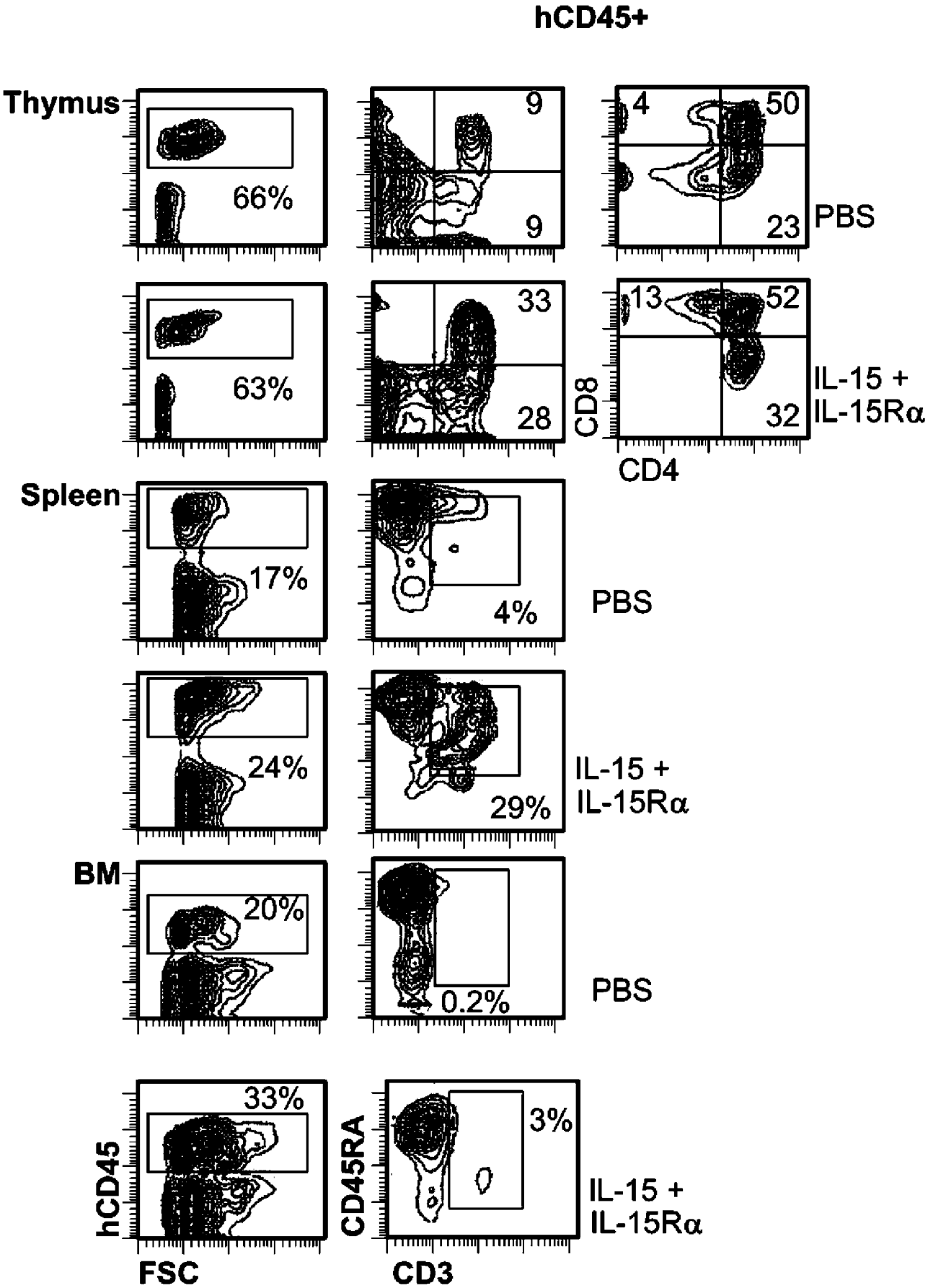

hCD45+
Thymus
66%
9
9
4
50
23
PBS
63%
33
28
13
52
32
IL-15 +
IL-15Rα
CD8
CD4
Spleen
17%
4%
PBS
24%
29%
IL-15 +
IL-15Rα
BM
20%
0.2%
PBS
33%
3%
0
IL-15 +
IL-15Rα
hCD45
FSC
CD45RA
CD3

FIG. 14B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/068826

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K67/027
ADD.

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)
A01K

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 practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEGRAND N ET AL: "Experimental models to study development and function of the human immune system in vivo" JOURNAL OF IMMUNOLOGY, AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 176, no. 4, 1 February 2006 (2006-02-01), pages 2053-2058, XP002428076 ISSN: 0022-1767 page 2054 page 2056 page 2057 figure 1	1-6

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* 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

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 2010	09/06/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Brero, Alessandro

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/068826

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GIMENO R ET AL: "Monitoring the effect of gene silencing by RNA interference in human CD34<+> cells injected into newborn RAG2<-/-> [gamma]c <-/-> mice: Functional inactivation of p53 in developing T cells" BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 104, no. 13, 15 December 2004 (2004-12-15), pages 3886-3893, XP002317351 ISSN: 0006-4971 cited in the application abstract page 3887 - page 3890 figure 3	1-3,5,6
X,P	HUNTINGTON NICHOLAS D ET AL: "IL-15 trans-presentation promotes human NK cell development and differentiation in vivo" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 206, no. 1, January 2009 (2009-01), pages 25-34, XP002571965 ISSN: 0022-1007 cited in the application the whole document	1-6, 16-20
A	HUNTINGTON NICHOLAS D ET AL: "Developmental pathways that generate natural-killer-cell diversity in mice and humans" NATURE REVIEWS IMMUNOLOGY, vol. 7, no. 9, September 2007 (2007-09), pages 703-714, XP002571966 cited in the application page 712	1-6, 16-20
A	RUBINSTEIN MARK P ET AL: "Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 103, no. 24, 13 June 2006 (2006-06-13), pages 9166-9171, XP002521796 ISSN: 0027-8424 cited in the application abstract page 9168 figure 2B	1-6, 16-20

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/068826

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MORTIER ERWAN ET AL: "IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation." THE JOURNAL OF EXPERIMENTAL MEDICINE 12 MAY 2008, vol. 205, no. 5, 12 May 2008 (2008-05-12), pages 1213-1225, XP002571967 ISSN: 1540-9538 cited in the application abstract	1-6, 16-20
A	HUNTINGTON N D ET AL: "Humanized immune system (HIS) mice as a tool to study human NK cell development" CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, SPRINGER, BERLIN, DE, vol. 324, 1 January 2008 (2008-01-01), pages 109-124, XP009130427 ISSN: 0070-217X cited in the application page 116 - page 120	1-6, 16-20
A	WO 2008/069659 A1 (AZ UNIV AMSTERDAM [NL]; WEIJER KEES [NL]; LEGRAND NICOLAS [NL]) 12 June 2008 (2008-06-12) abstract; claim 18 figures 1,2 page 13, line 24 - line 25	1-6, 16-20
A	DUBOIS SIGRID ET AL: "Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8(+)/CD44(high) T cells and its antitumor action" JOURNAL OF IMMUNOLOGY, vol. 180, no. 4, February 2008 (2008-02), pages 2099-2106, XP002582129 ISSN: 0022-1767 cited in the application abstract page 2100 figures 1,2	1-6, 16-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2009/068826

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.:
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:

see additional sheet

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 an 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.:

1-6, 16-20

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 4-6

A transgenic mouse (i) being deficient for murine T lymphocytes, B lymphocytes and NK cells, (ii) being engrafted with human hematopoietic cells and (iii) containing innate lymphocytes as well as as adaptive CD4 and CD8 T lymphocytes.

---

2. claims: 2, 3, 16-20

A transgenic mouse according to invention 1, wherein the innate lymphocytes as well as adaptive CD4 and CD8 T lymphocytes are induced through administration of an IL-15 or IL-15R[alpha] agonist, and subject-matter relating thereto.

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3. claims: 7-14

A method for increasing the number of innate lymphocytes as well as adaptive CD4 and CD8 T lymphocytes in a subject, comprising the administration of an IL-15 or IL-15-R agonist.

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4. claim: 15

A method for identifying a compound that induces maturation of innate human lymphocytes as well as adaptive CD4 and CD8 T lymphocytes comprising the steps set forth in claim 15.

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5. claims: 21-23

A method for preparing humanized antibodies, or fragments thereof, comprising the steps set forth in claim 21, and subject-matter relating thereto.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2009/068826

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008069659 A1	12-06-2008	EP 2088854 A1	19-08-2009
		US 2010115642 A1	06-05-2010