TITLE: Development of human CD4+FoxP3+ regulatory T cells in human stem cell factor, GM-CSF and interleukin 3 expressing NOD SCID IL2Rγnull humanized mice

Running title: Regulatory T cell development in humanized mice

Eva Billerbecka, Walter T. Barrya, Kathy Munia, Marcus Dornera, Charles M. Ricea, Alexander Plossa,1

aCenter for the Study of Hepatitis C, Laboratory of Virology and Infectious Disease, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

1 To whom correspondence should be addressed:
Alexander Ploss, Ph.D.
Center for the Study of Hepatitis C, Laboratory of Virology and Infectious Disease
The Rockefeller University
1230 York Avenue, Box 64
New York, NY 10065, USA
Phone: (212) 327-7899
Fax: (212) 327-7048
Email: aploss@rockefeller.edu
Abstract:
Human hemato-lymphoid mice have become valuable tools to study human hematopoiesis and uniquely human pathogens in vivo. Recent improvements in xenorecipient strains allow for long-term reconstitution with a human immune system. However, certain hematopoietic lineages, e.g. the myeloid lineage, are underrepresented possibly due to the limited cross-reactivity of murine and human cytokines. Therefore, we created a NOD/SCID/IL2Rγnull mouse strain expressing human stem cell factor (SCF), granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3), termed NSG-SGM3. Transplantation of CD34+ human hematopoietic stem cells (HSC) into NSG-SGM3 mice lead to robust human hematopoietic reconstitution in blood, spleen, bone marrow and liver. Human myeloid cell frequencies, specifically myeloid dendritic cells (DCs), were elevated in the bone marrow of humanized NSG-SGM3 compared to non-transgenic NSG recipients. Most significantly however was an increase in the CD4+FoxP3+ regulatory T (Treg) cell population in all compartments analyzed. These CD4+FoxP3+ Treg cells are functional as evidenced by their ability to suppress T cell proliferation. In conclusion, humanized NSG-SGM3 mice might serves as a useful model to study human regulatory T cell development in vivo but this unexpected lineage skewing also highlights the importance of adequate spatio-temporal expression of human cytokines for future xenorecipient strain development.
Introduction:
Humanized mice are amenable small animal models transplanted with human cells or tissues (and/or equipped with human transgenes). In particular animals conditioned to support engraftment of human immune cells have emerged as powerful tools to analyze human hematopoiesis and to study pathogens with unique human tropism. Since the earliest attempts to engraftment of human immune cells in mice in the late 1980s the field has progressed substantially and improved highly immunocompromized xenorecipient strains now allow for high level engraftment of human immune cells. Currently, the most advanced strains are the non-obese diabetic severe combined immunodeficiency (NOD SCID) mouse with either truncated (NOG) or complete (NSG) disruptions in the IL2 receptor common γ chain (IL2Rγ_NULL) as well as Balb/C Rag2/-/- IL2Rγ_NULL (BRG) mice. Injection of human hematopoietic stem cells (HSC) isolated from human cord blood or fetal liver tissue results in robust engraftment of a human hematolymphoid system. Consequently, such human immune system (HIS) mice have opened new opportunities to analyze human immunity in vivo and to study pathogens with unique human tropism, including Epstein Barr virus (EBV), human immunodeficiency virus (HIV) and Dengue virus (DENV). However, current humanized mouse models suffer from several shortcomings, which need to be overcome to advance towards a robust and predictive model for human immune responses. Specifically, the total amount of human cells in HIS mice is below the desired levels. HSC are insufficiently maintained, and differentiation into particular lineages, such as erythromyeloid cells, is impaired. Further, the inadequate formation of higher order lymphoid structures may be central to the limited immune response in HIS mice. Modifications to the humanization protocol and xenorecipients resulted in improved human hematopoiesis in specific compartments. For example, co-transplantation of small pieces of human fetal liver and thymus together with the injection of HSC into irradiated NOD/SCID mice leads to improved T cell selection in so called bone marrow/liver/thymus (“BLT”) mice. HLA class I expressing humanized NSG mice generate functional human T cell subsets with HLA-restricted T cell responses against EBV and DENV. Limited biological cross-reactivity between murine and human orthologs of cytokines, has been proposed as a contributing factor to inadequate representation of certain human hematopoietic lineages in humanized mice. In fact, administration of recombinant IL15/IL15R fusion protein, or transient expression of IL15
and Flt-3/Flk-2L boosts NK cell frequencies in HIS mice. Administration of human IL-7 enhances thymic human T cell development without affecting peripheral T cell homeostasis. Similarly, transient expression of human GM-CSF and IL-4, macrophage colony stimulating factor, or erythropoietin and IL-3 results in significantly enhanced reconstitution of dendritic cells, monocytes/macrophages, or erythrocytes, respectively.

In this study we describe the development and characterization of the NSG-SGM3 strain, an immunodeficient strain expressing transgenes for human stem cell factor (SCF)/KIT ligand (KITLG), granulocyte-macrophage colony stimulating factor (GM-CSF)/colony stimulating factor 2 (CSF2) and interleukin 3 (IL-3). It was recently shown that AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. In accordance with previous studies using NOD/SCID-SGM3 mice the reconstitution of human immunity in NSG-SGM3 recipients through transplantation of purified human HSCs resulted in a significant increase of human myeloid cells in the bone marrow as compared to NSG recipients. Specifically, we detected elevated numbers of myeloid dendritic cells. However, the most striking phenotype was a selective increase in the frequency of human CD4+ T cells in all organs analyzed as compared to non-transgenic NSG mice. Within the CD4+ T cell population we observed a significant increase of regulatory T cells (TReg) but not T helper 1, 2 or 17 cells (Th1, Th2, Th17). Such in vivo developed TReg cells expressed the lineage specific transcription factor FoxP3, CD25 and CTLA-4 and were able to suppress the proliferation of polyclonally activated T cells. Treg cell expansion most likely occurs in the periphery after thymic T cell development since the frequency of single positive CD4+FoxP3+ thymocytes was comparable in both mouse strains. Furthermore, human CD3+ T cells did not express detectable surface levels of CD116, CD123 and c-kit, the respective receptors for GM-CSF, IL3 and SCF indicating that the unexpected preferential lineage skewing in humanized NSG-SGM3 is more likely to be mediated via an indirect mechanism. In summary, our analysis of human HSC transplantation into NSG-SGM3 mice highlights the challenges of modeling human hematopoiesis in humanized mice but also provides a novel platform to study human regulatory T cell biology in vivo.
Methods

Mice. NOD.Cg-Prkdc<sup>scid</sup> IL2rg<sup>tm1Wjl</sup>/Sz (NSG) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and raised under specific pathogen-free conditions at the Rockefeller University. NOD.Cg-Prkdc<sup>scidTg(hSCF/hGM-CSF/hIL3)</sup> (NOD SCID SGM3)<sup>18</sup> were kindly provided by Dr. James Mulloy (University of Cincinnati, OH) and crossed onto the NSG background to homozygous transgene expression. Transgenic offspring were identified by gene specific PCR and homozygosity was confirmed by breeding with NSG mice resulting in the expected Mendelian distribution of 100% transgenic offspring. Homozygous NSG-SGM3 mice were then bred with NSG mice and the hemizygous offspring used for the generation of human immune system mice. Mice were bred and maintained under defined flora with irradiated food and acidified water at the Comparative Bioscience Center of the Rockefeller University according to guidelines established by the Institutional Animal Committee. All experiments in mice were performed at the Comparative Bioscience Center of the Rockefeller University under protocols approved by the Institutional Animal Committee.

Purification of human hematopoietic stem cells (HSC) and generation of HIS mice.
All experiments were performed with authorization from the Institutional Review Board and the IACUC at the Rockefeller University. Human fetal livers (16-22 weeks of gestational age) were procured from Advanced Bioscience Resources (ABR), Inc. (Alameda, CA) and the Human Fetal Tissue Depository at Albert Einstein College of Medicine (Bronx, NY). Fetal liver was homogenized and incubated in digestion medium (HBSS with 0.1% collagenase IV (Sigma), 40mM HEPES, 2M CaCl<sub>2</sub> and 2U/ml DNAse I (Roche)) for 30 min at 37°C. Human CD34+ HSC were isolated using a CD34+ HSC isolation kit (Stem Cell Technologies, Inc., Vancouver, Canada) according to the manufactures’ protocol. 1-5 days old NSG and NSG-SGM3 mice were irradiated with 100 cGy and 1.5-2 x 10<sup>5</sup> human CD34+ HSC were injected intrahepatically 6h after irradiation. 4, 8 or 12 weeks after HSC transplantation mice were sacrificed for the analysis of the human immune system reconstitution. Male and female mice transplanted with CD34+ HSC derived from various human donors were used in this study.
**Leukocyte Isolation.** Blood leukocytes were isolated from heparinized blood by Ficoll density gradient centrifugation (20 min, 2000 rpm). For isolation of intrahepatic leukocytes the liver was perfused with 20 ml cold phosphate buffered saline (PBS, Gibco, Invitrogen Carlsbad, CA), minced and pressed through a cell strainer (100 μm, BD Biosciences, San Jose, CA). The homogenized liver was resuspended in cold RPMI (Gibco) and centrifuged for 10 min at 1200 rpm. The pellet was resuspended in digestion medium (see above) and incubated at 37 °C for 40 min. After digestion the liver suspension was centrifuged for 10 min at 1200 rpm. The pellet was resuspended in RPMI, gently overlayed onto a Ficoll gradient (Cellgro, Manassas, VA) and centrifuged for 20 min at 2000 rpm. Leukocytes were collected from the interphase and washed twice in PBS. For the isolation of splenocytes the spleen was homogenized through a cell strainer (100 μm, BD) and digested for 20 min at 37 °C in digestion medium (see above). After digestion leukocytes were isolated by density gradient centrifugation as described above. To isolate bone marrow cells tibia and femur were flushed with PBS. The bone marrow was pressed through a cell strainer to obtain a single cell suspension and leukocytes were isolated as described above. For the analysis of human granulocytes the bone marrow was homogenized and directly stained for surface marker expression as described below. For the analysis of thymocytes the thymus was homogenized through a cell strainer (70 μm, BD) to obtain a single cell suspension.

**Antibody staining and flow cytometry.** The following anti-human antibodies were used: CD45-Pacific-Orange, CD45-PeCy5.5, CD14-Alexa700, CD19-Pacific-Blue, CD3-APC-Alexa750, CD3-PeTXR, CD38-PeTXR, HLA-DR-Qdot-605 (Invitrogen Corporation, Carlsbad, CA); CD8-APC, CD8-FITC, CD15-FITC, CD15-FITC, CD4-PE, CD56-Pe-Cy5.5, CD117-Pe-Cy7, CD33-PerCP-Cy5.5, CD86-PE, CD123-APC, CD133-APC, CD34-FITC, CD16-Pacific-Blue, CD25-PE, IL-10-PE and IFN-γ-FITC (BD Biosciences, San Jose, CA); CD11c-APC-Alexa750, CD1c-Pacific-Blue, CD127-Pe-Cy7, CD45RO-PerCP-eFluor710, CTLA4-PE, IL-17A-Alexa647 and IL-4-PE (eBioscience, San Diego, CA); CD116-PE, CD45RA-APC-Alexa750, CD4-Pacific-Blue, CD25-Pe-Cy5.5 and FoxP3-Alexa647 (Biolegend, San Diego, CA); BDCA3-FITC (Miltenyi Biotech, Bergisch Gladbach, Germany). Appropriate isotype controls were also purchased from each company. Anti-mouse-CD45-Pe-Cy7 was obtained from eBioscience.

For surface marker staining cells were blocked for 10 min at RT with anti-mouse Fc-Block (BD Biosciences), washed twice with staining buffer (PBS, 1% FBS), stained with
the appropriate antibodies for 15 min at RT, washed again twice with staining buffer and fixed with 4% paraformaldehyde. Intracellular FoxP3 staining was performed using the Biolegend FoxP3 Staining Kit according the manufactures’ instructions. To analyze intracellular cytokine production cells were stimulated with 10ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, MO) and 200ng/ml Ionomycin (Sigma) in the presence of Golgiplug (BD Biosciences) and incubated for 5h at 37°C. After incubation cells were blocked, stained for surface marker, permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained for intracellular IL-4, IFN-γ, IL-17 and IL-10. For cell counting experiments counting beads (Invitrogen) were added to the samples directly prior FACS analysis according to the manufactures’ instruction. FACS analysis was performed using a BD LSRII flow cytometer (BD Biosciences) and data was analyzed using FlowJo Software (Tree Star, Ashland, OR).

**In vitro suppression assays.** Regulatory T cells (T<sub>reg</sub> cells) were isolated from HIS-SGM3 mice blood-, spleen- and liver-derived pooled leukocytes using a human CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotech) according to the manufactures’ protocol. CD25-depleted autologous T cells were labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Invitrogen), stimulated with 0.1 μg/ml anti-CD3 (Biolegend) and 0.5 μg/ml anti-CD28 (BD Biosciences) and cocultured with either isolated CD4+CD25+ T<sub>reg</sub> cells or CD4+CD25- T cells on a 96 well plate at ratios of 1:1 and 2:1 in complete medium (RPMI with 10% FBS, 1.5% HEPES and 1% penicillin/streptomycin). After 4 days of culture proliferation of CFSE-labeled responder T cells was analyzed by flow cytometry.

**Analysis of cytokine concentrations.** The concentration of human cytokine levels in the serum and plasma of mice was determined by using GM-CSF and SCF ELISA construction kits (Antigenix America, Inc., Huntington Station, NY) or an IL-3 ELISA Kit (R&D Systems, Minneapolis, MN). The concentration of human TGF-ß, IL-10 and IL-2 was determined by performing Cytometric Bead Arrays (CBA) (BD Biosciences) at a LSRII flow cytometer.

**Statistical analysis.** Unpaired Student’s t test was used to evaluate statistically significant differences.
Results

Characterization of human hematopoiesis in humanized NSG-SGM3 mice. Stem cell factor (SCF), also known as steel factor (SF) or kit ligand (KITL) may serve as guidance cues that directs HSCs to their stem cell niche, and it plays an important role in HSC maintenance\textsuperscript{19}. GM-CSF and IL-3 stimulate the differentiation of HSCs in myeloid progenitor cells and IL-3 also stimulates proliferation of all cells in the myeloid lineage, i.e. erythrocytes, thrombocytes, granulocytes and monocytes. All of these cytokines are species specific\textsuperscript{9} and expression of human SCF, IL-3 and GM-CSF in NOD SCID mice (NS-SGM3) results indeed in larger number of myeloid cells in the bone-marrow, albeit at the cost of loss of multipotent progenitor cells in the bone-marrow\textsuperscript{20,21}. Given the overall positive effect on human myelopoiesis in these models, we crossed NS-SGM3 onto the NSG background resulting in NSG-SGM3 mice. As previously reported\textsuperscript{20,21} transgenic cytokine expression led to elevated levels of human SCF, GM-CSF and IL-3 levels in the serum of NSG-SGM3 mice (supplementary Fig.1). To comprehensively analyze the effect of human cytokine expression on human hematopoiesis on this improved immunodeficient background we injected sublethally irradiated neonatal NSG and NSG-SGM3 mice with human fetal liver derived CD34+ HSC. In both recipient strains we achieved a similarly high human hematopoietic chimerism of about 50% in blood (Fig.1A), which remained stable from 8 to 12 weeks following HSC transplantation. Comparable total numbers of human leukocytes were detected in liver, spleen and BM in both mouse strains 12 weeks after transplantation (Fig. 2D). In the following we refer to humanized NSG mice as “HIS” mice and humanized NSG-SGM3 mice as “HIS-SGM3” mice. In concordance with previous reports \textsuperscript{20} frequencies of CD33+ myeloid cells were significantly elevated in HIS-SGM3 as compared to HIS mice at week 8 but not at week 12 and achieved peak levels of approximately 20% in peripheral blood (Fig.1D and 1E). In all other tested organs (bone marrow, liver, spleen) CD33+ myeloid (Fig.2C, D and E) and CD14+ monocyte (data not shown) populations were equivalent in HIS and HIS-SGM3 mice. Since our leukocyte isolation protocol includes density gradient centrifugation, granulocytes are largely excluded from analysis. To compare granulocyte development, we also analyzed freshly isolated bone marrow directly for the presence of CD15+ granulocytes. As shown in supplementary figure 2A and B the levels of human granulocytes were slightly elevated in HIS-SGM3 as compared to HIS mice (p=0.05).
An analysis of human dendritic cell (DC) development revealed that all major subsets of human DCs are present in the bone marrow of HIS mice, specifically CD123\(^{\text{high}}\) CD11c\(^{\text{neg}}\) plasmacytoid DCs (pDCs), BDCA-3+ CD11c\(^{\text{low}}\) myeloid DCs (mDCs) and BDCA-1+CD11c\(^{\text{high}}\) myeloid DCs (Fig. 3A and B). Interestingly, myeloid CD1c (BDCA-1)+ DCs were about 3 fold increased in the bone marrow of HIS-SGM3 mice (Fig. 3C and D). These myeloid DCs displayed an activated phenotype as indicated by high expression of CD86 and HLA-DR (Fig. 3E). In spleen, liver and thymus equal levels of pDCs and mDCs were detected in HIS and HIS-SGM3 mice (supplementary Fig. 2C and D).

As previously documented\(^{20}\) the numbers of primitive HSC characterized by a CD38-CD34+ c-KIT+ phenotype were reduced in the bone marrow of humanized NSG mice expressing SCF, GM-CSF, IL-3 as compared to non-transgenic control mice (Fig. 3F and G). This finding can be explained by the ability of GM-CSF to mobilize HSC\(^{22}\).

In summary, HIS-SGM3 mice are comparable to HIS mice in their capacity for human immune cell engraftment with improved human myeloid cell development.

**Increased frequencies of CD4+ lymphocytes in HIS-SGM3 mice**

Interestingly, we detected the most significant differences in human hematopoiesis in HIS-SGM3 as compared to HIS mice in the lymphocyte population. Indeed, B cell frequencies significantly decreased in PBMC fractions from over 40% to about 20% at 8 weeks and slightly less pronounced from about 30% to 15% at 12 weeks in HIS versus HIS-SGM3 mice, respectively (Fig. 1C and E). Similar decreases in B cell frequencies and numbers were observed in spleen and bone marrow of HIS-SGM3 mice (Fig. 2B, D and E). This overall reduction in B cells was paralleled with a proportional increase in CD3+ T cell frequencies and numbers in all compartments analyzed (Fig. 1B and E and Fig. 2A, D and E). These significant changes in the lymphocyte populations had previously not been described\(^{20}\). To further characterize the increased T cell population in HIS-SGM3 mice we first compared CD4+ to CD8+ T cell ratios in both mouse strains. In peripheral blood the ratios of CD4+ to CD8+ T cells was approximately 2:1 in both transgenic and control mice (Fig.4A and E). However, within the CD3+ T cell population the CD4+ T cell subset increased significantly from about 40% to 80% in bone marrow and spleen and slightly less pronounced from 60-80% in the liver whereas the CD8+ T cell subset decreased proportionally in HIS-SGM3 and HIS mice, respectively (Fig 4B-
E). Taken together, our results show that the transgenic expression of human SCF, IL-3 and GM-CSF significantly promotes the expansion of human CD4+ T cells in HIS mice.

**Skewing towards the regulatory T cell lineage within the increased pool of CD4+ T cells in HIS-SGM3 mice**

Human CD4+ T cells can be phenotypically and functionally divided into minimally four sub-populations, Th1, Th2, Th17 and T\textsubscript{Reg} cells\textsuperscript{23,24}. Cell of the T helper (Th)1 lineage, which evolved to enhance eradication of intracellular pathogens are characterized by their production of interferon (IFN)-\(\gamma\). Cells of the Th2 lineage, which evolved to enhance elimination of parasitic infections (e.g. helminths), are characterized by production of IL-4, IL-5, and IL-13\textsuperscript{23}. More recently, Th17 cells have been identified, which primarily produce IL-17 seems to have a crucial role in mediating autoimmunity and inducing tissue inflammation. Regulatory T cells (T\textsubscript{reg}) suppress immune responses and play an important role in the maintenance of the immune system homeostasis. The expression of the lineage specific transcription factor FoxP3 is one major phenotypic characteristic of T\textsubscript{reg} cells\textsuperscript{25,26}.

In order to determine the relative distribution of the CD4+ T cell subsets within the increased T cell population we quantified the numbers of IFN-\(\gamma\)+, IL-4+, IL-17+ or FoxP3+ CD4+ T cells in HIS and HIS-SGM3 mice. All CD4+ T cell subsets could be detected in blood, liver, spleen and bone marrow of HIS and HIS-SGM3 mice (Figure 5). Interestingly however, CD4+FoxP3+ T\textsubscript{reg} cells were significantly increased (Fig. 5A and B) whereas all T helper subsets (Fig. 5C) were similarly distributed in SGM3 transgenic versus non-transgenic mice. These data suggest that transgenic expression of human SCF, GM-CSF and IL-3 leads to a specific development of human CD4+FoxP3+ T cells in HIS mice. It is unlikely, however, that the expansion of CD4+FoxP3+ T\textsubscript{reg} cells is due to a direct action of these cytokines. CD3+ T cells in HIS and HIS-SGM3 mice did not express CD116, CD123 and c-Kit, the receptors of GM-CSF, IL-3 and SCF, respectively (supplementary Fig. 3).

To examine whether the increase in T\textsubscript{reg} cells is caused by enhanced thymic generation of these cells in HIS-SGM3 mice, we comprehensively analyzed human thymocyte development in both mouse strains. Human CD3+ thymocyte numbers were low at week 4 and week 8 after human HSC transplantation but significantly increased at week 12 to comparable levels in HIS and HIS-SGM3 mice (Fig. 6A). At week 4, the majority of CD3+ thymocytes displayed an immature CD4-CD8- double negative phenotype whereas at
week 8 CD8+CD4+ double positive thymocytes were predominant in both, HIS and HIS-SGM3 mice (Fig.6B). 12 weeks after HSC transplantation lineage commitment into either CD8+ single positive or CD4+ single positive T cells was more advanced in the thymus of HIS-SGM3 mice as compared to non-transgenic HIS mice (Fig. 6B).

Human FoxP3+CD4+ single positive thymocytes were readily detectable only at week 12 after HSC transplantation at highly variable levels between individual mice (Fig. 6C and D). HIS-SGM3 mice displayed slightly higher frequencies of FoxP3+CD4+ thymocytes compared to HIS mice (Fig. 6C and B). In the thymus however, we did not detect the consistent and highly significant elevated levels of FoxP3+CD4+ Treg cells that we observed in the periphery. Taken together, equal numbers of human thymocytes and comparable levels of FoxP3+CD4+ cells in the thymus of both mouse strains suggest that the generation of regulatory T cells in HIS-SGM3 mice is more likely due to peripheral expansion rather than thymic development.

We further evaluated whether the increase of Treg cells in HIS-SGM3 mice correlates with increased serum levels of human IL-2, IL-10 or TGF-beta, three cytokines that play an important role in regulatory T cell expansion and homeostasis27. Although we were able to detect minimal concentrations of these cytokines in the serum no elevated levels were observed in HIS-SGM3 as compared to HIS mice (data not shown). These results however do not exclude the possibility that IL-2, IL-10 or TGF-beta are present at higher concentrations but only in local tissue environments, e.g. in spleen or liver, of HIS-SGM3 mice.

**CD4+FoxP3+ Treg cells developed in humanized NSG-SGM3 mice resemble human regulatory T cells in phenotype and function**

To determine whether CD4+FoxP3+ Treg cells developed in HIS-SGM3 mice indeed resemble “bona fide” human regulatory T cells we performed a phenotypic and functional analysis of these cells. Human CD4+FoxP3+ Treg cells are, amongst others, characterized by the constitutive expression of CD25, the IL-2 receptor α-chain, and CTLA-4 (cytotoxic T-lymphocyte antigen 4)27. Functionally they are characterized by their ability to suppress immune responses, e.g. the proliferation of activated T cells. This suppressive activity can either be mediated through direct cell-cell contact dependent mechanisms or the secretion of anti-inflammatory cytokines like IL-10 28. We found that CD4+FoxP3+ Treg cells in HIS as well as in HIS SGM3 mice express high levels of CD45RO, CD25 and CTLA-4 (Fig. 7A). Studies analyzing human PBMC have
recently defined T cells with this phenotype to be activated effector $T_{\text{reg}}$ cells with a potent in vitro suppressive capacity$^{29}$. Purification of CD4+ T cells derived from HIS-SGM3 mice according to CD25 expression resulted in an isolated CD4+FoxP3+ $T_{\text{reg}}$ cell population (Fig. 7B). These purified cells significantly suppressed the proliferation of effector T cells in co-culture experiments (Fig. 7C-F). Indeed, effector T cells from HIS-SGM3 mice proliferated significantly as indicated by CFSE dilution when stimulated with anti-CD3/CD28 alone (Fig 7C) or in the presence of isolated CD4+CD25- T cells (Fig. 7D). In the presence of isolated CD4+CD25+FoxP3+ $T_{\text{reg}}$ cells, however, effector T cell proliferation was strongly inhibited at effector to $T_{\text{reg}}$ cells ratios of 1:1 and 2:1 (Fig. 7E and F, respectively). CD4+FoxP3+ $T_{\text{reg}}$ cells from HIS and HIS-SGM3 mice did not produce IL-10 after stimulation with PMA/Ionomycin (data not shown) indicating that their suppressive activity is rather mediated through cell-cell contact dependent mechanisms. In summary, our analysis of in vivo expanded human CD4+FoxP3+ $T_{\text{reg}}$ cells revealed that they phenotypically and functionally resemble those regulatory T cells that have been described to be present in human blood and tissue$^{27}$. 
Discussion

Limited biological cross-reactivity among cytokines which are critical for hematopoietic development has been proposed as a major contributing factor to the misrepresentation of certain blood cell types in humanized mice\textsuperscript{8,9,30}. In fact, transient expression or exogenous administration of e.g. IL-3, -4, -7, -15, GM-CSF, EPO has been shown to boost human NK, T, myeloid cell and erythrocyte development in humanized mice\textsuperscript{13-15,31}. While such transient expression approaches allow for rapid testing of (combinations of) different human cytokines, trough levels decrease quickly after administration and requires frequent reinjection. Thus, mice expressing transgenically human SCF, GM-CSF and IL3\textsuperscript{20,21} allow analysis of the long-term effects of these cytokines on human hematopoiesis. Originally, aspects of human hematopoiesis were analyzed in SGM3 transgenic mice on the NOD SCID background. However, engraftment of NSG mice with human HSC generates substantially higher percentages of human CD45+ cells in host bone marrow than with similarly treated NOD SCID mice\textsuperscript{3,4}. Therefore, we crossed NOD SCID SGM3 mice to the NSG background. Overall engraftment was similarly robust in NSG and NSG-SGM3 mice and as previously reported we observed increases in myeloid cell and reduction of hematopoietic progenitor cell frequencies in the bone marrow\textsuperscript{20}. However, the most striking phenotype was the selective increase in CD4+FoxP3+ regulatory T cell numbers. These data suggests that the selective expression of human cytokines in humanized mice might affect the complex process of hematopoiesis in an unexpected way and should therefore be carefully considered. In NSG-SGM3 mice a constitutively active CMV promoter drives transgene expression. However, in order to accurately reproduce the complex spatio-temporal expression pattern of cytokines in vivo, it will be important to include bacterial artificial chromosome (BAC) transgenic and knock-in approaches in future strain development. Furthermore, it has to be considered that other factors may influence the ability of exogenous cytokines to function properly than their timing and site of expression such as other human-murine mismatches in humanized mice that will need to be understood and solved.

Regulatory T cells play a pivotal role in controlling immune responses and maintaining immune system homeostasis\textsuperscript{24}. They have been implicated in a number of pathologic processes including cancers, infectious diseases, as well as autoimmune diseases. T\textsubscript{reg} cells can be divided into two major subsets: natural T\textsubscript{reg} cells and induced T\textsubscript{reg} cells\textsuperscript{32}. While natural T\textsubscript{reg} cells develop in the thymus, induced T\textsubscript{reg} cells are generated from
effector T cell precursors in the periphery during an immune response. Regulatory T cell lineage development has been intensely studied but some aspects remain opaque. Genetic studies in both mice and humans have identified Scurfin or FoxP3, a fork-head transcription factor, as a determinant of T_{reg} cell development. Mice carrying spontaneous mutations in FoxP3, resulting in the scrufin phenotype, or with targeted disruptions in the FoxP3 gene lack functional T_{reg} cells and exhibit lymphoproliferative diseases and autoimmune phenotypes. Similarly, humans with mutations in the human FoxP3 gene lack of functional T_{reg} cells and consequently suffer from severe autoimmune symptoms in multiple organs. However, regulatory T cell populations, specifically those that are induced in the periphery, are very heterogeneous in phenotype and function and not all of them express FoxP3. The mechanisms of peripheral regulatory T cell induction are complex and may include a specific mode of antigen presentation or the exposure to anti-inflammatory cytokines like IL-10 or TGF-beta. Recent studies indicate that dendritic cells may play a major role in this process.

Human regulatory T cells development in humanized mice has not been studied in detail so far. It was previously reported that approximate 1-4% of human CD4+ T cells present in thymus, spleen, lymph nodes and blood of humanized BRG (Balb/c Rag2-/- IL2Rγ^NULL) mice display both T_{reg} phenotype (CD25+ FoxP3+) and T_{reg} function. In NSG recipients human CD4+ T cells preferentially develop into Th1 cells, although Th2 and Th17 cells are also generated. Here, we confirm these observations in both HIS and HIS-SGM3 mice and additionally provide evidence that also human CD4+FoxP3+ regulatory T cells develop in both strains albeit at significantly increased frequencies in HIS-SGM3 recipients. T_{reg} cells in HIS-SGM3 mice express high levels of CD25 and CTLA-4 and are fully functional as they can suppress T cells proliferation ex vivo. The mechanisms by which regulatory T cells are expanded in HIS-SGM3 mice remain to be determined. Our data demonstrate that human mature T lymphocytes in humanized mice do not express the receptors for SCF, GM-CSF, IL-3 and thus it is not likely that they respond directly these cytokines. Although, c-KIT, CD116 and CD123 may be expressed on developing thymocytes these receptors are usually not expressed in cells committed towards the lymphoid lineage. We found equal numbers of CD3+ thymocytes and comparable frequencies of CD4+FoxP3+ cells in the thymus of HIS and HIS-SGM3 mice. Although HIS-SGM3 mice showed higher levels of lineage committed single positive thymocytes at week 12 after HSC transplantation we did not observe a
skewing towards the CD4+ T cell lineage in the thymus. Thus, T_{reg} cell expansion in transgenic mice is more likely mediated via an indirect mechanism in the periphery rather than via enhanced thymic development. Future studies should address the question of whether the large increase in human regulatory T cells in HIS-SGM3 mice is due to peripheral proliferation of thymically derived natural T_{reg} cells or due to the conversion of effector T cells into induced regulatory T cells. Studying the mechanisms underlying this expansion might give important insights into human regulatory T cell biology and tolerance maintenance.

Dendritic cells play a pivotal role in maintaining peripheral tolerance. Studies in mice and humans demonstrated that several subsets of DCs are able to regulate T_{reg} cell expansion. For example, a DC subset in the gut characterized by expression of CD103+ is specialized to induced Foxp3+ T_{reg} cells in the absence of any exogenous factors and participates to maintain oral tolerance. Similarly, in mice it was shown that splenic CD8+ DEC-205+ DCs have the capacity to induce antigen specific Foxp3+ T_{reg} cells. The human equivalent of mouse CD8+ DCs was recently identified, characterized by a CD1- CD141+ Clerc9A+ Necl2+ XCR1+ surface phenotype. DCs in the skin, called Langerhans cells, may also play a role in maintaining tolerance by controlling Foxp3+ Treg cell frequencies. It was further demonstrated that a subset of more mature DCs in the human thymus, following stimulation with thymic stromal lymphopoietin (TSLP) can lead to expansion of FoxP3+ T_{reg} cells.

Previous studies and our data suggest that all major subsets of human DCs develop in humanized mice. In addition, we found increased levels of BDCA-1 (CD1c)+ myeloid DCs in the bone marrow of HIS-SGM3 recipients. Thus, human DC subsets capable of inducing T_{reg} cells might be one possible mechanism for the increased number of human CD4+FoxP3+ T_{reg} cells in HIS-SGM3 mice.

In conclusion, the humanized NSG-SGM3 mouse model might be a useful in vivo system to study human T_{reg} cell development and the possible mechanism underlying their peripheral generation.
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Correspondence: Alexander Ploss, Laboratory of Virology and Infectious Disease, The Rockefeller University, 1230 York Ave, New York, NY 10065; e-mail: aploss@rockefeller.edu.
References


**Figure Legends**

**Figure 1**

**Peripheral human immune system reconstitution in HIS versus HIS-SGM3 mice.** HIS mice and HIS-SGM3 mice were generated by transplantation of human CD34+ HSC into newborn NSG (NOD/SCID/IL2Rγnull) mice and NSG mice transgenic for human GM-CSF, IL-3 and SCF (NSG-SGM3). 8 and 12 weeks after transplantation blood was analyzed for human immune system reconstitution by flow cytometry. (A) Isolated leukocytes from HIS (white dots) and HIS-SGM3 (black dots) mice were counter-stained with anti-mouse CD45 and anti-human CD45 antibodies to determine the overall human immune cell chimerism. Within the human CD45+ cell population the frequency of T cells (B) B cells (C) and myeloid cells (D) in HIS versus HIS-SGM3 mice was determined. Representative FACS dot plots are shown on the right (E). Unpaired student’s t test: * p ≤ 0.05; ** p ≤ 0.005; *** p ≤ 0.0001.

**Figure 2**

**Human immune system reconstitution in spleen, liver and bone marrow of HIS versus HIS-SGM3 mice.** 12 weeks after human CD34+ HSC engraftment various organs from HIS and HIS-SGM3 mice were analyzed from human immune cell subset reconstitution by flow cytometry. Frequencies of human CD3+ T cells (A), CD19+ B cells (B) and CD33+ myeloid cells (C) in spleen, liver and bone marrow of HIS (white dots) and HIS-SGM3 (black dots) are shown. Total cell counts of indicated human immune cell subsets in the liver, spleen and bone marrow are displayed in (D). Representative FACS dot plots for bone marrow and spleen are shown on the right (E). Unpaired student’s t test: * p ≤ 0.05; ** p ≤ 0.005; *** p ≤ 0.0001. Error bars indicate SEM.

**Figure 3**

**HIS-SGM3 mice show increased levels of myeloid DCs and decreased levels of primary HSC in the bone marrow.** Bone marrow derived leukocytes from HIS and HIS-SGM3 mice were stained for the expression of human CD45, CD123, CD11c, CD1c (BDCA-1), BDCA-4, HLA-DR and CD86 to analyze the development of human dendritic
cells (DCs). Representative FACS plots in (A) demonstrate the presence of CD123+ plasmacytoid DCs and BDCA-3+ or BDCA-1+ myeloid DCs in HIS mice. Histogram in (B) show CD11c expression on CD123+ (light grey), BDCA-4+ (black) and BDCA-1+ (grey solid) DCs. Frequencies of CD1c+ (BDCA-1+) myeloid DCs are shown in (C) and original FACS plots are displayed in (D). Histograms in (E) show CD86 and HLA-DR expression on CD1c+ myeloid DCs in HIS mice (grey solid) and HIS-SGM3 mice (black open) compare to isotype control (light black). To compare the maintenance of primary human hematopoietic stem cells (HSC) in the bone marrow of HIS and HIS-SGM3 mice human leukocytes were analyzed for the expression of CD34, CD38, c-KIT and CD133. Group data of human CD34+CD38-c-KIT+ HSC frequencies are shown in (F) and original FACS plots are displayed in (D). Unpaired student’s t test: ** p ≤ 0.005

Figure 4

**Elevated levels of CD4+ T cells in HIS-SGM3 mice.** 12 weeks after human CD34+ HSC engraftment HIS and HIS-SGM3 mice were analyzed for human CD4+ to CD8+ T cell ratios in blood (A), spleen (B), liver (C) and bone marrow (D). Original FACS plots are shown in (E). Cells were gated on human CD45+CD3+ T cells. Unpaired student’s t test: * p ≤ 0.05; ** p ≤ 0.005; *** p ≤ 0.0001

Figure 5

**CD4+FoxP3+ regulatory T cells are selectively expanded in HIS-SGM3 mice.** To determine the proportion of T_{reg} cells within the CD3+CD4+ T cell population human leukocytes from 6 HIS and 6 HIS-SGM3 mice were analyzed for expression of transcription factor FoxP3 by flow cytometry. Frequencies of human CD4+FoxP3+ T cells in blood, spleen, liver and bone marrow of HIS and HIS-SGM3 mice are shown in (A), representative FACS plots from blood, spleen and liver in (B) To determine the frequency of T helper subsets Th1, Th2 and Th17 within the CD4+ T cell population cells were stimulated ex vivo with PMA/Ionomycin for 5 hours at 37°C and subsequently stained for intracellular IFN-γ (Th1), IL-4 (Th2) and IL-17 (Th17). Group data of a total of 6 HIS and 6 HIS-SGM3 mice is shown in (C). Unpaired student’s t test: * p ≤ 0.05; ** p ≤ 0.005; *** p ≤ 0.0001. Error bars: SEM.
Figure 6

**Thymocyte development in HIS and HIS-SGM3 mice.** Human thymocyte development was analyzed 4, 8 and 12 weeks after human HSC transplantation by staining thymus-derived cells for the expression of human CD45, CD3, CD4, CD8 and FoxP3. (A) Total cell numbers of hCD45+CD3+ thymocytes at week 4, 8 and 12 in HIS and HIS-SGM3 mice are shown. (B) Frequencies of human CD4-CD8- double negative (DN), CD4+CD8+ double positive (DP), CD4+ single positive (CD4 SP) and CD8+ single positive (CD8 SP) within the CD3+ thymocyte population at week 4, 8 and 12 are displayed. (C) The frequencies of single positive CD3+CD4+FoxP3+ thymocytes at indicated time points in HIS and HIS-SGM3 mice are shown. (D) Original FACS plots showing FoxP3 expression in human CD4 SP thymocytes 12 weeks after HSC transplantation are depicted. Error bars: SEM

Figure 7

**Phenotypic and functional characteristics of CD4+FoxP3+ T_{reg} cells expanded in HIS-SGM3 mice.** Leukocytes isolated from blood, liver and spleen of HIS and HIS-SGM3 mice were analyzed for the expression of human CD45, CD3, CD4, FoxP3, CD25, CD45RA, CD45RO and CTLA-4 to determine the phenotype of in vivo expanded human T_{reg} cells. (A) Representative histograms show CD45RO, CD25 and CTLA-4 expression on CD4+FoxP3- (solid grey) and CD4+FoxP3+ (black open) T cells from the blood of HIS and HIS SGM3 mice compared to isotype control (light black). To test their suppressive capacity CD4+FoxP3+ T_{reg} cells from HIS-SGM3 mice were purified according to their CD25 expression (B) and co-cultured with CFSE-labeled autologous CD3+ T cells in the presence of anti-CD3/CD28. After 4 days of culture the proliferation of human CD3+ T cells alone (C) in the presence of isolated CD4+CD25- T cells (D, ratio 1:1) or in the presence of CD4+CD25+ T_{reg} cells (E, ratio 1:1; F, ratio 2:1) was determined by flow cytometry. One out of four individual experiments are shown.
Figure 1

A. Leukocytes

B. T cells

C. B cells

D. Myeloid cells

E. HIS

HIS-SGM3

CD3 vs. CD45

CD19 vs. CD45

CD33 vs. CD45
Figure 3

A

![Bar charts showing CD123 and BDCA-4, BDCA-1 levels](#)

B

![Histograms showing CD11c](#)

C

![Graph showing % of CD1c+CD123 cells](#)

D

![Flow cytometry plots for HIS SGM3](#)

E

![Flow cytometry plots for CD86 and HLA-DR](#)

F

![Graph showing % of CD38-CD34+c-KIT+ cells](#)

G

![Flow cytometry plots for HIS SGM3 and HIS CD34+CD38-](#)
Figure 6

A. Bar graph showing the number of human CD3+ thymocytes over weeks 4, 8, and 12. The bars are divided into two categories: HIS and HIS SGM3.

B. Column chart showing the percentage of human CD3+ thymocytes at weeks 4, 8, and 12. The chart is divided into five categories: DN, DP, CD4 SP, CD8 SP.

C. Scatter plot showing the percentage of SP CD4+FoxP3+ thymocytes over weeks 4, 8, and 12. Points are differentiated by HIS and HIS SGM3.

D. Flow cytometry plots for HIS and HIS SGM3 at week 12, showing the distribution of FoxP3 and CD4.
Figure 7

A

HIS

HIS SGM3

CD45RO

CD25

CTLA4

B

CD4+CD25-

CD4+CD25+

FoxP3

CD4

C

D

E

F

CFSE

15.9  84.1

44.1  55.9

15.9  84.1

15.8  84.2
Development of human CD4+FoxP3+ regulatory T cells in human stem cell factor, GM-CSF and interleukin 3 expressing NOD SCID IL2Rγnull humanized mice

Eva Billerbeck, Walter T. Barry, Kathy Mu, Marcus Dorner, Charles M. Rice and Alexander Ploss