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A novel Flt3-deficient HIS mouse model with selective enhancement of human DC development

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Humanized mice harboring human immune systems (HIS) represent a platform to study immune responses against pathogens and to screen vaccine candidates and novel immunotherapeutics. Innate and adaptive immune responses are suboptimal in HIS mice, possibly due to poor reconstitution of human antigen-presenting cells, including dendritic cells (DCs). DC homeostasis is regulated by cytokine availability, and Flt3-ligand (Flt3L) is one factor that conditions this process. Mouse myelopoiesis is essentially normal in most current HIS models. As such, developing mouse myeloid cells may limit human DC reconstitution by reducing available Flt3L and by cellular competition for specific "niches." To address these issues, we created a novel HIS model that compromises host myeloid cell development via deficiency in the receptor tyrosine kinase Flk2/Flt3. In Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*Flt3*$^{-/-}$ (BRGF) recipients, human conventional DCs and plasmacytoid DCs develop from hCD34^{+} precursors and can be specifically boosted with exogenous Flt3L. Human DCs that develop in this context normally respond to TLR stimulation, and improved human DC homeostasis is associated with increased numbers of human NK and T cells. This new HIS-DC model should provide a means to dissect human DC differentiation and represents a novel platform to screen immune adjuvants and DC targeting therapies.

Keywords: Adjuvants · Cytokines · Dendritic cells · Humanized mice · Innate immunity

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

Recently, humanized mouse models have undergone a renaissance by virtue of their ability to model human diseases caused by infectious pathogens and malignant tumors [1–4]. Mice harboring human hematopoietic cells, hepatocytes, myoblasts, skin, and/or other tissues allow a wide variety of human pathophysiologies to be studied in vivo and can provide insights into interactions between different cell types (immune cells and epithelial cells) that operate within tissues. As such, humanized mouse models constitute a powerful platform for testing novel therapies, including vaccines and other immunomodulators.

Human immune system (HIS) mice can be generated by several different methods [1, 5]. In one version, HIS mice are generated following engraftment of newborn or adult immunodeficient strains (based on SCID and γ_c mutants on the NOD background such as NOG and NSG (NOD/SCID/γ_c) or carrying the Sirpa^{NOD} allele (Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ *Sirpa*^{NOD} or BRGS) with human HSC from fetal liver or cord blood; this approach is simple and robust. Another version of HIS mice (bone marrow, liver, thymus, or "BLT") involves grafting of fetal thymus, liver, or bone fragments to boost human hematopoietic engraftment and function [6]. In either case, human lymphopoiesis dominates in HIS mice, comprising mainly T and B cells that can stably reconstitute the recipients (> 1 year). In contrast, NK and diverse myeloid compartments develop poorly in these recipients.

The reasons for the lymphoid dominance of HIS models are multiple and include competition with host elements, lack of species-specific cytokines and other growth factors and poor maintenance of human HSC and other myeloid lineage precursors [7, 8]. Recently, substantial efforts have been devoted to improving the development and function of innate lymphocytes, monocytes, myeloid subsets, and macrophages in HIS mice resulting in several improved models [7, 9–11]. Still, the extent of human DC development in HIS mice, while permissive, remains relatively modest [12]. As DC are essential sentinels of pathogen invasion and are critical orchestrators of immune responses, optimal human DC development, and function in HIS mice would appear critical for the ability of these models to appropriately mimic human immunity.

Considering the poor development of human DC in HIS mice, we reasoned that the defect might be secondary to (i) a limited supply of essential human cytokines, (ii) the inability or inefficiency of mouse cross-reacting cytokines, and/or (iii) competition from mouse DCs for cellular resources and "niches." Critical cytokines for DC development include GM-CSF and Flt3 ligand (Flt3L) and DC progenitors differentially express the receptors for these factors [13, 14]. Mice lacking GM-CSF or GM-CSF receptor show a marginal reduction of DCs in lymphoid organs and substantial reduction of migratory DCs in skin and gut, indicating the dispensable role of GM-CSF for lymphoid resident DC subset [15], while deletion of Flt3 has a broad effect, reducing both conventional CD11c$^+$ DC as well as CD123$^+$ plasmacytoid DC [16].

Mouse and human Flt3L are cross-reactive and administration of human Flt3L expands both human and mouse DCs in HIS mice [17]. In this report, we devise a novel approach to promote development of human DC subsets in HIS mice. A new HIS mouse model was engineered in the Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ strain by eliminating the *Flk2/Flt3* receptor (BRGF). BRGF mice harbored a defective mouse DC compartment and enhanced human DC development could be selectively achieved after hHSC engraftment and administration of exogenous human Flt3L. This novel HIS-DC model based on BRGF mice should find multiple applications in the study of human DC biology and for screening of novel immunomodulators and vaccine candidates.

Results

Novel immunodeficient mouse strain with compromised mouse DC homeostasis

Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (BRG) recipient hosts can be efficiently engrafted with human hematopoietic stem cells (hHSC: CD34$^+$CD38$^-$) allowing for the generation of diverse adaptive lymphocyte lineages including CD4$^+$ and CD8$^+$ T cells as well as IgM$^+$IgD$^+$ mature B cells. In contrast, innate lymphoid cells (including NK cells) as well as different myeloid cells develop to a lesser degree [9, 18]. One possible explanation for the limited human DC development in this model might relate to competition with endogenous mouse DCs that normally develop in the absence of *Rag*- and *Il2rg*-dependent pathways. We therefore created a novel host mouse strain that included a genetic deficiency in the fetal liver kinase-2/fms-like tyrosine kinase 3 (*Flk2/Flt3*) known to be critical for conventional and plasmacytoid DC development [16, 19].

After extensive backcrossing (> eight generations) of the *Flk2/Flt3* null allele [20] onto the BRG background, resultant *Flt3*$^{+/-}$ mice were intercrossed to generate Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*Flt3*$^{-/-}$ (BRGF) mice. We first evaluated the effect of Flt3 deficiency on development of mouse myeloid cells in BRG mice. Twelve week-old BRGF and BRG mice were sacrificed and hematopoietic cells in the BM and spleen were compared. Flt3 deficiency led to the expected loss of Flt3-expressing lineage-negative precursor cells (CD19$^-$NK1.1$^-$CD11b$^-$CD11c$^-$Gr-1$^-$F4/80$^-$) in the BM (Fig. 1A). Both conventional DC (cDC: CD19$^-$NK1.1$^-$ CD11c$^+$ PDCA-1$^-$) and plasmacytoid DC (pDC: CD19$^-$NK1.1$^-$CD11c$^+$PDCA$^-$1$^+$) were decreased in spleen and BM of BRGF mice (Fig. 1B). Consistent with previous reports [16], polymorphonuclear neutrophils (PMN: CD19$^-$NK1.1$^-$CD11b$^+$CD11c$^-$Gr-1$^+$), and monocytes (mono: CD19$^-$NK1.1$^-$CD11b$^+$CD11c$^-$Gr$^-$1^{lo}) were only slightly affected by the absence of Flt3 (Fig. 1D). Levels of Flt3L were substantially elevated in BRGF mice compared to BRG mice (Fig. 1E), presumably due to lack of utilization of this factor by Flt3$^+$ hematopoietic cells.

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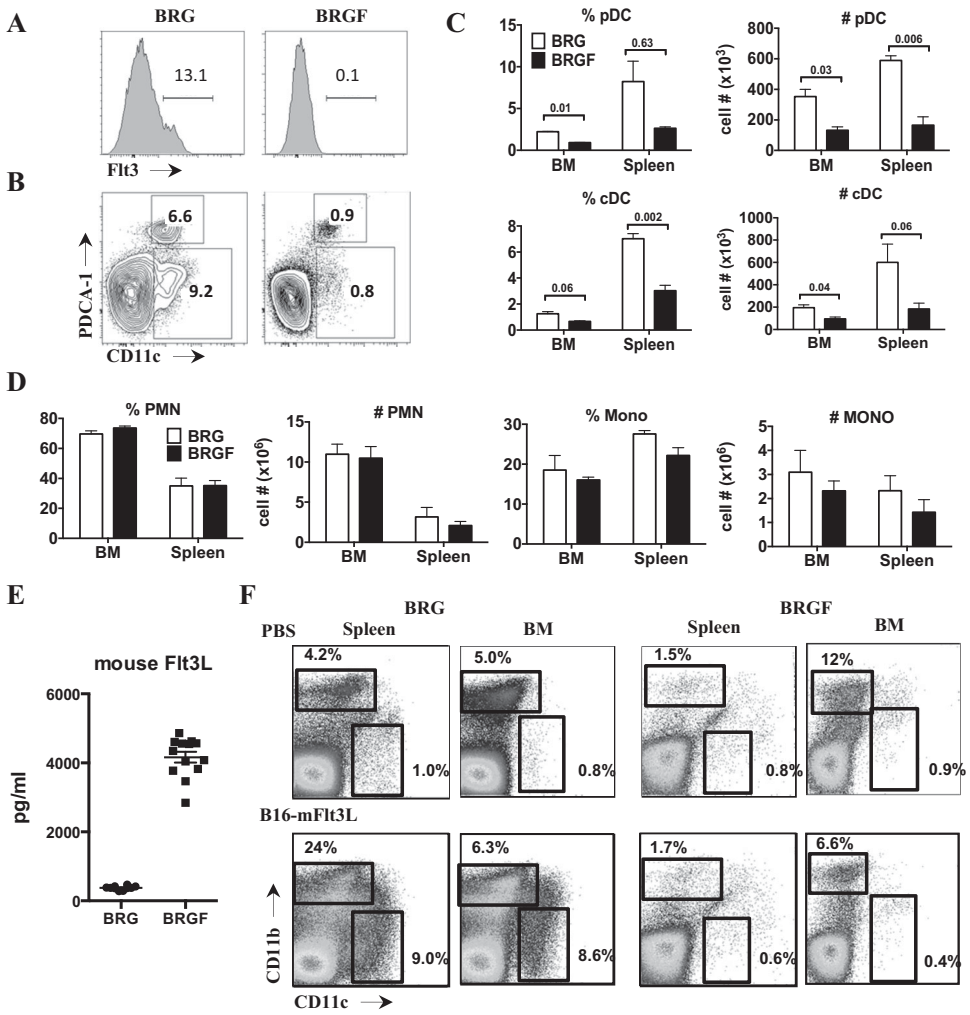

Figure 1. Mouse myeloid cell development in BRGF mice. (A–F) Twelve week-old BRGF mice were analyzed for mouse myeloid subsets. (A) BM cells were isolated from (left) BRGF and (right) BRG mice ($n = 4$ mice/group). Mouse Flt3 expression in CD19$^-$NK1.1$^-$CD11b$^-$CD11c$^-$Gr-1$^-$F4/80$^-$ lineage negative cells was analyzed by FACS. Numbers indicate the percentages of Flt3$^+$ cells. (B) Splenocytes were isolated from (left) BRGF and (right) BRG mice ($n = 4$ mice/group), and CD19$^-$NK1.1$^-$ cells were analyzed for CD11c$^+$PDCA-1$^+$ pDCs and CD11c$^+$PDCA-1$^-$ cDCs. Numbers indicate the percentages of pDCs and cDCs. (C) Percentages and (D) absolute numbers of pDCs, cDCs, PMN (CD19$^-$NK1.1$^-$CD11b$^+$CD11c$^-$GR-1$^+$), and monocytes (CD19$^-$NK1.1$^-$CD11b$^+$CD11c$^-$GR-1^{lo}) within mouse CD45.2 population were determined in the spleen by FACS. (C and D) Data are shown as mean +SEM ($n = 4$ mice/group) and are representative of three independent experiments. Two-tailed unpaired Mann–Whitney test was used for statistical analysis; p values shown are considered significant when less than 0.05. (E) Serum levels of mFlt3L were determined by ELISA in adult BRG and BRGF mice ($n = 9$–13). Each dot represents an individual mouse. Shown is the mean ± SEM/SD. (F) Myeloid cells in BRG or BRGF mice ($n = 4$) were analyzed two weeks after s.c. injection of PBS or 2×10^6 B16 melanoma cells expressing mouse Flt3L. Flow cytometric analysis of CD11b and CD11c expression within total spleen and BM cells are shown. (F) Data are representative of two independent experiments.

In order to confirm the absence of Flt3 function in BRGF mice, we injected irradiated mouse Flt3L-secreting B16 melanoma cells [21] subcutaneously and analyzed the impact on BM and splenic myeloid cells 2 weeks after injection. As expected [21], exogenous mouse Flt3L dramatically increased percentages of mouse CD11c$^+$ DCs both in the spleen and BM of BRG mice, but not in BRGF mice (Fig. 1F). Mouse pDC (pDCA-1$^+$B220$^+$) and CD11b$^+$ cells were increased in the LN and spleen, respectively of Flt3L-treated BRG mice, but not in similarly treated BRGF mice (Fig. 1F, Supporting Information Fig. 1). We conclude that BRGF mice have reduced cDC and pDC compartments, increased Flt3L levels and a hematopoietic cell compartment that cannot respond to Flt3L stimulation due to a germ-line deficiency in Flt3.

Generation of human myeloid cell subsets in HIS mice made in BRGF recipients

To assess the impact of reduced endogenous DC homeostasis and elevated host Flt3L levels on HIS mouse development, BRGF mice were xenografted with hCD34$^+$ HSC and were analyzed 12 weeks after transplantation. Percentages of hCD45$^+$ cells were slightly

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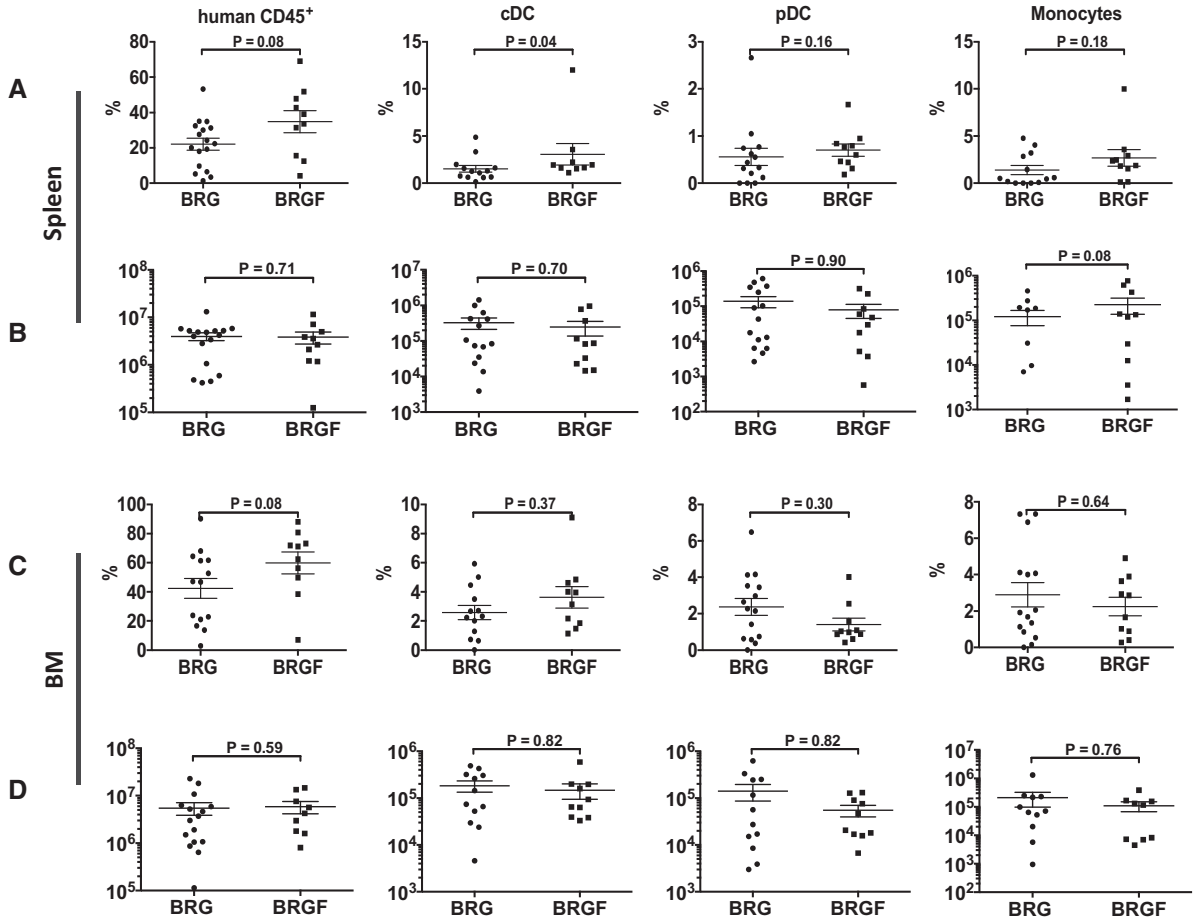

Figure 2. Human hematopoietic cell reconstitution in BRGF mice. (A–D) Purified human CD34$^+$CD38$^-$ fetal liver cells were injected into newborn BRGF pups irradiated at 3 Gy. Human hematopoietic cell reconstitution was analyzed after 12 weeks, defined as a ration between the hCD45$^+$mCD45.2$^-$ percentages over hCD45$^+$ percentages plus mCD45.2 percentages. Within CD45$^+$mCD45.2$^-$ population are CD14$^-$CD11c$^+$ cDC, CD123$^+$BDCA2$^+$ pDC, and CD14$^+$ monocytes. (A, C) Percentages and (B, D) absolute numbers of human cells, cDCs, pDCs, and monocytes in (A, B) spleen or (C D) BM of BRGF HIS mice were measured by FACS. Each symbol represents an independent HIS mouse. Results are compiled from three independent experiments. Error bars indicate ±SEM. Two-tailed unpaired Mann–Whitney test was used for statistical analysis; *p* values shown for each plot are considered significant when less than 0.05.

increased in the spleen and BM of engrafted BRGF mice compared to BRG recipients suggesting a better overall reconstitution when endogenous mouse DC were reduced (Fig. 2), although this difference was not statistically significant. In addition, slightly higher percentages of human CD14$^-$CD11c$^+$ cDCs, CD123$^+$BDCA2$^+$ pDCs, and CD14$^+$ monocytes/macrophages were observed in spleens of engrafted BRGF mice, although significant differences were only found for percentages of human cDC (Fig. 2A and B). No significant differences were observed between BRG mice and BRGF mice in the percentages or absolute numbers of other human myeloid cells in the BM (Fig. 2C and D). These findings demonstrate that the reduction of host myeloid cells and DC subsets secondary to Flt3 deficiency in BRG mice creates a novel immunodeficient mouse strain that can be robustly engrafted with human hematopoietic cell precursors. Nevertheless, the reduction in host DC in BRGF mice does not appear to broadly impact on overall human DC reconstitution after the transfer of hCD34$^+$ HSC.

Previous studies have shown that human NK cell reconstitution in HIS mice is limited due to poor reactivity of mIL-15 with IL-15-responsive human cells [9, 11]. Delivery of exogenous hIL-15/hIL-15Rα complexes in HIS mice allowed for efficient "transpresentation" of this cytokine and dramatic boosting of human NK cell differentiation [9]. We next assessed whether hFlt3L administration could improve human DC development and homeostasis in BRGF recipients.

Specific boosting of functional human DCs in hFlt3L-treated BRGF mice

Both mouse and human DCs can be robustly expanded following exposure to Flt3L in vivo [17, 19]. To determine whether exogenous human Flt3L could specifically affect human DC development in HIS mice, we administered hFlt3L over a course of two weeks

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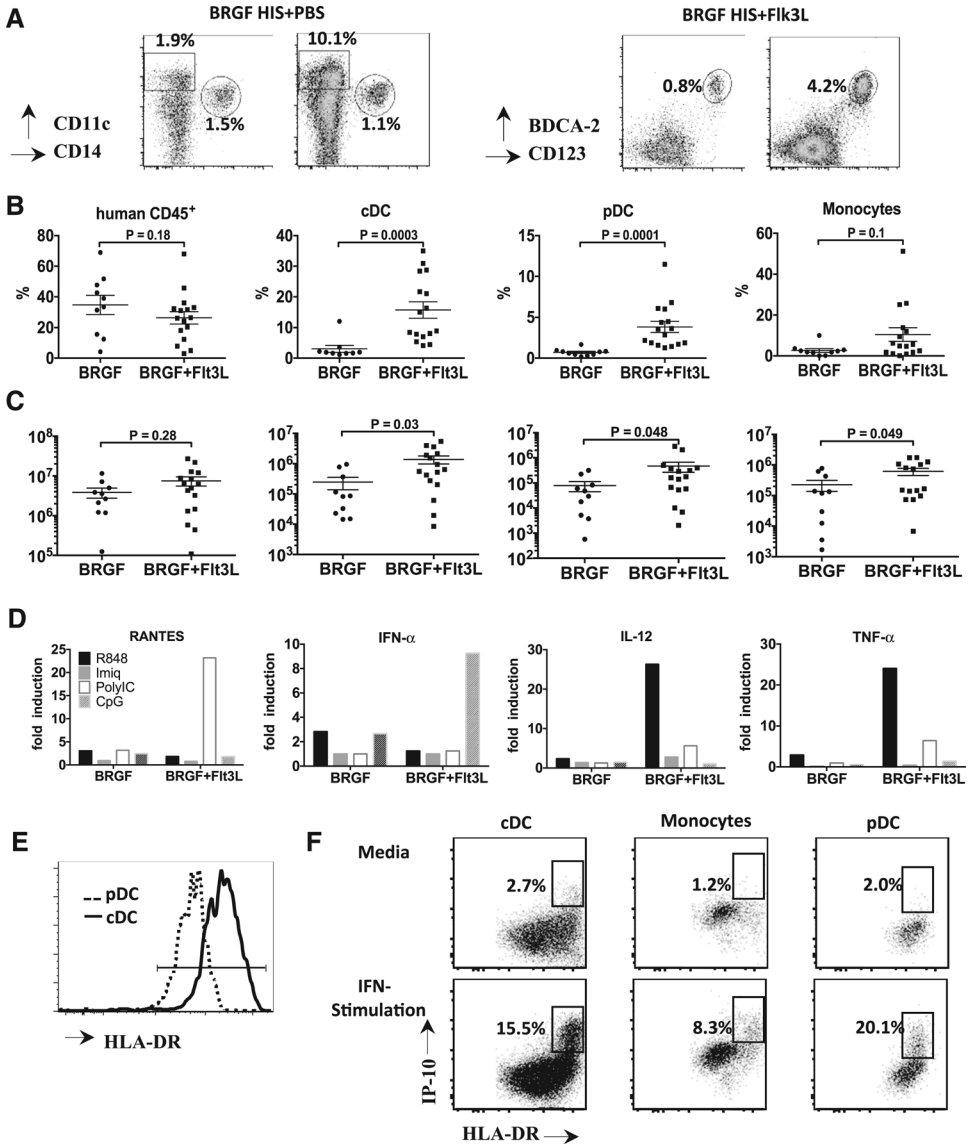

Figure 3. Functional human DC subsets in BRGF mice after human Flt3L treatment. (A–F) Three months after hHSC engraftment in BRGF mice, 5 μg human Flt3L was administered i.p. 3 times a week for 2 weeks, and mice were analyzed 1 week after the last injection. (A) Representative plots of $CD45^+CD45.2^-$ cells from the spleen of hFlt3L- or PBS-treated BRGF HIS mice. (B) Percentages and (C) absolute numbers of human cells ($hCD45^+/(hCD45^+ + CD45.2)$), cDCs ($CD14^-CD11c^+$), pDCs ($CD123^+BDCA2^+$), and monocytes ($CD14^+$) in spleen were analyzed by FACS. (B and C) Each symbol represents an independent HIS mouse. Results are compiled from three independent experiments. Error bars indicate ±SEM. Two-tailed unpaired Mann–Whitney test was used for statistical analysis; *p* values shown for each plot are considered significant when less than 0.05. (D) Fold increase of TNF-α, RANTES, IFN-α, and IL-12 in culture supernatant following 24 h in vitro stimulation of splenocytes from BRGF HIS mice and BRGS HIS mice treated with hFlt3L was measured by Luminex. (E) HLA-DR expression in pDCs and cDCs from splenocytes of BRGS HIS mice treated with hFlt3L was measured by FACS. Histogram is representative of three independent experiments. (F) Intracellular staining of IP-10 after IFN-γ stimulation of cDCs, monoyctes, and pDCs from splenocytes of hFlt3L treated BRGF HIS mice were measured by FACS. Data are representative of three independent experiments.

to BRGF mice that had been previously transplanted with $hCD34^+$ HSC. Mice were sacrificed for analysis one week after the end of the hFlt3L treatment. Human Flt3L treatment did not increase the overall percentages or absolute numbers of $hCD45^+$ cells in BRGF recipients (Fig. 3B, C; Supporting Information Fig. 2A, B); however, some changes in the proportions of different hematopoietic lineages were apparent. Monocytes were significantly increased in number in BM and spleen, but the most obvious expansion was observed for the human pDC and cDC compartments (Fig. 3A–C). Both frequency and absolute numbers of human cDC and pDC were increased in these organs. Moreover, hFlt-3L induced cDC express higher levels of HLA-DR than induced pDC (Fig. 3E). These data suggest that hFlt3L administration into BRGF mice can specifically amplify human DC reconstitution in a

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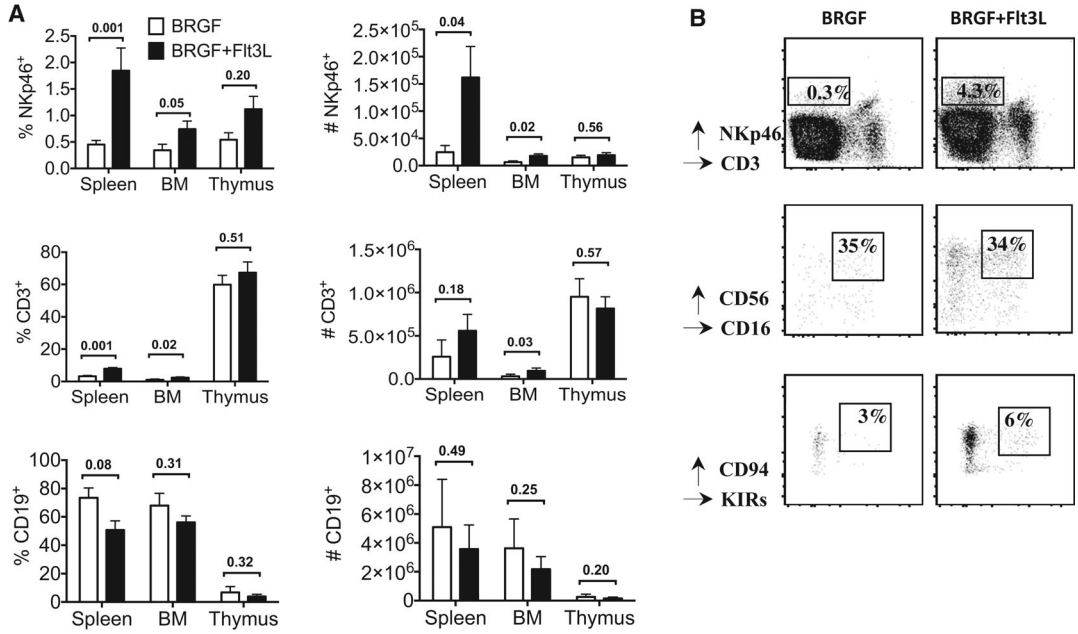

Figure 4. Increased human T NK-cell homeostasis in BRGF HIS mice. (A) Percentages and absolute numbers of human NK cells (NKp46$^+$CD3$^-$), T cells (NKp46$^-$CD3$^+$), and B cells (CD19$^+$) in the spleen of BRGF HIS mice with or without exogenous Flt3L were calculated by FACS. Data are shown as mean +SEM ($n = 10$ mice/group) and are pooled from three independent experiments. Two-tailed unpaired Mann–Whitney test was used for statistical analysis; p values shown are considered significant when less than 0.05; all other comparisons were not significant. (B) Representative plots of human CD45$^+$ cells in the spleen of BRGF HIS mice with or without exogenous Flt3L. Numbers indicate the percentages of NK cells (NKp46$^+$CD3$^-$), cytotoxic NK subsets (CD56$^+$CD16$^+$), mature NK cells (CD94$^+$KIRs$^+$). Plots are representative of two independent experiments.

similar fashion to that previously reported for the effect of mFlt3L on mouse myeloid cells [16, 19].

We next tested the function of hFlt3L-induced human DCs generated in BRGF mice in response to TLR ligands. Splenocytes and BM cells from hFlt3L-treated BRGF HIS mice were stimulated in vitro with different TLR agonists and responses were compared with cells from PBS-treated BRGF HIS mice. After stimulation, supernatants were analyzed for cytokine production, and the results were normalized to media control (Fig. 3D and Supporting Information Fig. 2C). In general, cytokine production from hFlt3L-boosted human DC was substantially increased compared to DC derived from PBS-treated HIS mice. For example, TLR7/8 triggering with R848 induced substantial and diverse cytokine production, including TNF-α and IL-12, mainly from BRGF mice treated with hFlt3L, whereas these cytokines were barely detectable in R848 stimulated cultures from PBS-treated HIS mice. Similarly, activation of TLR3 via poly I:C increased TNF-α, IL-12, and RANTES production in cultures from hFlt3L-boosted HIS mice, while TLR9 triggering with CpG promoted strong production of IFN-α (likely due to the significantly expanded pDC population) in hFlt3L-treated but not PBS-treated BRGF mice. These data demonstrate that the human cDCs and pDCs induced by hFlt3L treatment in BRGF mice express broad range of functional TLRs.

IFN-γ induced protein 10 (IP-10) is secreted by a variety of cell types, including monocytes and DCs, in response to type I or type II IFN and serves as an important chemoattractant for monocytes/macrophages, NK and activated T lymphocytes [22]. We next examined the capacity of splenocytes from hFlt3L-treated BRGF mice to produce IP-10 in response to IFN-γ stimulation (Fig. 3F). We found that both pDC and cDC produced similar high levels of IP-10 after incubation with IFN-γ, in contrast with IFN-γ stimulated monocytes. These results confirm the robust functional capacity of human DC subsets in hFlt3L-boosted HIS mice.

Improved homeostasis of human T lymphocytes and NK cells in DC-boosted HIS mice

Previous studies have shown that in vivo administration of Flt3L in mice could increase resident cDCs that promote mature NK cell survival via trans-presented IL-15 [23]. We have reported a specific increase of human NK cells in HIS mice supplemented with trans-presented IL-15 [9]. Moreover, increased IL-15 in this context could also improve human CD8$^+$ T cell, and surprisingly, human CD4$^+$ T-cell homeostasis [24]. We next assessed whether hFlt3L administration that improves human DC homeostasis in this novel HIS mouse model would impact on human NK and T-cell reconstitution. Spleen, BM, and thymus from hFlt3L-treated BRGF HIS mice were analyzed for the overall levels of T, B, and NK cell engraftment compared with PBS-treated BRGF HIS mice. We found a significant increase in the absolute numbers of human NKp46$^+$ NK cells in the spleen and BM of hFlt3L-treated BRGF HIS mice (Fig. 4A) suggesting an effect of the enhanced human DC generation. In addition, we found a selective increase in the proportions of human CD3$^+$ T cells in the spleen and BM, whereas

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$CD19^+$ B cells were reduced. Overall, the administration of hFlt3L to BRGF HIS mice resulted in a redistribution of human lymphoid cells within lymphoid tissues that more closely resembled the situation observed in normal humans [25].

We further examined the phenotype of NK cells generated after hFlt3L treatment in BRGF HIS mice. Although the overall numbers of $NKp46^+CD3^-$ NK cells were increased, the relative frequency of $CD16^+$ and $CD16^-$ NK subsets remained unchanged (Fig. 4B). Interestingly, the percentage of KIR^+ NK cells was slightly increased (3–6%) suggesting a selective effect of enhanced DC homeostasis on more differentiated NK cells. These data demonstrate that hFlt3L treatment broadly expands NK populations in HIS mice without dramatic skewing of the NK cell repertoire.

Discussion

Previous studies have shown that in vivo Flt3L administration can dramatically boost both $CD11c^+$ cDC and CD11c-pDC numbers in human volunteers [26, 27]. Recently, human Flt3L treatment in humanized NOD/SCID mice could increase human $CD141^+$ DC numbers, that could produce IFN-λ and cross-present protein antigens to human $CD8^+$ T cells after activation with polyI:C [17]. Still, the strong cross-reactivity of Flt3L in mouse and human systems means that exogenous hFlt3L delivery will expand both mouse and human DCs that are present in humanized mice (Fig. 3 and Supporting Information Fig. 3A, B). It should be noted that the absolute numbers of mouse DC present in untreated BRG HIS mouse exceed the number of human DCs by four to fivefold (Fig. 1 and Supporting Information Fig. 2). Exogenous treatment of BRG hosts with Flt3L (mouse or human) expands mouse DC by a factor of 10 (Supporting Information Fig. 3). Similarly, administration of hFlt3L expands human DC in BRGF-based HIS mice tenfold (Fig. 3). As such, use of Flt3L supplementation in existing HIS models (based on BRG, NSG, NOG) will not change the relative abundance of human versus mouse DCs in this context but will simply increase overall DC numbers (both mouse and human). In our new HIS mouse model, Flt3 deficiency effectively reduces the generation of mouse DC subsets and eliminates the possibility that residual mouse DC will respond to exogenously delivered Flt3L. As such, BRGF HIS mice supplemented with exogenous human Flt3L generate a human DC compartment that exceeds mouse DC by a factor of 5 (Figs. 1 and 3). This new DC-boosted HIS mouse model offers a powerful platform to discover the mechanisms that regulate human DC development and to evaluate potential DC-based vaccine strategies and therapies.

Human DC can be differentiated in vitro from monocytes in the presence of GM-CSF and IL-4 [28]. In contrast, the bioactivity of murine cytokines for cognate receptors on human DC and their progenitors is not completely understood. Exogenous delivery of DC-promoting growth factors can be used to increase human DC development in HIS mice. Hydrodynamic injection of GM-CSF/IL-4 in HIS mice greatly increased the numbers of DC-sign expressing DCs [29]. However, human T and B cells become activated by this approach, probably due to the inflammatory properties of GM-CSF/IL-4 induced DCs.

A survey of potential cross-reactivity between mouse and human hemopoietins revealed that several critical myeloid factors in mice lacked the ability to stimulate human cells [7]. As such, mice in which cytokine gene loci are replaced with human coding sequences have been developed. HIS mice with human GM-CSF and IL-3 knock-in showed increased number of human myeloid colony forming cells, alveolar macrophages, but not monocytes and DCs under steady-state conditions [30]. In contrast, MISTRG hosts with human M-CSF, IL-3, GM-CSF, and TPO knock-in were reported that high levels of human myeloid cells reconstitution, in which cell counts of human cDC and pDC were also increased [31]. Together these reports provide improved HIS mouse models whereby human DC can be studied, although some concerns about the lifespan of these models has been raised due to the strong suppression of mouse erythropoiesis.

While both hematopoietic and nonhematopoietic stromal cells produce Flt3L, DC development in the BM and peripheral maintenance of DC within lymphoid organs is thought to mainly rely on Flt3L production by hematopoietic cells [32]. In BRG mice, availability of Flt3L may be reduced due to the absence of Flt3L producer lymphocytes (including NK cell and T cells) and to consumption by mouse DC precursors and mature DC subsets. In BRGF mice, mFlt3L levels are sharply increased (Fig. 1E), suggesting that consumption of this factor by developing DC far outweighs any loss due to absence of Flt3L-producing lymphocytes. The increased Flt3L levels in BRGF mice could theoretically provide human DC precursors and mature DC more access to endogenous mFlt3L. Still, human DC development is not markedly different in BRGF compared with BRG recipients suggesting that residual mFlt3L is not sufficiently potent to drive human DC development in the absence of competition from mouse Flt3L-responsive myeloid cells.

NK cell–DC interactions provide a bilateral "cross-talk" that is critical for survival, maturation, and function of both NK and DC in vivo. Flt3L produced by NK cells can promote DC progenitor differentiation in lymphoid organs [32]. Moreover, activated NK cells kill immature DCs to expand the most immunogenic DCs [33]. On the other hand, IL-15 trans-presented by DCs appears to play a crucial role in mouse and human NK and T-cell homeostasis [9, 24]. MHC molecules expressed on DCs, and cytokines such as IFN-α secreted by activated pDCs and IL-12 by cDCs promote NK cell licensing and activation [34, 35]. Hence, the increased NK and T-cell homeostasis, differentiation and function in BRGF mice treated with hFlt3L may be the result of enhanced IL-15 trans-presentation by human DCs. Further studies are necessary to unravel the mechanisms by which activated NK cells edit DC functions during infections or antitumor responses. DC-boosted HIS mice may provide a novel means to address these questions in a physiologically relevant context.

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Materials and methods

Generation of HIS mice in a novel BRGF recipient strain

Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*Flt3*$^{-/-}$ (BRGF) mice were generated by extensive backcrossing of *Flt3*$^{-/-}$ mice ([20]; generously provided by Dr. Ihor Lemischka) onto the Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (BRG) strain for >eight generations. Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$*Flt3*$^{+/-}$ mice were subsequently intercrossed to create the BRGF strain. HIS mice were generated as previously described [9, 24]. Briefly, fetal liver CD34^{+} cells were isolated using affinity matricies according to manufacturer's instructions (Miltenyi Biotec) and subsequently phenotyped for CD38 expression. Newborn (3–5 day old) received sublethal irradiation (three Gy) and were injected intrahepatically with 5×10^4 CD34^{+}CD38^{-} human fetal liver cells. All manipulations of HIS mice were performed under laminar flow conditions. Experiments were approved by an ethical committee at the Institut Pasteur (Reference # 2007–006) and validated by the French Ministry of Education and Research (Reference # 02162.01).

In vivo Flt3L treatment

HIS mice were injected i.p. three times a week for 2 weeks with 5 μg hFlt3L-Fc (BioXcell), commencing at 12 weeks after reconstitution. Control mice were injected with the same volume of PBS. HIS mice were analyzed one week after the last hFlt3L injection. Experiments analyzing Flt3L responsiveness of mouse myeloid cells in BRGF mice utilized B16 melanoma cells overexpressing mFlt3L ([21]; 2×10^6 cells injected subcutaneously).

FACS analysis for cell surface and intracellular proteins

Single cell suspensions of crushed bones, spleen, and thymus were labeled with mAbs against the following human surface markers: mCD11b, mCD11c, mCD45-2, CD45, CD14, CD11c, CD123, BDCA2, CD3, CD19, NKp46. All washings and reagent dilutions were done with PBS containing 2% FCS. All acquisitions were performed using BD Fortessa and data were analyzed by FlowJo software. For detecting IP-10 expression after IFN-γ stimulation, intracellular staining was performed after fixation and permeabilization of cellular suspensions using BD Perm/Wash and BD Cytofix/Cytoperm reagents from BD Bioscience according to manufacturer's instructions.

Cytokine secretion assay

Splenocytes were cultured in RPMI 1640 GLUTAMAX supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen) with various TLR agonists, including poly I:C (20 μg/mL), CpG (10 μg/mL), R848 (5 μg/mL), and Imiquimod (10 μg/mL) (all from Invivogen). Supernatants were harvested after 24 h of stimulation. Cytokine human 25-plex panel luminex assays were performed on cell-free supernatants (Invitrogen).

Statistical analysis

GraphPad Prism (GraphPad Software) was used to perform statistical analysis. Two-tailed unpaired Mann–Whitney test was applied to all the data. The obtained p values were considered significant when $p < 0.05$.

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Conflict of interest: J.P.D. is a stakeholder in AXENIS (founder, member of the executive board). The remaining authors declare no conflict of interest.

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Abbreviations: **BRG**: Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ · **HIS**: human immune system

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