IL-15 transpresentation promotes both human T-cell reconstitution and T-cell–dependent antibody responses in vivo

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Cytokine immunotherapies targeting T lymphocytes are attractive clinical interventions against viruses and tumors. In the mouse, the homeostasis of memory α/β CD8$^+$ T cells and natural killer (NK) cells is significantly improved with increased IL-15 bioavailability. In contrast, the role of "transpresented" IL-15 on human T-cell development and homeostasis in vivo is unknown. We found that both CD8 and CD4 T cells in human immune system (HIS) mice are highly sensitive to transpresented IL-15 in vivo, with both naïve (CD62L$^+$CD45RA$^+$) and memory phenotype (CD62L$^-$CD45RO$^+$) subsets being significantly increased following IL-15 "boosting." The unexpected global improvement in human T-cell homeostasis involved enhanced proliferation and survival of both naïve and memory phenotype peripheral T cells, which potentiated B-cell responses by increasing the frequency of antigen-specific responses following immunization. Transpresented IL-15 did not modify T-cell activation patterns or alter the global T-cell receptor (TCR) repertoire diversity. Our results indicate an unexpected effect of IL-15 on human T cells in vivo, in particular on CD4$^+$ T cells. As IL-15 promotes human peripheral T-cell homeostasis and increases the frequency of neutralizing antibody responses in HIS mice, IL-15 immunotherapy could be envisaged as a unique approach to improve vaccine responses in the clinical setting.

In vivo studies of lymphocyte development, homeostasis, and immune responses upon infection, antigenic challenge, or following vaccination have been largely characterized in mice. Although this line of experimentation is valuable, 60 million years of evolution have generated important differences between murine and human immune systems, and therefore some of the knowledge derived from mouse models may not be directly applicable to humans. 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 involves engraftment of newborn BALB/c Rag2$^{-/-}$ $\gamma_c{}^{-/-}$ mice with human hematopoietic stem cells (HSCs) from fetal liver or cord blood, which generates human innate and adaptive lymphocytes and dendritic cell subsets required for immune responses (1–3). HIS mice are proving to be a very powerful biotechnology, and although they are successfully used to model human hematopoiesis, their capacity for studying human immune responses is suboptimal (2). This is likely due to perturbed homeostasis of human T cells in BALB/c Rag2$^{-/-}$ $\gamma_c{}^{-/-}$ HIS mice, as these cells exhibit an abnormally high turnover rate and fail to accumulate (1–5). Not surprisingly, many current efforts are focused on improving human T-cell reconstitution and homeostasis in HIS mice with the ultimate goal of inducing robust and consistent human immune responses in vivo.

T-cell homeostasis comprises T-cell generation in the thymus, export to the periphery, maintenance of the peripheral naïve T-cell pool, and regulation of activated effector and memory T-cell compartments (6). Several signals have been implicated in controlling T-cell homeostasis, including those emanating from the T-cell receptor (TCR) following interactions with self-peptide + major histocompatibility complex (pMHC) and those induced by growth factors, including cytokines (6). The common cytokine receptor gamma chain (γ_c) family of cytokines (which comprises IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) in particular have been demonstrated to play a role in T-cell homeostasis in mammals (7). Humans and mice possessing mutations in genes encoding the γ_c, Jak3 (both critical for signal transduction following binding γ_c cytokines) or the alpha chain of the IL-7 receptor (IL-7Rα), display a severe block in T-cell development and resulting severe combined immunodeficiency (8, 9). The γ_c-dependent cytokine IL-15 is unusual because its bioactive form is a functional complex associated with the IL-15Rα chain. Thus, cells expressing IL-15 such as monocytes, dendritic cells, and stromal cells must also coexpress the IL-15Rα to "transpresent" IL-15 to IL-15–responsive cells (that express the IL-2Rβ/γ_c complex). Accordingly, both IL-15 and IL-15Rα are up-regulated on myeloid cells following inflammation, thereby increasing IL-15 bioavalability (10–12).

We demonstrated that transpresented murine IL-15 inefficiently triggered human natural killer (NK) cells in vitro and in vivo providing an explanation for the poor human NK cell reconstitution in BALB/c Rag2$^{-/-}$ $\gamma_c{}^{-/-}$ HIS mice (3). Exogenous administration of a potent human IL-15R agonist (referred to as RLI, consisting of human IL-15 covalently linked to an extended human IL-15Rα "sushi" domain thus mimicking IL-15 transpresentation) (13–15) was sufficient to restore human NK cell development in HIS mice (3). Whereas memory CD8$^+$ T cells in mice are highly responsive to exogenous IL-15 (6, 11–14), naïve CD4$^+$ and CD8$^+$ T cells are not thought to require IL-15 for normal homeostasis (6, 11–14). However, in vivo effects of human

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IL-15 on human T cells have not been studied, and it remained possible that the poor reactivity of human T cells to mouse IL-15 might also contribute to the low human T-cell reconstitution in the HIS mouse model. Here we show that human IL-15 transpresentation increases human T-cell reconstitution and the frequency of T-cell–dependent antibody responses in HIS mice. These studies provide a first preclinical trial of transpresented human IL-15 on human T cells in vivo and indicate that increased IL-15 bioavailability globally boosts human naïve and memory T-cell homeostasis in this humanized mouse model. Our findings offer a unique approach to study human T-cell immune responses in vivo and suggest that IL-15 immunotherapy may be useful to promote global T-cell reconstitution in humans.

Results

Improved Development of Human CD4$^+$ and CD8$^+$ T Cells in HIS Mice Receiving RLI. We and others have recently reported that human fetal liver HSCs (CD34$^+$CD38$^-$) engrafted into newborn BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice develop into mature myeloid and lymphoid cells (1–5). We used this approach to investigate the effect of transpresented human IL-15 on human T-cell development in vivo. Eight weeks after HSC engraftment, HIS mice were injected with the potent human IL-15/IL-15Rα agonist, RLI (13–15) (Fig. 1*A*). Administration of RLI resulted in a significant increase of human hematopoietic cells in the thymus and spleen and a specific increase in CD3$^+$ cells in the bone marrow (Fig. 1*B*). CD8$^+$ T cells were significantly augmented in the spleen ($P = 0.025$), blood ($P = 0.027$), and thymus ($P = 0.033$) following RLI treatment in HIS mice, whereas CD4$^+$ T cells were also augmented in spleen ($P = 0.017$) and lymph node ($P = 0.025$) (Fig. 1 *B* and *C*). In addition, NK cells were also augmented as previously reported (3), whereas B-cell numbers were not significantly altered (Fig. S1*A*). Thus, the increase in total human hematopoietic cells in the thymus ($P = 0.0005$) and spleen ($P = 0.002$) following RLI treatment is attributed to significant increases in total T-cell numbers (Fig. 1*B*).

RLI Promotes Proliferation of Naïve and Memory Phenotype Peripheral CD4$^+$ and CD8$^+$ T Cells in HIS Mice. Because RLI was effective in increasing both CD4$^+$ and CD8$^+$ T cells, we next

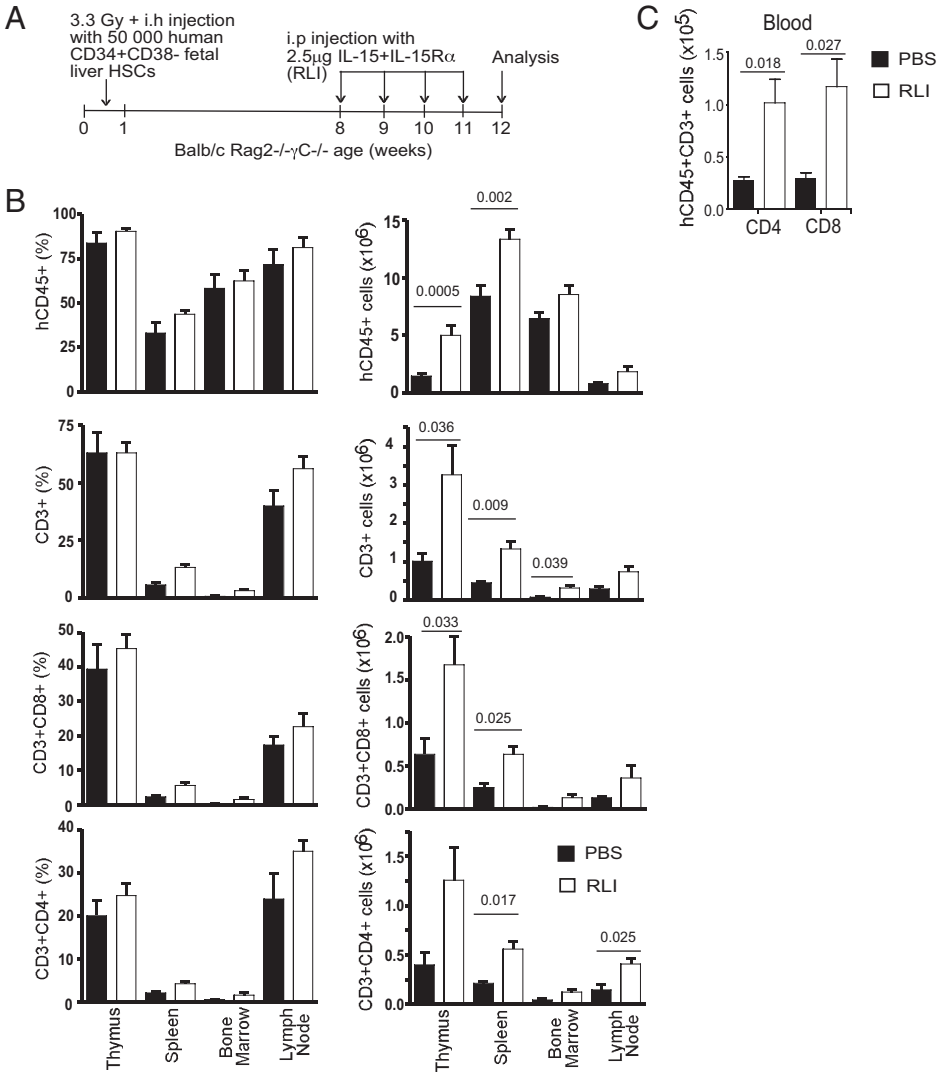

Fig. 1. RLI enhances human CD4$^+$ and CD8$^+$ T-cell development and reconstitution of lymphoid organs in HIS mice. (*A*) Experimental scheme. Newborn Rag2$^{-/-}\gamma_c^{-/-}$ mice were irradiated with 3.3 Gy injected intrahepatically (i.h.) with 5×10^4 CD34$^+$CD38$^-$ human fetal liver cells. At 8, 9, 10, and 11 wk of age, HIS mice were injected intraperitoneally (i.p) with 2.5 μg IL-15-IL–15Rα fusion protein (RLI) or PBS. Mice were killed and analyzed at 12 wk. (*B*) Lymphoid organs and (*C*) peripheral blood from HIS mice were analyzed for either total human hematopoietic cell (human CD45; hCD45$^+$) and human T-cell (hCD45$^+$CD3$^+$ and CD4$^+$ or CD8$^+$) reconstitution by flow cytometry, and cellularity was enumerated. hCD45$^+$ (%) is the percentage of total cells, whereas CD3$^+$, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ (%) are the percentages within the hCD45$^+$ population. Values represent mean ± SEM of nine mice per group.

investigated whether any phenotypic changes were induced on the expanded T-cell populations. We failed to observe any differences in classical T-cell markers (CD27 and CD28) or any change in the proportion of naïve markers (CD62L and CD45RA) on $CD4^+$ and $CD8^+$ T cells in the bone marrow, spleen, mesenteric lymph node (mLN), liver, and lungs of RLI-treated HIS mice (Fig. S1 *B* and *C* and Fig. S2 *A* and *B*). The expression of receptors for γ_c cytokines (including CD122, CD127, and CD25) were unchanged following RLI treatment as were the populations of regulatory T cells ($CD4^+CD25^+$) (Fig. S1 *B* and *C* and Fig. S2*B*). Because γ_c cytokines such as IL-2 and IL-7 influence T-cell proliferation and survival, we analyzed the serum concentration of these and other cytokines that are produced by IL-15 responsive lymphocytes such as NK cells. The serum concentration of IL-2 and IL-7 were unchanged in mice treated with RLI and were only slightly above the level of Luminex detection (Fig. S2*C*). Furthermore, we failed to observe any increase in IFN-γ or TNF-α in RLI-treated mice, suggesting that increases in T cells and NK cells are not associated with hyperproduction of proinflammatory cytokines (Fig. S2*C*).

The clear augmentation in T-cell numbers without a change in the relative frequency of naïve T cells suggested that RLI potentially targeted both naïve and memory phenotype T cells. To test this hypothesis, analysis of BrdU incorporation into T cells in RLI-treated HIS mice was performed. Proliferation of naïve ($CD45RA^+$) and memory phenotype ($CD45RA^-$) $CD4^+$ and $CD8^+$ T cells in the spleen and mesenteric lymph node was significantly increased following RLI treatment in vivo (Fig. 2 *A*–*C*). $CD8^+$ T cells, in particular those with a naïve phenotype ($CD45RA^+$), showed the greatest fold increase in proliferation after RLI treatment followed by naïve $CD4^+$ T cells, memory phenotype $CD8^+$ T cells, and, lastly, memory phenotype $CD4^+$ T cells (Fig. 2 *A*–*C*). The relative responsiveness of T-cell populations to IL-15 treatment can be compared with that of NK cells that are highly IL-15 dependent (Fig. 2 *A*–*C*). Intracellular staining for prosurvival proteins in peripheral T cells revealed increased expression of Bcl-x_L and Bcl-2 protein levels in $CD8^+$ T cells, whereas levels of these proteins appeared unchanged in $CD4^+$ T cells following RLI treatment (Fig. S1*D*).

In addition to soluble factors, MHC molecules have been shown to play an important role in peripheral T-cell homeostasis (6). Interactions with MHC class I molecules expressed on nucleated cells and MHC class II molecules expressed on antigen-presenting cells provide signals that maintain peripheral $CD8^+$ and $CD4^+$ T cells, respectively (6). We found that human cells in RLI-treated HIS mice expressed increased levels of MHC class I and class II compared with PBS-treated control HIS mice (Fig. S3). The enhanced human MHC expression could provide an explanation for the improved T-cell homeostasis in IL-15/IL-15Rα–treated HIS mice, although the precise mechanism behind this observation remains to be elucidated.

Analysis of TCR β Repertoires in IL-15 "Boosted" HIS Mice. The maintenance of a highly diverse TCR repertoire is essential for efficient immune responses against the extensive number of foreign and altered-self antigens encountered during our lifetime. We next examined the effect of RLI on the TCR repertoire by analyzing complementarity-determining region 3 (CDR3)-length profiles of the β, γ, and δ TCR chains expressed by splenic T cells. Although a limited number of peripheral T cells are generated in

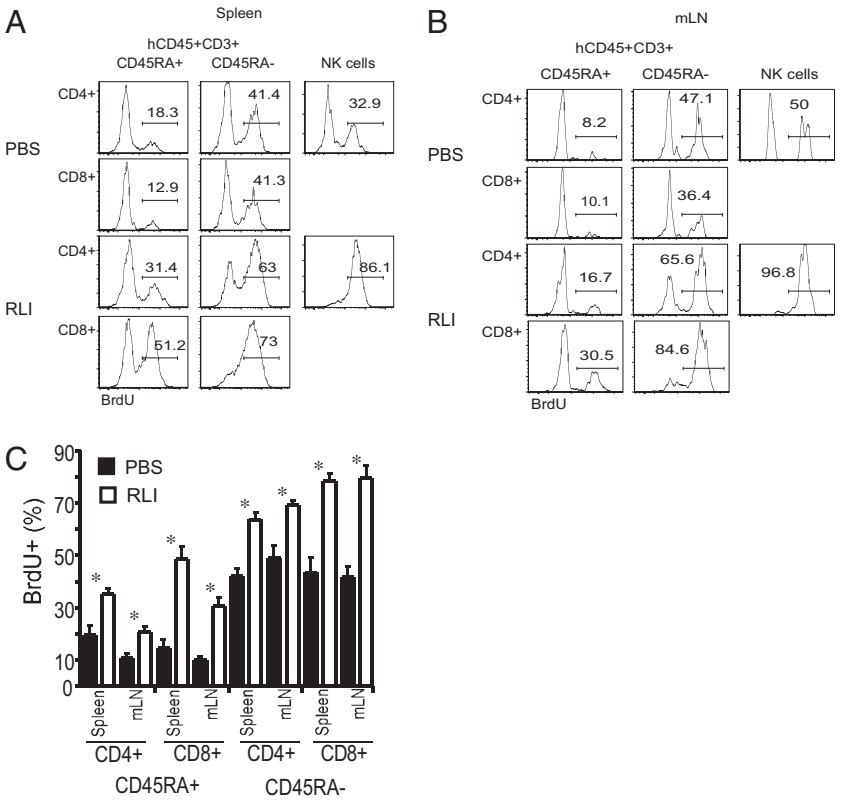

Fig. 2. RLI treatment in vivo enhances both $CD8^+$ and $CD4^+$ naïve and memory phenotype T-cell proliferation. HIS mice treated as in Figure 1*A* were injected intraperitoneally with 1 mg BrdU daily on the last 2 d before being killed. $CD4^+$ and $CD8^+$ human T cells ($hCD45^+CD3^+$) from (*A*) spleen and (*B*) mLN were analyzed by flow cytometry for surface antigens corresponding to activation status (CD45RA) and incorporation of BrdU. FACS plots are representative of three individual experiments. BrdU uptake by human NK cells ($hCD45^+NKp46^+$) in the same organ is shown as a positive control for IL-15 responsiveness. (*C*) Summary histograms of $BrdU^+$ T-cell populations shown in *A* and *B* from three individual experiments. $^*P < 0.05$.

HIS mice, the TCR repertoire of the peripheral T-cell pool appears diverse, with T cells bearing TCRs from all Vβ family members and demonstrating varying CDR3 lengths (Fig. S4 *A* and *B*). Importantly, RLI administration did not skew the TCR repertoire compared with control HIS mice, suggesting that IL-15 globally boosts the available broad TCR pool. This is a valuable observation considering our model of humanized mice is becoming more popular for the study of T-cell immune responses and suggests that an extensive TCR repertoire is generated providing a cellular substrate that could react against a large number of peptide antigens.

Using CDR3 immunoscope analysis of γ and δ variable chains we found that most human γ/δ T cells found in the periphery of HIS mice use Vδ2 (similar to human peripheral blood mononuclear cells, PBMCs); this preferential use is unaffected by RLI treatment (Fig. S4 *C* and *D*). Interestingly, we observed two large populations of $V\gamma8^+$ and $V\gamma9^+$ T cells that each represent between 40% and 60% of total γ/δ T cells and who pair almost exclusively with Vδ2, although we observed occasional use of Vδ3, -5, and -8 (Fig. S4*C*). In vivo administration of RLI did not influence the relative γ and δ chain use nor the variability in CDR3 length for any given Vγ or Vδ chains (Fig. S4*D*).

Improved Development of Human $CD4^+$ and $CD8^+$ T Cells in HIS Mice Receiving RLI Results in Improved Humoral Responses Following Immunization. We next investigated whether improved human T-cell reconstitution impacted on immune functions in HIS mice. Because antigen-specific T-cell responses, particularly cytotoxic T-cell responses (cytotoxic T lymphocytes, CTLs) remain poorly elicited in HIS mice, we characterized T-dependent antigen-specific B responses that can be evoked following vaccination of HIS mice. To this end, HIS mice cohorts were immunized with commercial hepatitis B virus (HBV) and tetanus toxoid (TT) vaccines. Although serum IgM levels remained unchanged following RLI treatment, significant increases in total IgG levels were observed in RLI-treated immunized HIS mice (Fig. 3*A*). Using a diagnostic ELISA, we were able to detect anti-TT–specific IgG and more frequently anti-HBV surface antigen (HbsAg)-specific Ig in the sera of immunized HIS mice (Fig. 3 *B* and *C*). Interestingly, although RLI treatment did not modify the concentration of antigen-specific antibody in mice that mounted an antigen-specific Ig response (Fig. 3 *B* and *C*), the frequency of "responders" (HIS mice with either >0.5 IU/mL anti-TT–specific IgG or >7 IU/L anti-HbsAg–specific Ig) was significantly enhanced following RLI treatment ($P < 0.05$; $c^2 = 3.85$) (Fig. 3*D*). Thus, the improvement in human T-cell homeostasis in RLI-treated HIS mice increased the likelihood of inducing antigen-specific humoral responses following immunization.

Discussion

HIS mouse models have been continually improved over the past 30 y and are now at a stage where both antibody and cellular human immune responses can be elicited (16–19). Given that the immune system in this HIS mouse model is skewed toward B-cell development, there is a clear need to improve T lymphopoiesis and homeostasis (1, 2). We recently reported a beneficial effect on human thymopoiesis following human IL-7 treatment in HIS mice; however, peripheral T-cell numbers remained unchanged despite transient increases in thymocyte numbers, suggesting that other factors are involved (5). Given that human lymphocytes are poorly triggered by murine IL-15 (3), we hypothesized that a similar mechanism may explain the poor T-cell homeostasis that is observed in this HIS mouse model.

Previous studies in mice demonstrated increased $CD8^+$ T-cell numbers following treatment with transpresented mouse IL-15 (14, 21). Mouse $CD4^+$ T cells do not respond to IL-15 in vivo, but human $CD4^+$ T cells can express IL-2Rβ and γ_c, raising the possibility that these cells could respond to IL-15 in vivo. In this manuscript, we validate this hypothesis by showing that transpresented human IL-15/IL-15Rα complexes (RLI) can induce both naïve and memory human $CD4^+$ and $CD8^+$ T cells to proliferate in vivo, thereby identifying a fundamental difference between mouse and human T cells with respect to cytokine-regulated T-cell homeostasis. The RLI treatment in HIS mice resulted in a clear increase in global T-cell numbers by promoting both the survival and proliferation of peripheral human T cells.

The observation that IL-15 receptor ligation promotes naïve $CD4^+$ T cell proliferation in vivo differs from in vitro studies where IL-15 only promoted the generation of effector memory phenotype $CD4^+$ T cells from central memory phenotype $CD4^+$ T cells (20). Whereas previous studies of IL-15 actions on human T cells were performed in vitro, our results represent a first preclinical study of transpresented human IL-15 on human T cells in vivo. Transpresented human IL-15 may directly stimulate human naïve and memory T cells in vivo in HIS mice or, alternatively, may activate myeloid cells that then indirectly influence human T-cell homeostasis. Some evidence for the latter was provided by the observation of increased human MHC class I and II expression in RLI-treated HIS mice. Thus, our study highlights the value of the HIS model to uncover differences between mouse and human T-cell biology and to study human immunology in vivo.

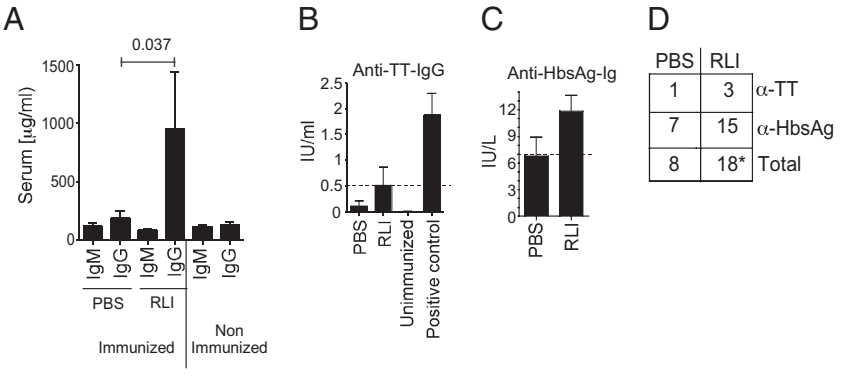

Fig. 3. Increased frequency of T-dependent B-cell responses following RLI treatment in vivo. (*A*) Total serum IgM and IgG concentrations were determined by ELISA in nonimmunized HIS mice and HIS mice immunized with vaccines against hepatitis B virus (HBV) and tetanus toxoid (TT). Values represent mean ± SEM of 16 mice per group. (*B*) TT-specific serum IgG or (*C*) HBV surface antigen (HbsAg)-specific serum Ig from immunized HIS mice were determined by ELISA. Values represent mean ± SEM of 29 mice per treatment group. (*D*) Frequency of "vaccine responders" (mice with serum values >0.5 IU/mL anti-TT IgG or >7 IU/L anti-HbsAg-Ig) as determined in *B* and *C*. χ^2 value *$c^2 = 3.85$, $P < 0.05$.

IL-15–treated Macaques displayed increases in CD8 T cells, NK cells, and to a lesser extent CD4 T cells, which 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 nonspecific dermatitis, which was avoided when IL-15 was given intermittently (22). In contrast, we failed to detect any obvious signs of toxicity in HIS mice using our treatment regime of transpresented hIL-15. This observation may signal a fundamental difference between the bioavailability of IL-15 when administered as a soluble cytokine or as an IL-15Rα-coupled complex.

We have previously shown that RLI is effective in an NK cell-dependent murine metastatic melanoma and colorectal cancer model (23), whereas others have reported similar effects with $CD8^+$ T-cell–dependent systems (24, 25). Furthermore, IL-15 has been shown to induce antigen-independent expansion of naïve, virus-specific, and tumor-specific cytotoxic $CD8^+$ T cells in vitro (14, 26–29) and rescue tolerant $CD8^+$ T cells for use in adoptive immunotherapy of established tumors (30). IL-15 has also been shown to enhance number, function, and survival of HIV-specific $CD8^+$ T cells (31, 32), suggesting that transpresented 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. Increased peripheral $CD4^+$ T-cell numbers may account for the more frequent T-dependent antibody responses observed in RLI-treated HIS mice, as the frequency of antigen-reactive T cells may be increased. Our results suggest that the globally improved human T-cell homeostasis obtained following hIL-15 transpresentation could substantially extend the use of this immunomodulator for treating human immunodeficiency states and for augmenting response rates in human vaccine trials.

Materials and Methods

Mice. BALB/c $Rag2^{-/-}\gamma_c^{-/-}$ mice were generated by intercrossing B6 $Rag2^{-/-}$ (33) and B6 $\gamma_c^{-/-}$ (34) and backcrossing these mice to the BALB/c background. Mice were maintained in isolators with autoclaved food and water. Mice with an HIS were generated as previously described (1–3). Briefly, newborn (3–5 d old) $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice received sublethal (3.3 Gy) total body irradiation from a cesium source, and were injected intrahepatically (i.h.) with 5×10^4 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.

In Vivo Assays and Treatments. HIS mice were injected intraperitoneally (i.p.) with 100 μL of 2.5 μg RLI or PBS alone, commencing at either 5–6 wk after reconstitution or 8 wk after reconstitution. A total of 100 μL of 10 mg/mL of BrdU (BD Bioscience) was injected i.p. daily 2 d before analysis. HIS mice were immunized by intramuscular route (biceps femoris) using a 29-gauge needle, three times, on weeks 12, 14, and 16 with 100 μL of the HBV vaccine (Engerix-B; GlaxoSmithKline) or 50 μL of the tetanus vaccine (vaccin tétanique pasteur; Sanofi Pasteur). These amounts correspond to 1/10th of the normal human dose. Negative controls received the same volume of PBS buffer. Two weeks after the last immunization, HIS mice were killed for analysis.

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), CD86 (2331), CD127 (hIL-7R-M21), HLA-DR (G46-6), HLA-A/B/C (G46-2.6), CD45 (2D1), CD69 (L78), Bcl-2 (Bcl-2/100), IFN-γ (XMG1.2), BrdU (B44) from BD Bioscience; KIR2DL2/L3 (DX27), KIR3DL1 (DX9), CD28 (CD28.2), CD27 (O323), CD62L (DREG-56) from Biolegend; KIR2DS4 (FES172), KIR2DL1/DS1 (EB6B), KIR2DL2/L3/DS2 (GL183), KIR3DL1/DS1 (Z27.3.7), Vγ9 (IMMU 360), Bcl-xL (7B2.5) from Beckman Coulter; Granzyme-B (GB11) from Caltag (Invitrogen); and CD25 (BC96), γ/δ TCR (B1.1), CCR7 (3D12), and CD45RA (HI100) from eBioscience. Intracellular staining was performed after fixation and permeabilization of the cellular suspensions using BD Perm/Wash and BD Cytofix/Cytoperm reagents from BD Bioscience according to manufacturer instruction. For BrdU detection, cells were incubated for 1 h at 37 °C with 30 μg DNase from BrdU flow kits (BD Bioscience). All washings and reagent dilutions were done with PBS containing 2% 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).

Cell Preparation. Human fetal liver was obtained from elective abortions, with gestational age ranging from 14 to 20 wk. Experiments using human fetal liver cells were approved by the medical and ethical committees at the Institut Pasteur and Academic Medical Center–University of Amsterdam and performed in full compliance with French law. Single-cell suspensions of fetal material was achieved by mechanical disruption using a Stomacher Biomaster laboratory system (Seward). Magnetic enrichment of $CD34^+$ cells (>98% pure) was performed by using the CD34 Progenitor Cell Isolation kit (Miltenyi Biotech), after preparation of single-cell suspension and isolation of mononuclear cells by density gradient centrifugation over Ficoll-Hypaque (Nycomed Pharma). Cell suspensions were prepared in RPMI medium with 2% FCS. Single-cell suspensions of murine organs were prepared as previously described (35).

TCR Vβ,γ,δ and CDR3 Immunoscope Analysis. Twelve weeks after $CD34^+CD38^-$ HSC engraftment, HIS mice were killed and single-cell suspensions of splenocytes were prepared. Red cell 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 manufacture instructions. TCR Vβ immunoscope was performed as previously described (36). Briefly, cDNA was prepared and real-time PCR performed by combining primers for the different Vβ,γ,δ 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.

ELISA. The plasma harvested from HIS mice was screened by ELISA for the presence of antigen-specific and total antibodies. The 96-well plates were coated either with Engerix B (GlaxoSmithKline) (10× diluted in PBS) or 10 μg/mL goat antihuman IgM or 10 μg/mL goat antihuman IgG (Jackson ImmunoResearch Laboratories) in PBS for 1 h at 37 °C or overnight 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 antihuman IgM and IgG antibodies (Jackson ImmunoResearch Laboratories) followed by TMB substrate/stop solution (Biosource). Alternatively, HBsAg specific Ig or tetanus toxoid-specific IgG were measured using Monolisa (Biorad) or RIDASCREEN (R-Biopharm) ELISA kits, respectively, according to manufacturer instructions.

Luminex Cytokine Detection. Sera from HIS mice were prepared from total blood at time of sacrifice by centrifugation at 13,000 rpm for 10 min at 4 °C. Cytokine human 25-plex panel Luminex assays were performed according to manufacture instructions (Invitrogen).

Statistical Analysis. Statistical analyses were performed using GraphPad Prism (GraphPad Software). All data were subjected to two-tailed unpaired Student's *t* test analysis. In addition, data concerning vaccine responders were subjected to χ^2 analysis with 1 degree of freedom. The obtained *P* values were considered significant when $P < 0.05$.

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