Lymphoid development; a dynamic interplay of timing and dosing
van Lent, A.U.G.

Citation for published version (APA):
van Lent, A. U. G. (2009). Lymphoid development; a dynamic interplay of timing and dosing
IL-7 enhances thymic human T cell
development in “Human Immune System”

Rag2\(^{-/-}\)γc\(^{-/-}\) mice without affecting
peripheral T cell homeostasis

Anja U. van Lent, Wendy Dontje, Maho Nagasawa, Rachida Siamari,
Arjen Q. Bakker, Stephan M. Pouw, Kelly A. Maijoor, Kees Weijer,
Jan J. Cornelissen, Bianca Blom, James P. Di Santo, Hergen Spits
and Nicolas Legrand

Submitted
Abstract

Interleukin-7 (IL-7) is a central cytokine in the development of hematopoietic cells, although interspecies discrepancies have been reported. By co-culturing human post-natal thymus hematopoietic progenitors and OP9-huDL1 stromal cells, we found that murine IL-7 is around 100-fold less potent than human IL-7 for supporting human T cell development in vitro. We investigated the role of human IL-7 in newborn BALB/c Rag2⁻/⁻γc⁻/⁻ mice transplanted with human hematopoietic stem cells (HSC) as an in vivo model of human hematopoiesis, using three approaches to improve IL-7 signaling: administration of human IL-7; ectopic expression of human IL-7 by the transplanted human HSC; or enforced expression of a murine/human chimeric IL-7 receptor binding murine IL-7. We show that premature IL-7 signaling at the HSC stage, prior to entrance in the thymus, impeded T cell development, whereas increased intra-thymic IL-7 signaling significantly enhanced the maintenance of immature thymocytes. Increased thymopoiesis was also observed when we transplanted BCL-2- or BCL-XL-transduced human HSC. Homeostasis of peripheral mature T cells was not improved by any of these strategies. Overall, our results provide evidence for an important role of IL-7 in human T cell development in vivo and highlight the notion that IL-7 availability is but one of many signals that condition peripheral T cell homeostasis.
Introduction

IL-7 is a major cytokine for the control of lymphocyte differentiation, the maintenance of cell viability and the regulation of lymphocyte homeostasis (1, 2). The receptor for IL-7 comprises two chains, the IL-7 receptor alpha chain (IL-7Rα/CD127) and the common gamma chain (γc/CD132) that is shared by the receptors for IL-2, IL-4, IL-9, IL-15 and IL-21. IL-7 is produced in a constitutive manner by stromal cells located in a variety of non-lymphoid and lymphoid tissues, including thymus, spleen, bone marrow, lymph nodes, skin and intestine, but is not expressed by hematopoietic cells (3). Lymphopenia in mice, humans and non-human primates is associated with elevated levels of IL-7 in the blood and in tissues (4-7). In contrast, the expression of CD127 is tightly regulated during development and maturation of hematopoietic cells, leading to the concept that availability of IL-7 is critically conditioned by its utilization rather than regulation of its production (8).

The expression of CD127 is mostly restricted to hematopoietic cell lineages, e.g. lymphoid progenitors, developing T and B cells, mature T cells and bone marrow-derived macrophages (3, 8-10). The expression of CD127 is dynamically regulated during hematopoiesis. Constitutive expression of CD127 impacts negatively on thymopoiesis, probably due to binding of IL-7 by double-positive thymocytes leading to starvation of their double-negative progenitors (11). Mice transgenic for IL-7 exhibit enhanced numbers of T and B lymphocytes in peripheral lymphoid organs (12, 13), and can eventually develop B cell lymphomas (14). Consequently, IL-7 has been implicated in induction of cell proliferation, control of cell metabolism, maintenance of cell survival and antigen receptor V(D)J rearrangements in developing T and B cells (3, 8, 15). As far as lymphocyte survival is concerned, IL-7 enhances B cell lymphoma (BCL)-2 expression in freshly isolated mouse T cells (16), and mouse T cell cultures are protected from dexamethasone-induced apoptosis in presence of IL-7, correlating with enhanced expression of BCL-2 and BCL-XL anti-apoptotic factors (17). Overall, mouse experiments indicate that the homeostatic maintenance of naïve T cells is strongly dependent on IL-7 levels, especially naive CD8+ T cells, but other signals are also proven important, e.g. TCR/MHC interactions (1, 18, 19).

Genetic ablation of IL-7 or CD127 expression in mice leads to severe reduction in numbers of thymocytes, B cell progenitors in the bone marrow, and consequently peripheral T and B cells (20, 21). The peripheral B and T cell lymphopenia observed in CD127−/− mice can be at least partially recovered by a Bcl-2 transgene (22, 23). In contrast, human patients with
deficiencies for the expression of CD127 or the downstream Janus kinase (JAK)3 completely lack T cells, but exhibit normal or increased numbers of NK cells and circulating B cells (24-28). Altogether, these discrepancies between mouse and human studies highlight the existence of major inter-species differences for the role of IL-7 in lymphocyte development and homeostasis. In order to further clarify the role for IL-7 in human lymphopoiesis, in vitro experimental models such as fetal thymic organ cultures (FTOC) (29) or Notch ligand-expressing stromal cells (OP9-hDL1) (30) have been used. Alternatively, the role of IL-7 can be addressed in vivo, using mouse models of human hematopoiesis and immunity (31-33). We and others have constructed such “human immune system” (HIS) mice by transplanting human hematopoietic stem cells (HSC) into conditioned newborn BALB/c Rag2−/−γc−/− recipient mice, and the resulting HIS (BALB-Rag/γ) mice exhibit multilineage reconstitution by human lymphoid and myeloid cells – although in a sub-optimal fashion (34, 35). Limited de novo human thymopoiesis and peripheral T cell accumulation is a common feature of several mouse models humanized for the immune system. Peripheral human T cell numbers represent ~1% of what is found in Rag2−/−γc−/− mice reconstituted with murine fetal liver stem cells (36). To some extent, the sub-optimal amount of human T lymphocytes in HIS (BALB-Rag/γ) mice resembles what is reported in mice with deficient IL-7 signaling (20, 21).

In this study, we examined the in vivo role of IL-7 on human hematopoiesis – and particularly T cell development and peripheral maintenance – in HIS (BALB-Rag/γ) mice. We asked whether poor cross-reactivity of murine IL-7 in vivo could explain at least partially the suboptimal levels of human reconstitution in mouse xenograft recipients. We therefore used several approaches to enhance IL-7 signaling in the human cells generated in the HIS (BALB-Rag/γ) mice, and we analyzed their respective impact on generation and maintenance of human immune cells.

Material and methods

Constructs and production of viral supernatants
The cDNA sequence encoding human CD127 and a chimeric murine-human CD127 were inserted into the multiple cloning site of the LZRS vector upstream of an internal ribosomal entry site (IRES) and enhanced green (GFP) or yellow (YFP) fluorescent protein, respectively (37). Control vectors were empty LZRS IRES-GFP and LZRS IRES-YFP. Retroviral supernatants were produced as described (38), using the 293T-based Phoenix
packaging cell line (39). Similarly, the cDNA sequence encoding human BCL-2 or BCL-XL was inserted in the LZRS vector. The cDNA sequence encoding human interleukin-7 (huIL-7) was inserted into the multiple cloning site of a modified pCDH1 self-inactivated lentiviral vector (System Biosciences), downstream of the EF1α promoter and upstream of IRES and GFP sequences. Lentiviral supernatants were produced on 293T cells as previously reported (40, 41). Lentivirus stocks were concentrated with ultra-concentrator columns (Amicon), aliquoted and stored at -80°C.

Isolation of human hematopoietic progenitors

Human thymic progenitors were isolated from post-natal thymus (PNT) obtained from children (<3 years of age) undergoing open-heart surgery. Human hematopoietic progenitors were isolated from fetal liver (FL) tissue samples obtained from elective abortions, with gestational age of 14-18 weeks. The use of these human tissues was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam (AMC-UvA) and was contingent on informed consent.

Single-cell suspensions were prepared from PNT and FL tissues, and progenitor cell populations were isolated as previously described (42, 43). In brief, the CD34+ cells were enriched by immunomagnetic cell sorting, using either the direct (PNT) or the indirect (FL) CD34 human progenitor cell isolation kit (Miltenyi Biotec). The CD34+CD1a-CD56-CD1a- BDCA2- (further referred to as CD34+CD1a-) PNT cells and the CD34+CD38- FL cells were further sorted using a FACS Aria (BD Bioscience), to purity always ≥ 99%.

Transduction of human hematopoietic progenitors

Human thymic or hematopoietic progenitors were transduced with retroviral or lentiviral vectors before OP9 co-cultures or inoculation into recipient animals. The CD34+CD1a- PNT progenitors were cultured overnight in IMDM (Invitrogen) supplemented with Yssel's medium (44), 5% normal human serum (NHS), 20 ng/mL human stem cell factor (huSCF; PeproTech), and 20 ng/mL huIL-7 (Cytheris). The following day, cells were incubated for 6 to 8 hours with virus supernatant in fibronectin-coated plates (30 µg/mL; Takara Biomedicals). An identical procedure was used with FL CD34+CD38- cells, except that the medium was supplemented with 20 ng/mL human thrombopoietin (huTPO; PeproTech).

Co-cultures of human progenitor and OP9 cells

Murine bone marrow stromal OP9-control and OP9-huDL1 cell lines (45) were maintained
in MEMα (Invitrogen) supplemented with 20% FCS (Hyclone). Development of human T cells was assessed by co-culturing 5x10^4 PNT CD34^+CD1a^- progenitor cells with 5x10^4 OP9-huDL1 cells in MEMα (Invitrogen) with 20% FCS (Hyclone), 5ng/mL human Flt-3 ligand (huFlt-3L; PeproTech) and variable amounts huIL-7 (Cytheris) or murine IL-7 (muIL-7; PeproTech). Co-cultures were supplemented every 2 to 3 days with fresh medium, and progenitor cells were transferred to fresh stromal cells every 4-5 days of culture (45).

**Generation of HIS (BALB-Rag/γ) mice and huIL-7 inoculations**

BALB/c H-2d Rag2^-/-gc^-/- mice (46) were bred and maintained in self-ventilated isocages, and were fed autoclaved food and water. Mice with a human immune system (HIS (BALB-Rag/γ)) were generated with minor modifications as compared to previously described (34, 35, 42). Newborn (<1 week old) Rag2^-/-gc^-/- mice received sub-lethal (3.5 Gy) total body irradiation with a 137Cs source, and were injected intra-hepatic (i.h.) with 5-10x10^4 sorted CD34^+CD38^- human FL cells. When FL cells were transduced with retroviral or lentiviral vectors, the bulk cell culture was injected i.h. to the newborn recipients immediately after transduction. All manipulations of HIS (BALB-Rag/γ) mice were performed under laminar flow.

When indicated, 2-week and 10-week old HIS (BALB-Rag/γ) mice received 200-250 μg huIL-7/kg (Cytheris) i.p. every other day (3 injections per week), i.e. 1-5 μg of huIL-7 per injection depending on the age. In some experiments, 10-week old HIS (BALB-Rag/γ) mice received a 5-fold higher dose (±1mg huIL-7/kg; 25 μg per injection). The huIL-7 was diluted in PBS, and control animals were injected with the same volume of PBS (100-200μL depending on the size of the animals).

**Flow cytometry analysis for cell surface markers**

Cell suspensions were labeled with FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, Alexa Fluor-647, APC-Cy7 or Alexa Fluor-700 coupled anti-human mAb targeting the following cell surface markers: CD3 (SK7), CD4 (SK3), CD8 (SK1), CD11c (B-ly6), CD19 (HIB19), CD34 (8G12), CD38 (HB7), CD45 (2D1), CD45RA (HI100), CD123/IL-3Rα (9F5) (BD Biosciences), BDCA2 (AC144) (Miltenyi Biotech), CD127/IL-7Rα (eBioRDR5) (eBioscience), and CD1a (T6-RD1) (Beckman Coulter). Dead cells were excluded based on DAPI incorporation. All washings and reagent dilutions were done with PBS containing 2% FCS and 0.02% sodium azide (NaN3). All acquisitions were performed with a LSR-II cytometer interfaced to a FACS Diva software system (BD Biosciences).
Statistical analyses
Data were subjected to two-tail unpaired Student’s t-test analysis where indicated in the figure legends. The obtained p values were considered significant when p<0.05.

Results

Human IL-7 is 100-fold more potent than mouse IL-7 in promoting human hematopoietic progenitor cell proliferation and differentiation in vitro.
Mouse IL-7 binds to the huIL-7 receptor (32), thereby allowing some degree of functional redundancy in chimeric experimental systems such as human/mouse FTOC (29). We analyzed to which extent muIL-7 was able to sustain the development of human lymphoid precursors in the presence of mouse bone marrow stromal OP9 cells expressing the human NOTCH ligand Delta-like1 (Fig.1A). Human CD34^+CD1a^- PNT lymphoid progenitors cultured in these conditions expand extensively and progress towards the T cell lineage (43, 45).

OP9-huDL1/PNT co-cultures were supplemented either with muIL-7 or with huIL-7, at concentrations ranging from 0.5 to 50ng/mL. In contrast to huIL-7, muIL-7 induced limited expansion of the human PNT progenitors in the co-culture assay over a 3-week period (Fig.1B). We used the expression of CD1a and CD3 to evaluate the degree of T cell commitment induced by muIL-7 and huIL-7 in the co-culture assay. Acquisition of CD1a expression is associated with T cell commitment, and human pro-T cells (CD1a^+CD3^-) further differentiate into immature thymocytes (CD1a^-CD3^+) and mature thymocytes (CD1a^-CD3^+) (10). As already described (47), the frequency of human progenitors committed into the T cell lineage (expressing CD1a and/or CD3) and the number of CD3^+CD1a^- mature T cells were increased by huIL-7 in a concentration-dependent manner (Fig.1C). In contrast, muIL-7 enhanced T cell commitment only at high dose (50ng/mL), leading to a modest accumulation of mature T cells (Fig.1C). The extent of cell expansion and T cell differentiation was similar when 50ng/mL muIL-7 and 0.5ng/mL huIL-7 were used, suggesting that huIL-7 is approximately 100-fold more potent than muIL-7 to promote human PNT proliferation and differentiation.

Heterogeneous expression of human IL-7 receptor by human cells in HIS (BALB-Rag/γ) mice
To study the role of IL-7 in human lymphoid development and maintenance in vivo, we
Figure 1. In vitro development of human T cells in presence of muIL-7 or huIL-7. (A) Human CD34^+CD1a^- lymphoid progenitors were purified from post-natal thymocytes and co-cultured with stromal OP9 cells expressing huDL1 ligand of NOTCH. The co-cultures were supplemented with huFlt-3L and variable amounts of either huIL-7 or muIL-7. (B) The graphs show the global human cell expansion in the co-cultures over time, relative to the original CD34^+CD1a^- PNT progenitors input (50x10^3 cells, rate=1). Typical cell expansions obtained with no IL-7 (open circles, dotted line), 0.5ng/mL (open triangles), 5ng/mL (closed diamonds) and 50ng/mL (open squares) of either huIL-7 (left) or muIL-7 (right) are shown. (C) The fraction of human cells committed
transplanted human HSC into sub-lethally irradiated immuno-compromised newborn BALB/c Rag2−/−γc−/− mice to generate “human immune system” HIS (BALB-Rag/γ) mice (Fig.2A) that are multilineage repopulated by human immune cells in primary and secondary lymphoid organs, but in a suboptimal fashion. We asked whether poor cross-reactivity of muIL-7 in vivo could explain at least partially the suboptimal levels of human reconstitution in human HSC-transplanted mouse recipients.

We analyzed CD127 expression on human cells found in HIS (BALB-Rag/γ) mice 2, 6 and 10 weeks after human HSC transplantation. At two weeks post-graft, human cells are mostly immature progenitors located in the liver and the bone marrow, with limited colonization of the mouse thymic rudiments. At 6 weeks of age, HIS (BALB-Rag/γ) mice exhibit robust human B cell development in the bone marrow and human T cell differentiation in the thymus. Circulating, mature human T and B lymphocytes are observed in the blood and secondary lymphoid organs starting 6-8 weeks after HSC transplantation (34, 35). CD127 was expressed on a limited proportion (1.7±0.8% at week 2; 5.5±0.5% at week 6; 4.3±0.9% at week 10) of CD34+CD38− bone marrow cells, which are enriched for HSC (Fig.2B). A larger fraction of CD34+CD38+ multi-lineage committed precursor cells expressed CD127 at their surface (17.5±8.7% at week 2; 32±3.7% at week 6; 29±12.4% at week 10) (not shown). Around 20% of the CD19+ B cell-committed cell population in the bone marrow expressed CD127 on the cell surface at all analyzed time points (Fig.2C). At week 2, the bone marrow of HIS (BALB-Rag/γ) mice also contained a CD34+CD7+CD45RA− cell population (4.5±2.1% of human cells) of which the majority (66±16%) was CD127-negative (not shown). This CD34+CD7+CD45RA−CD127− cell population might correspond to human thymic seeding progenitors (10). In the thymus, a large fraction (50-75%, depending on the age) of the human thymocytes expressed CD127 (Fig.2D). Surface CD127 expression was observed on various thymocytes subsets such as immature CD3−CD1a+CD4−CD8+, early thymic precursors, immature (CD3−CD1a+) single positive CD4+ cells, CD4+CD8+ double positive thymocytes, and single positive (CD4+CD8− or CD4−CD8+) mature CD3+CD1a+ thymocytes (not shown). In peripheral lymphoid organs 10 weeks post-graft, CD127 was expressed on the surface of T cells, and not on other lineages such as B cells or plasmacytoid dendritic cells (pDCs) (Fig.2E-F).
ROLE OF IL-7 IN HIS (BALB-RAG/γ) MICE

huIL-7 administered to HIS (BALB-Rag/γ) mice generates a transient increase in human thymocytes numbers

To investigate the role of IL-7 in early human thymopoiesis, we treated 2-week old HIS (BALB-Rag/γ) mice with huIL-7 over 4 weeks (Fig.3A). Thymus cellularity was increased approximately 3-fold after 2 weeks of huIL-7 treatment, but was similar to PBS-injected animals at weeks 1, 3 and 4 of treatment (Fig.3B). A more detailed analysis of thymic subsets revealed that the absolute number of CD4CD8CD3 triple negative thymocytes was increased 3-fold after 1 week of huIL-7 treatment (Fig.3C). The increase of thymus size at 2 weeks was due the accumulation (3-fold increase) of CD4+CD8+ double-positive thymocytes (Fig.3C). After 4 weeks of huIL-7 treatment, i.e. in 6-week old HIS (BALB-Rag/γ) mice, we finally observed the accumulation of CD3+CD1a+ mature thymocytes (Fig.3C), which did not result in increased numbers of peripheral mature T cells (data not shown). In the bone marrow, we could not observe any significant impact of repeated huIL-7 injection on B cell ontogeny (Fig.3D), which is consistent with the fact that B cell numbers are normal in CD127-deficient patients (24-28). In contrast, we observed a transient accumulation of human pDCs in the liver of huIL-7 treated HIS (BALB-Rag/γ) mice, which peaked at around 2-3 weeks after the onset of treatment (Fig.3E).

We asked whether an increased availability of huIL-7 would lead to accumulation of human cells in adult (≥ 10 weeks after xenograft transplantation) HIS (BALB-Rag/γ) mice, which already contain significant amounts of human cells in all peripheral lymphoid organs. We injected adult HIS (BALB-Rag/γ) mice for 2 weeks with high doses of huIL-7 (up to 25 μg/mouse; 3 injections per week) and analyzed the effect on human cell development and peripheral homeostasis.
Figure 3. Injection of huIL-7 to young HIS (BALB-Rag/γ) mice. (A) Schematic representation of the experimental set-up. Two weeks after human HSC transplantation, the animals received every other day 200-250 μg huIL-7/kg i.p., over a 4-week period (from 1 μg per injection during the 1st week to 4 μg during the 4th week). The control animals received the same volume of PBS. Some animals were sacrificed and analyzed every week after the onset of the treatment. (B) The number of human thymocytes was compared between PBS (open circles)
The number of human cells did not increase in huIL-7 treated animals in the thymus (Fig.4A), spleen (Fig.4B), liver, bone marrow or blood (data not shown). No significant effect was observed on thymopoiesis (Fig.4A) or frequency of T cells (Fig.4B), B cells, pDCs, NK cells or myeloid cells (not shown) in the periphery.

Altogether, these results show that supplementation of young HIS (BALB-Rag/γ) mice with huIL-7 enhanced human T cell and pDC development in a transient manner, whereas huIL-7 treatment had no effect in adult animals.
Enforced expression of human IL-7 increases human thymopoiesis, but does not affect peripheral human cell homeostasis

Considering that exogenous delivery of huIL-7 to HIS (BALB-Rag/γ) mice might not result in optimal IL-7 bioavailability, we assessed whether constitutive expression of huIL-7 by the human cells would be more effective. We therefore generated HIS (BALB-Rag/γ) mice using HSC transduced with a huIL-7-encoding lentiviral vector (Fig.5A). An aliquot of transduced HSC was maintained in culture to measure the expression of the reporter GFP gene (transduction efficiency ±20% for both control and huIL-7 vectors). When comparing the transduction efficiency with the frequency of GFP+ cells in HIS (BALB-Rag/γ) mice 8-10 weeks after transplantation, we observed a competitive advantage of huIL-7-expressing thymocytes (Supp.Fig.1A). The tendency towards increased thymus size in huIL-7-expressing animals correlated with higher numbers of CD1a+CD3- T cell-committed progenitors and CD4+CD8+ thymocytes (Fig.5B). In contrast, the frequencies of peripheral human CD45+ cells and T cells were unaltered in adult huIL-7-expressing HIS (BALB-Rag/γ) mice (Fig.5C), as well as the CD4/CD8 T cell ratio (not shown). Overall, we conclude that cell-autonomous huIL-7 expression results in a selective advantage for the transduced cells, with specific benefits for subsets of immature thymocytes.
ROLE OF IL-7 IN HIS (BALB-RAG/γ) MICE

Figure 5. Enforced expression of huIL-7 in HIS (BALB-Rag/γ) mice. (A) Experimental scheme including HSC manipulation by lentiviral transduction prior to xenograft transplantation. (B) The number of human thymocytes was compared between HIS (BALB-Rag/γ) mice generated with control-transduced (closed squares) or huIL-7-transduced (closed triangles) HSC, 8-10 weeks after transplantation (left). The relative frequencies of thymic cellular subsets are shown (middle and right), as depicted in the legend of Figure 4. (C) The number of human splenocytes is shown (left), as well as the frequency of mature T cells in spleen (right). Two independent experiments were performed and the results were pooled to perform the statistical analysis. Unpaired Student's t-test analysis: * p<0.05; ** p<0.01; *** p<0.001.

Transplantation of HSC over-expressing a chimeric mu/huCD127 results in enhanced recovery of human cells, except T cells

We next assessed whether muIL-7 – which is abundantly available in recipient BALB/c Rag2−/− IL-2Rγ−/− mice due to the absence of mature murine lymphocytes and their precursors (6, 8) – can promote human lymphopoiesis in HIS (BALB-Rag/γ) mice. To allow optimal interaction of muIL-7 with human cells, we transduced the human progenitors with a retroviral vector encoding a chimeric mu/huCD127 composed of mouse CD127 extracellular domain and human CD127 transmembrane-cytoplasmic domain. We transduced human PNT progenitors with the mu/huCD127-encoding vector and cultured them on OP9-huDL1 cells (Fig.1A). The responsiveness of human cells to muIL-7 was markedly enhanced, with a 15-fold increase in cell expansion and mature T cell generation, as compared to control-transduced PNT (Supp.Fig.2). These results indicate that the chimeric mu/huCD127 sustained proper binding of muIL-7 and enhanced signal transduction within human cells.

We next investigated whether endogenous muIL-7 could enhance the human hematopoiesis in HIS (BALB-Rag/γ) mice by injecting human HSC transduced with the chimeric mu/huCD127-expressing vector into newborn BALB/c Rag2−/−γc−/− mice. We produced control groups either with an empty vector, or with a vector expressing human CD127. The resulting HIS (BALB-Rag/γ) mice were analyzed around 10 weeks after xenograft transplantation,
when human cell repopulation in peripheral lymphoid organs was optimal. Human CD45+ leukocyte reconstitution was equivalent in the thymus of all groups (Fig.6A), whereas it was significantly improved in the spleen (Fig.6B), the bone marrow, the liver and peripheral blood (not shown) of HIS (BALB-Rag/γ) mice injected with mu/huCD127-transduced HSC. Improved human reconstitution in these organs correlated with selective advantage for the GFP+ mu/huCD127-expressing human cells, whereas no effect was noted in the thymus (Supp.Fig.1B). Engraftment of mu/huCD127-transduced HSC resulted in increased numbers of human CD11c BDCA2+ pDCs, CD11c+BDCA2 cells – which contain conventional dendritic cells (cDC) and monocytes – and CD19+ B cells in the bone marrow, spleen, liver and blood of these animals (Fig.6C-D and data not shown). The numbers of peripheral T cells in HIS (BALB-Rag/γ) mice injected with HSC transduced either with the chimeric mu/huCD127, the huCD127 or the control GFP expressing vectors were similar (Fig.6E). Consistently, thymopoiesis was not enhanced in mu/huCD127-expressing HIS (BALB-Rag/γ) mice at 4 weeks after HSC transplantation, whereas a significant increase in peripheral (non-T cell) reconstitution was already apparent at this early time point (Supp.Fig.3A). The enforced expression of the chimeric mu/huCD127 chain resulted in a reduction of the frequency of CD3 CD1a+ thymocytes (12% in GFP vs. 5% in GFP+ on average, p<0.05), suggesting that commitment to the T cell lineage was impaired (Supp. Fig.3B).
Overall, increasing the responsiveness of human cells to the environmental IL-7 (muIL-7) globally enhanced the levels of human hematopoietic reconstitution in HIS (BALB-Rag/γ) mice, with the exception of thymocytes and peripheral T cells.

Over-expression of anti-apoptotic genes in HSC does not improve their T cell differentiation capacity

We previously reported that human T cells in HIS (BALB-Rag/γ) mice exhibit high turnover rate and susceptibility to apoptosis (48), suggesting that low T cell numbers may result at least partly from limited survival capacity. We hypothesized that it might be the consequence of an inadequate balance between pro-apoptotic stimuli and IL-7 induced anti-apoptotic factors. To test this possibility, we investigated the impact of human BCL-2 or BCL-XL.
gene over-expression on human hematopoiesis in the HIS (BALB-Rag/γ) mouse model.

Ectopic expression of BCL-2 in human HSC favored the survival of human cells in the thymus, as illustrated by an increased recovery of GFP+ cells (Fig. 7A). In particular, the maintenance of CD3+CD1a+ T-committed progenitors and more specifically immature CD4+CD8+ double positive thymocytes was favored by BCL-2, although the total number of human thymocytes was not enhanced (Fig. 7A). No effect of BCL-2 gene over-expression was observed on the numbers of total human leukocytes, T cells or B cells in the peripheral lymphoid organs were noted (Fig. 7B). In contrast, over-expression of BCL-XL gene resulted in a 2 to 3-fold increase of the thymus, which was almost fully repopulated by BCL-XL-transduced human cells (>90% GFP+), indicating a strong competitive advantage of BCL-XL-transduced HSC (Fig. 7C). Consequently, peripheral T cells were mostly GFP+ BCL-XL-expressing cells, and the frequency of T cells among BCL-XL GFP+ cells was significantly enhanced (Fig. 7D). However there was no significant increase of the total number of human peripheral T cells since only rare T cells were observed in the GFP cellular fraction (not shown). We conclude that the high frequency of BCL-XL GFP+ T cells in the periphery is due to the over-representation of GFP+ cells in the thymus, and not to a selective survival advantage of BCL-XL GFP+ cells in the periphery.

Discussion

In this study, we explored the dependency of human hematopoiesis on IL-7, using an experimental system mimicking human immune system development in vivo. In adult HIS (BALB-Rag/γ) mice, the generation and maintenance of human lymphoid and myeloid cell lineages are suboptimal, in particular for human thymocytes and peripheral T cells. Although muIL-7 interacts with the huIL-7 receptor (32), it is around 100-fold less potent than huIL-7 to promote human PNT progenitors proliferation and differentiation towards the T cell lineage in vitro. However, as shown here, this low efficacy cannot explain the sub-optimal human T cell development observed in vivo in HIS (BALB-Rag/γ) mice.

Using several approaches to improve IL-7 signaling in the developing human cells, we made the following observations: (a) enforced expression of the mu/huIL-7Ra chain, which ensures constitutive binding of muIL-7 in an optimal manner, gave rise to enhanced production of B cells, pDC and myeloid cells, but not thymocytes or peripheral T lymphocytes, suggesting a role for IL-7 in the generation of non-T cell-committed progenitor cells; (b) IL-7
ROLE OF IL-7 IN HIS (BALB-Rag/γ) MICE

Figure 7. Over-expression of anti-apoptotic BCL2/BCL-XL genes in HIS (BALB-Rag/γ) mice. HIS (BALB-Rag/γ) mice reconstituted with BCL-2-transduced human HSC were analyzed once adult (≥10 weeks post-transplantation). (A) The two graphs on the top show the number of human thymocytes and the recovery ratio of GFP+ cells in the thymus, as compared to the original transduction efficiency (at 100%, the frequency of GFP+ cells in the animal is identical to the GFP+ frequency of originally transplanted HSC). The two bar graphs on the bottom show the relative frequencies of thymic cellular subsets, as depicted in the legend of Figure 4. (B) The two graphs on the top show the number of human splenocytes and the recovery ratio of GFP+ cells in the spleen, as compared to the original transduction efficiency. The bar graph on the bottom shows the relative frequencies of T cells (CD3+), B cells (CD19+) and other subsets in the spleen. (C, D) The same analysis was performed with HIS (BALB-Rag/γ) mice containing BCL-XL over-expressing human cells. Three (BCL-2) to six (BCL-XL) independent experiments were performed and the results were pooled to perform the statistical analysis. Unpaired Student's t-test analysis: * p<0.05; ** p<0.01; *** p<0.001.
supplementation (either injected or over-expressed) and promotion of human cell survival (over-expression of BCL-2 or BCL-XL genes) consistently improved human thymopoiesis, in particular by enhancing the maintenance of immature thymocytes subsets (CD1a+CD3- T cell-committed progenitors; CD4+CD8+ double-positive thymocytes), confirming a role for IL-7 in early intra-thymic T cell development; and (c) the number of human peripheral T cells was not increased by any of the approaches tested, suggesting that IL-7 alone is not sufficient to sustain the survival of human peripheral T cells in HIS (BALB-Rag/γ) mice.

Premature expression of the mu/huCD127 chain in HIS (BALB-Rag/γ) mice hinders the engraftment of the thymus by human cells, as indicated by the effect on early committed T cell precursors (CD3-CD1a+), whereas non-T cell lineages benefit from increased access to murine IL-7. This observation suggests that the maintenance and expansion of an early hematopoietic progenitor cell population is mediated by IL-7, and/or that premature expression of the IL-7 receptor directs these progenitors away from the T cell lineage. Alternatively, the reduced frequency of mu/huCD127+ T cell lineage-committed (CD1a+CD3) thymocytes might reflect an intrinsic defect of mu/huCD127+ thymic seeding progenitors (TSP) to enter the thymus. In humans, little is known about the nature of the TSP, largely due to experimental limitations. It was initially proposed that B, NK, and T lymphoid cells derive from an CD127+ common lymphoid progenitor (CLP) (49), but this concept is now challenged by the recent identification of CD127- intrathymic T cell progenitors (50, 51). Haddad et al. reported that the most immature (CD34hiCD1a-) human fetal thymocytes do not express CD127, like fetal BM CD34hiCD45RAhiCD7+ hematopoietic progenitor cells (51), suggesting that the most efficient T cell progenitor seeding the thymus is CD127- and is not a progeny of the CLP (52). As shown in mice, transgenic expression of CD127 results in a decreased thymic cellularity (11), which is not corrected by Bel-2 enforced expression (53). It was speculated that the constitutive IL-7 consumption by CD127-transgenic CD4+CD8+ thymocytes deprived the early CD4-CD8- progenitor compartment from IL-7, therefore resulting in impaired thymopoiesis (11). An alternative, non-exclusive explanation to reduced thymopoiesis in this context might be a reduced entry of TSP into the thymus, either due to reduced generation of TSP or to impaired cell migration into the thymus. Overall, these observations highlight the fact that a strict regulation of CD127 expression is a key element for proper T cell development (54).

Limited de novo human thymopoiesis is a common feature of many humanized mouse models (32, 33, 48). It is known that IL-7 administration in conditioned recipients receiving
ROLE OF IL-7 IN HIS (BALB-RAG/γ) MICE

A bone marrow transplant improves T cell production (55, 56). Similarly, intra-thymic T cell development was consistently enhanced in HIS (BALB-Rag/γ) mice with improved IL-7 signaling, in particular through better maintenance of immature T cell subsets (CD4⁺CD8⁺ and CD3⁻CD1a⁺). Enforced expression of genes that recapitulate the anti-apoptotic effects of IL-7 also had a positive outcome on human thymopoiesis, although BCL-2 and BCL-XL differentially impacted on this process. When a survival advantage was provided through BCL-XL gene over-expression, which should decrease T cell susceptibility to apoptosis (17), human thymocytes (this work) and NK cells (57) were selectively favored. BCL-2 increased the proportion of immature thymocytes, most obviously CD3⁻CD1a⁺ thymocytes, whereas BCL-XL enhanced all thymic subsets equally. Exogenous huIL-7, however, induced a transient wave of thymocytes generation in young HIS (BALB-Rag/γ) mice. Such a phenomenon is also observed in mice with non-optimal thymopoiesis, e.g. in newborn animals or during recovery after bone marrow transplantation. For instance, in 2-week old mice receiving muIL-7 daily during 3 weeks, the number of thymocytes is increased transiently 1 week after onset of treatment (58). The transient effect observed in HIS (BALB-Rag/γ) mice may be the consequence of proliferation and/or differentiation of huIL-7-responsive human thymic progenitors, which have become huIL-7 insensitive and/or are insufficiently replenished with TSP from the bone marrow.

Thymic subsets recovered from adult (8 to 10 week old) HIS (BALB-Rag/γ) mice contain relatively high numbers of mature T cells, supporting the notion that the early thymic progenitor supply is suboptimal in the HIS (BALB-Rag/γ) mice. In line with this observation, exogenous huIL-7 administration to adult HIS (BALB-Rag/γ) mice was ineffective. In contrast, in presence of constitutive huIL-7 expression, we observed a long-term enhancement of human thymopoiesis. One explanation may be an insufficient bioavailability of exogenous huIL-7 in the thymus, e.g. due to short half-life in vivo. Alternative strategies for IL-7 delivery in vivo make use of IL-7/anti-IL-7 mAb complexes or IL-7-Fc fusion proteins, which intrinsically possess improved biological activity and/or stability as compared to IL-7. Two to three injections of IL-7/anti-IL-7 mAb complexes (1.5 µg + 15 µg every other day i.p.) are sufficient to transiently enhance thymopoiesis in wild-type mice (15-20% increase in thymus size) or in IL-7⁻/⁻ lymphopenic mice (50-100 fold increase in thymus size) (59). Humanized NOD-scid IL-2Rγ null mice transplanted with human CD34⁺ mobilized peripheral blood cells and immediately treated with IL-7-Fc fusion protein (20 µg weekly i.v.) also exhibited enhanced thymopoiesis, with marked maintenance of CD4⁺CD8⁺ immature thymocytes (60).
Despite numbers of studies linking peripheral IL-7 levels and T cell survival (8, 19, 56, 61), we did not observe enhanced human peripheral T cell accumulation in HIS (BALB-Rag/γ) mice by genetically providing huIL-7 or survival advantage, even when human thymopoiesis was improved. It is likely that, once they have left the thymus, mature human T cells lack factors required for their survival in the mouse peripheral lymphoid organs (48). We show that IL-7 alone is not enough to overcome this human T cell survival defect, which probably results from a combination of missing factors. It may be that molecules involved in cell migration between lymphoid organs, cell adhesion, co-stimulation or cross-talk with epithelial and stromal components, function in a sub-optimal fashion due to interspecies incompatibilities, thereby negatively impacting on the generation of lymphoid progenitor cells, T cell differentiation and mature T cell survival. As shown here for IL-7, cross-reactivity between mouse cytokines and the corresponding human receptors is not optimal (32). Similarly, human NK reconstitution in HIS (BALB-Rag/γ) mice is hindered by poor reactivity of human cells to mouse IL-15, which can be overcome by IL-15/IL-15Ra treatment (57). The construction of xenograft-permissive mouse recipients expressing human cytokines instead of the corresponding mouse molecules (gene swapping) will provide valuable insights on their functions, but our results already indicate that combination of cytokines will be necessary to obtain significant effects on the extent of human reconstitution. Considering that MHC-TCR interactions are required for optimal survival of murine naive T cells (61), humanized mouse models transgenic for human MHC molecules (e.g. HLA-A2, HLA-DR2) are good candidates to dissect the mechanisms driving the peripheral maintenance of human T cells. Similarly, improving human DC accumulation in HIS (BALB-Rag/γ) mice should be beneficial to human T cell homeostasis. Human T cells in HIS (BALB-Rag/γ) mice could also be particularly sensitive to active mechanisms of elimination by the remaining components of the murine immune system, e.g. macrophages or monocytes (48). Considering that defective interaction between human CD47 and mouse SIRPa expressed on phagocytes might be a major determinant of xenograft rejection (62), SIRPa humanization in mouse recipients should provide a more permissive background for human HSC transplantation. These few examples illustrate that future generations of HIS mice will have to combine the expression of several human gene products, both soluble and on stromal cellular components, in order to insure improved levels of human cellular reconstitution and proper function of human immune cells.

Overall, we conclude that IL-7 alone stimulates human T cell development, but is by itself unable to adequately support peripheral T cell maintenance. Our results provide further
evidence for an important role of human IL-7 in human T cell development in vivo and highlight the notion that IL-7 availability is but one of many signals that condition peripheral T cell homeostasis.

Acknowledgments

We thank Mireille Centlivre for helpful comments on the manuscript and Berend Hooibrink for cell sorting and maintenance of the AMC-UvA flow cytometry facility. We acknowledge the Bloemenhove Clinic (Heemstede, The Netherlands) for providing fetal tissues and the staff of the Animal Research Institute Amsterdam for animal care.
CHAPTER 4

References

17. Amos, C. L., A. Woetmann, M. Nielsen, C. Geisler, N. Odum, B. L. Brown, and P. R. Dobson.


ROLE OF IL-7 IN HIS (BALB-RAG/γ) MICE

Supplementary figures

Supplementary figure 1. Recovery of GFP+ cells in HIS (BALB-Rag/γ) mice generated with huIL-7-transduced HSC or IL-7Rα-transduced HSC. Recovery of GFP+ cells (= % of GFP+ cells in harvested cells / % GFP+ cells transplanted into the mouse recipients) in the thymus 8 to 10 week-old HIS (BALB-Rag/γ) mice that received HSC transduced either with (A) the huIL-7-GFP lentiviral vector, or (B) the mu/huIL-7Rα-GFP or huIL-7Rα-GFP retroviral vectors. Control vectors only express GFP.

Supplementary figure 2. In vitro evaluation of improved muIL-7 usage in human post-natal thymocytes with enforced expression of a mu/huIL-7Rα chimeric chain. Control transduced or mu/huIL-7Rα chimeric
chain transduced human CD34+CD1a- post-natal thymocytes (transduction efficiency was 17% and 10% respectively) were co-cultured with stromal OP9-DL1 cells. The co-cultures were supplemented with huFlt-3L and variable amounts of either huIL-7 or muIL-7. (A) The graphs show the total number of GFP+ cells developing in the co-cultures over time, starting from 10,000 GFP+ cells at day 0. Typical cell numbers obtained with 0.5ng/mL (closed diamonds) huIL-7 and 5ng/mL (open triangles) or 50ng/mL (open squares) of muIL-7 are shown for control transduced cells (left) and mu/huIL-7Rα chimeric chain transduced cells (right). (B) The accumulation of GFP+ mature T cells (CD3+CD1a-) over time. The results show one representative experiment out of 3.

Supplementary figure 3. Analysis of mu/huIL-7Rα transduced HIS (BALB-Rag/γ) mice 4 weeks after HSC transplantation. (A) The left graph shows the number of human thymocytes harvested from 4 week old HIS (BALB-Rag/γ) mice produced either with control-transduced or chimeric mu/huIL-7Rα-transduced human HSC. The graph on the right show the total number of human cells in the spleen. To correct for variations in transduction efficiencies between retroviral supernatants and experiments, the results are normalized for a fixed (18,000) amount of GFP+ progenitors. (B) This graph shows the proportion of thymic early committed T cell precursors (CD3-CD1a+) in the populations with or without the enforced mu/huIL-7Rα-expression in the HSC. Student's t-test analysis: * p<0.05.