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Robust Expansion of Dendritic Cells *in vivo* by Hydrodynamic FLT3L-FC Gene Transfer

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Abstract

Due to low numbers of endogenous dendritic cells (DC) in vivo, exogenous DC-poietin Fms-like tyrosine kinase 3-ligand (FLT3L) is routinely used to generate DC for subsequent studies. We engineered a novel FLT3L-FC DNA construct that, when combined with hydrodynamic gene transfer (HDT), induced robust DC expansion in mice. DC generated in vivo by FLT3L-FC HDT produced cytokines in response to stimulation by an array of TLR agonists and promoted T cell proliferation. The FLT3L-FC protein produced in vivo spontaneously homodimerized to enable effective FLT signaling and the FC-domain enhanced its plasma half-life, providing an improved reagent and method to boost DC numbers.

Keywords

cytokine; progenitor; pharmacokinetics; pharmacodynamics

1. Introduction

Owing to their capacity to control both tolerance and immune responses, DC are the cellular fulcrum of the immune system. Indeed, DC are being tested in separate clinical trials for efficacy in suppressing autoimmune type one diabetes on one hand, and in boosting anti-tumor immune defenses (cancer vaccines) on the other. Although highly potent in processing and presenting antigens, DC are relatively rare in vivo compared with other leukocyte populations. Their scarcity presents an ongoing challenge for basic science

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research in defining their myriad properties, as well as in developing therapeutic DC for testing in preclinical disease models.

To generate clinically or experimentally useful numbers of DC, investigators routinely use standardized protocols [1] to culture and stimulate DC proliferation *in vitro* with DC-poietins, typically FLT3L [2-4] or a combination of interleukin-4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) [5]. Certain cytokine combinations can drive the generation of DC subsets with specific functional properties: for example, transforming growth factor-beta (TGF-β), M-CSF and IL-4 combine to generate tolerogenic DC from human cord blood monocytes [6,7]. FLT3L used alone generates bone marrow cultured DC that share many features with freshly isolated DC from normal mice, including morphology, surface marker expression, and production of inflammatory mediators in response to stimulation [8].

Due to its short plasma half-life (1.4 h following 10 μg i.p. injection, Fig. 1A); note a slightly longer $t_{1/2}$ of 5.2 h following 5 μg FLT3L injected i.m. was reported[9]), repeated daily injections with recombinant FLT3L protein [10] or implantation of FLT3L-secreting tumor cells [11] is necessary to achieve sufficient levels of FLT3L for effective DC expansion in vivo. Limitations to these approaches include expense and labor for the former, or generating DC in the context of rapidly growing tumors that can secrete factors and alter DC function [12], for the latter. We therefore sought to engineer FLT3L to extend its in vivo half-life, and then combine this improved reagent with a simple and cost-effective recombinant DNA-based overexpression method (hydrodynamic gene transfer) to effectively boost DC numbers in vivo.

2. Material and methods

2.1. Mice and animal care

Eight week-old C57Bl/6 female mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and used for all experiments. All experiments and procedures were approved by the Institutional Animal Use and Care Committee.

2.2 Hydrodynamic gene transfer

Mouse FLT3L and FLT3L-FC chimera were cloned into pLEV113 DNA vector (LakePharma, Inc.; Belmont, CA, USA) for mammalian gene expression in vivo. HDT was performed as previously described [13,14]. In brief, plasmid DNA was diluted in 2 ml physiological saline solution and rapidly injected *i.v.* (tail vein) in 3-5s.

2.3 Pharmacokinetic analysis by ELISA for human FC and FLT3L

Mice were injected with 10μg of either FLT3L (Peprotech; Oak Park, CA) or FLT3L-FC (LakePharma, Inc., Belmont, CA, USA) protein *i.p.* in PBS, or 10μg FLT3L-FC DNA by HDT. Blood plasma was collected at various time points and levels of FLT3L and FLT3L-FC protein determined by mouse FLT3L ELISA (R&D Systems; Minneapolis, MN, USA) per manufacturer's protocol.

2.4 Western Blot

Mice were injected with human FC-control or FLT3L-FC chimera DNA by HDT. Twelve days post-HDT, blood plasma was collected and proteins separated via SDS-PAGE. Western blot was performed using anti-mouse FLT3L antibody (R&D Systems; Minneapolis, MN, USA) and images captured using Odyssey software (LI-COR Biosciences; Lincoln, NE, USA).

2.5 Tissue harvest and flow cytometry

Mice were injected with human FC-control or FLT3L-FC vector by HDT, or *s.c.* with 5×10^6 FLT3L-secreting B16 melanoma cells. 12 days post-injection, mice were euthanized by CO2 asphyxiation. Inguinal lymph nodes and spleen were collected, processed for single cell isolation, counted by hemocytometry (trypan blue exclusion to identify live cells), and stained for flow cytometry analysis. The following antibodies were used: biotinylated lineage marker antibodies (CD3, CD19, Ter119, Ly-6G/C, and NK1.1), anti-CD11c, -MHCII, -PDCA1, -CD11b, -CD8α, -CD80, -CD86. Flow cytometry was performed using FACSDiva software on a LSRII (BD Biosciences; San Jose, CA, USA) and analyzed using FlowJo software (Tree Star, Inc.; Ashland, OR, USA).

2.6 Cell isolation for functional studies

DC were isolated from spleen, mesenteric lymph nodes and peripheral lymph nodes by positive selection of CD11c+ cells using CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. CD11c+ DC were further separated into total cDC as Lin (CD3 CD19, Ter119, Ly6G/C, NK1.1)-CD11c+MHCII +PDCA1- using a FACSAria. pDC were isolated using plasmacytoid dendritic cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Spleen $CD4^+$ T cells were isolated by negative selection using CD4+ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.7 Cell culture and CFSE labeling

DC (2×10^6 cells/ml) were cultured in complete RPMI 1640 supplemented with 10% FCS alone or in the presence of R837 (0.5 μg/ml), LPS (5 μg/ml), CpGA-ODN1585 (5 μg/ml), or polyinosinic:polycytidylic acid [poly(I:C); 5 μg/ml] (all from InvivoGen, San Diego, CA). Cytokine secretion was determined at 20 h of culture by ELISA. For co-culture experiments, MACS purified (Miltenyi Biotech, San Diego, CA, USA) CD4+ T cells were labeled with CFSE (1 μM; R&D Systems; Minneapolis, MN, USA) for 5 min at RT, followed by extensive washing in complete RPMI 1640, prior to culture. DC were cultured at a ratio of 1:20 with CD4+ T cells (1.25×10^5/ml) in the presence of anti-CD3 (1 μg/ml). Cells were collected on day 3 of culture and stained with CD4 antibody for detection of T cells and analyzed by Flow cytometry using FACSDiva Software on an LSRII (BD Biosciences; San Jose, CA, USA).

2.8 ELISA

The following reagents were used for ELISA: mouse IL-6 ELISA set, mouse IL-12p40 ELISA set, (BD Biosciences; San Jose, CA, USA). IFN-α ELISA was performed using anti

μ–IFNα (Rmma-1) as capture Ab and rabbit-PAb against μ–IFN-α for detection (PBL InterferonSource, Piscataway, NJ, USA).

3. Results and Discussion

To improve DC expansion in vivo, we first sought to enhance the pharmacokinetics of FLT3L. While others have reported success with FLT3L incorporation into a sustained-release polaxamer-based matrices [9], we pursued cytokine fusion to human immunoglobulin FC to prolong plasma half-life, as reported for G-CSF [15]. Indeed, the C_{max} of engineered chimeric mouse FLT3L-human FC (IgG_1 isotype, FLT3L-FC) was ∼5-fold higher than FLT3L, the $t_{1/2}$ was extended ∼16-fold, and the total cytokine exposure over 24 h ($AUC_{0\rightarrow24}$) was ∼32-fold higher than unmodified FLT3L (Fig. 1 A).

We next added hydrodynamic gene transfer (HDT), where plasmid DNA is rapidly injected (3-5 s) i.v. by tail vein in a large volume (2 ml) of isotonic buffer[13]. The supraphysiologic venous pressure drives hepatocyte uptake of DNA and transient protein overexpression [13,14]. A strong constitutively active mammalian promoter (in this case, elongation factor 1α) is necessary for high level gene expression by HDT (H.T. unpublished observations). FLT3L-FC protein expression following HDT peaked on day 6 with a C_{max} of 42.6 μg /ml plasma, ∼7-fold higher than the C_{max} following recombinant protein injection (10 μg), and the levels were durable and remained elevated for at least 12 days with a post-C_{max} $t_{1/2}$ of 34.3 h (Fig. 1 A). Following FLT3L-FC HDT, cytokine exposure over 12 days ($AUC_{0\rightarrow288}$) was vastly higher (∼140-fold) than the extrapolated exposure following daily dosing (10 μg) with unmodified FLT3L protein.

Due to the importance of dimeric FLT3L in binding to FLT3 to trigger receptor dimerization and autophosphorylation [16,17], we investigated the quaternary structure of engineered FLT3L-FC. While endogenous FLT3L is a non-covalently-linked homodimer, the cysteines present in the engineered FC domain of FLT3L-FC are predicted to enable spontaneous assembly into covalently-linked (FC-disulfide-FC) stabilized homodimers, similar to G-CSF-FC [15]. Indeed, by western analysis FLT3L-FC formed disulfide-bonded homodimers that were reduced in the presence of p-mercaptoethanol (Fig. 1B). The reduced monomer has a predicted M_r of 44 kDa; the apparent M_r of the protein was ∼50 and 35 kD due to glycosylation (Fig. 1B) [18]. FLT3L-FC expressed in vivo by HDT in plasma was also a covalently-linked homodimer (Fig. 1B); the bulk of plasma FLT3L-FC (∼70%) was ∼50 kDa under reducing conditions. Thus the engineered FC domain likely stabilizes the FLT3L-FC homodimer to enable efficient FLT3 signaling.

Splenomegaly is a simple pharmacodynamic (PD) readout for the in vivo effects of leukocyte-targeted growth factors such as FLT3L [10]. We investigated the PD profile of various doses of FLT3L-FC DNA administered by HDT. Just 1-10 μg of DNA induced a 4-5-fold increase in spleen size and splenocyte numbers by day 12 post-HDT, with an EC_{50} of 0.1 μg (Fig. 1C).

We next assessed the expansion of two basic DC subsets: $CD11c^{hi}MHCII^{+}$ conventional DC (cDC) and $CD11c^{int}PDCA1^{+}$ plasmacytoid DC (pDC) in the spleen and lymph nodes. We compared the in vivo efficacy of our platform technology (engineered cytokine FLT3L-FC

combined with HDT) vs. conventional *s.c.* injection of FLT3L-secreting B16 tumor cells for DC expansion. Twelve days post-injections we harvested spleens and inguinal lymph nodes and isolated single cells for antibody staining and flow cytometry. In all treatment conditions, the percentage and absolute numbers of cDC and pDC increased compared to mice injected with FC-control (Fig. 1D,E). Importantly, FLT3L-FC HDT administration expanded cDC and pDC with similar, if not improved efficacy to that of conventional FLT3L-secreting tumors (Fig. 1D,E).

DC expanded by HDT or FLT3L-secreting tumors expressed low levels of CD80 and CD86, and intermediate levels of MHC class II, consistent with an immature DC phenotype (Fig. 1E). To analyze the subsets of cDC expanded by FLT3L-FC HDT, we used CD11b and CD8α to define myeloid DC (CD11b+CD8α-, known for enhanced phagocytic activity and Th2 priming) and lymphoid DC (CD11b-CD8α+, characterized by maintenance of self-antigenic immune tolerance in the steady state, and reduced phagocytic activity and Th1 priming in the activated state). Most of the cDC expanded by FLT3L-FC HDT and by FLT3L-secreting tumors were CD11b+CD8α- (∼60%), and 10-20% were CD11b-CD8α+ (Fig. 1E).

We next assessed the functional activity of DC expanded by FLT3L-FC HDT. First we purified cDC and pDC and evaluated cytokine production in response to an array of TLR ligands. cDC produced significant levels of IL-12p40 and IL-6 following CpGA (TLR9 agonist) stimulation (Fig. 2A). LPS (TLR4 agonist) triggered a similar response, although with reduced amplitude (Fig. 2A). cDC also secreted significant levels of IL-6 in response to pIC (TLR3 agonist, Fig. 2A). pDC produced significant levels of IFNα in response to R837 (TLR7 agonist) compared to unstimulated cells (Fig. 2A). TLR-ligand stimulation elicited a similar cytokine secretion profile from purified DC expanded by the FLT3L tumor model, although in most cases the level of cytokine secretion was slightly higher than FLT3L-FC HDT-expanded DC (Fig. 2A). Finally, we evaluated the ability of FLT3L-FC HDT expanded DC to promote naive T cell proliferation, a typical APC function. When combined with polyclonal anti-CD3 stimulation, purified cDC induced robust proliferation of CFSE-labeled CD4 T cells (Fig. 2B). T cell mitogenesis was similarly induced by DC generated in vivo by FLT3L-secreting tumors (Fig. 2B). Thus, FLT3L-FC HDT generates functionally active DC that secrete cytokines in response to TLR agonists and stimulate T cell proliferation, and are comparable to DC expanded by the FLT3L tumor model.

Ultimately, the total number of splenic cDC increased to 26 million (from 0.5 million in untreated mice), and the total number of splenic pDC increased to 28 million (from 0.3 million) following a single i.v. injection of FTL3L-FC DNA by HDT. This inexpensive and simple approach enabled a 50-100 fold expansion of DC in vivo, and will facilitate basic science and preclinical DC-centric investigations. Furthermore, because of its reliance on standard molecular biology techniques and not on in vitro expression and purification of recombinant proteins, this approach can easily be extended to evaluate cytokines or engineered cytokine mutants for leukocyte subset expansion in vivo, or to directly test for cytokine efficacy in mouse disease models.

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References

1. Weimershaus M, van Endert P. Preparation of dendritic cells by in vitro cultures. Methods Mol Biol. 2013; 960:351–357. [PubMed: 23329498]
2. Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol. 2007; 7:19–30. [PubMed: 17170756]
3. Brasel K, De Smedt T, Smith JL, Maliszewski CR. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood. 2000; 96:3029–3039. [PubMed: 11049981]
4. Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, Fuchsberger M, Lahoud MH, O'Keeffe M, Shao QX, Chen WF, Villadangos JA, Shortman K, Wu L. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. J Immunol. 2005; 174:6592–6597. [PubMed: 15905497]
5. Scheicher C, Mehlig M, Zecher R, Reske K. Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. J Immunol Methods. 1992; 154:253–264. [PubMed: 1401959]
6. Li G, Abediankenari S, Kim YJ, Campbell TB, Ito S, Graham-Evans B, Cooper S, Broxmeyer HE. TGF-beta combined with M-CSF and IL-4 induces generation of immune inhibitory cord blood dendritic cells capable of enhancing cytokine-induced ex vivo expansion of myeloid progenitors. Blood. 2007; 110:2872–2879. [PubMed: 17585053]
7. Li G, Kim YJ, Broxmeyer HE. Macrophage colony-stimulating factor drives cord blood monocyte differentiation into IL-10(high)IL-12absent dendritic cells with tolerogenic potential. J Immunol. 2005; 174:4706–4717. [PubMed: 15814695]
8. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. J Immunol. 2007; 179:7577–7584. [PubMed: 18025203]
9. Robinson SN, Chavez JM, Pisarev VM, Mosley RL, Rosenthal GJ, Blonder JM, Talmadge JE. Delivery of Flt3 ligand (Flt3L) using a poloxamer-based formulation increases biological activity in mice. Bone Marrow Transplant. 2003; 31:361–369. [PubMed: 12634727]
10. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. J Exp Med. 2003; 198:305–313. [PubMed: 12874263]
11. Mach N, Gillessen S, Wilson SB, Sheehan C, Mihm M, Dranoff G. Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. Cancer Res. 2000; 60:3239–3246. [PubMed: 10866317]
12. Polak ME, Borthwick NJ, Jager MJ, Cree IA. Melanoma vaccines: The problems of local immunosuppression. Hum Immunol. 2009; 70:331–339. [PubMed: 19405172]
13. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 1999; 6:1258–1266. [PubMed: 10455434]
14. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther. 1999; 10:1735–1737. [PubMed: 10428218]
15. Cox GN, Smith DJ, Carlson SJ, Bendele AM, Chlipala EA, Doherty DH. Enhanced circulating half-life and hematopoietic properties of a human granulocyte colony-stimulating factor/immunoglobulin fusion protein. Exp Hematol. 2004; 32:441–449. [PubMed: 15145212]
16. Hannum C, Culpepper J, Campbell D, McClanahan T, Zurawski S, Bazan JF, Kastelein R, Hudak S, Wagner J, Mattson J, et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of

haematopoietic stem cells and is encoded by variant RNAs. Nature. 1994; 368:643–648. [PubMed: 8145851]

17. Savvides SN, Boone T, Andrew Karplus P. Flt3 ligand structure and unexpected commonalities of helical bundles and cystine knots. Nat Struct Biol. 2000; 7:486–491. [PubMed: 10881197]
18. Verstraete K, Savvides SN. Extracellular assembly and activation principles of oncogenic class III receptor tyrosine kinases. Nat Rev Cancer. 2012; 12:753–766. [PubMed: 23076159]

Abbreviations

AUC	area under curve
cDC	conventional DC
C_{max}	maximum concentration
DC	dendritic cell
EC_{50}	half-maximal effective concentrations
FLT3L	Fms-like tyrosine kinase 3-ligand
HDT	hydrodynamic gene transfer
$t_{1/2}$	half-life
PD	pharmacodynamics
pDC	plasmacytoid DC
PK	pharmacokinetics

Highlights

- We engineered a novel FLT3L-immunoglobulin FC fusion construct
- The FC-domain improved the plasma pharmacokinetics profile of FLT3L
- Hydrodynamic gene transfer using FLT3L-FC DNA induced robust DC expansion in vivo
- FLT3L-FC protein produced in vivo spontaneously dimerized to enable FLT3 signaling
- Splenomegaly is an effective pharmacodynamic readout for FLT3L action in vivo

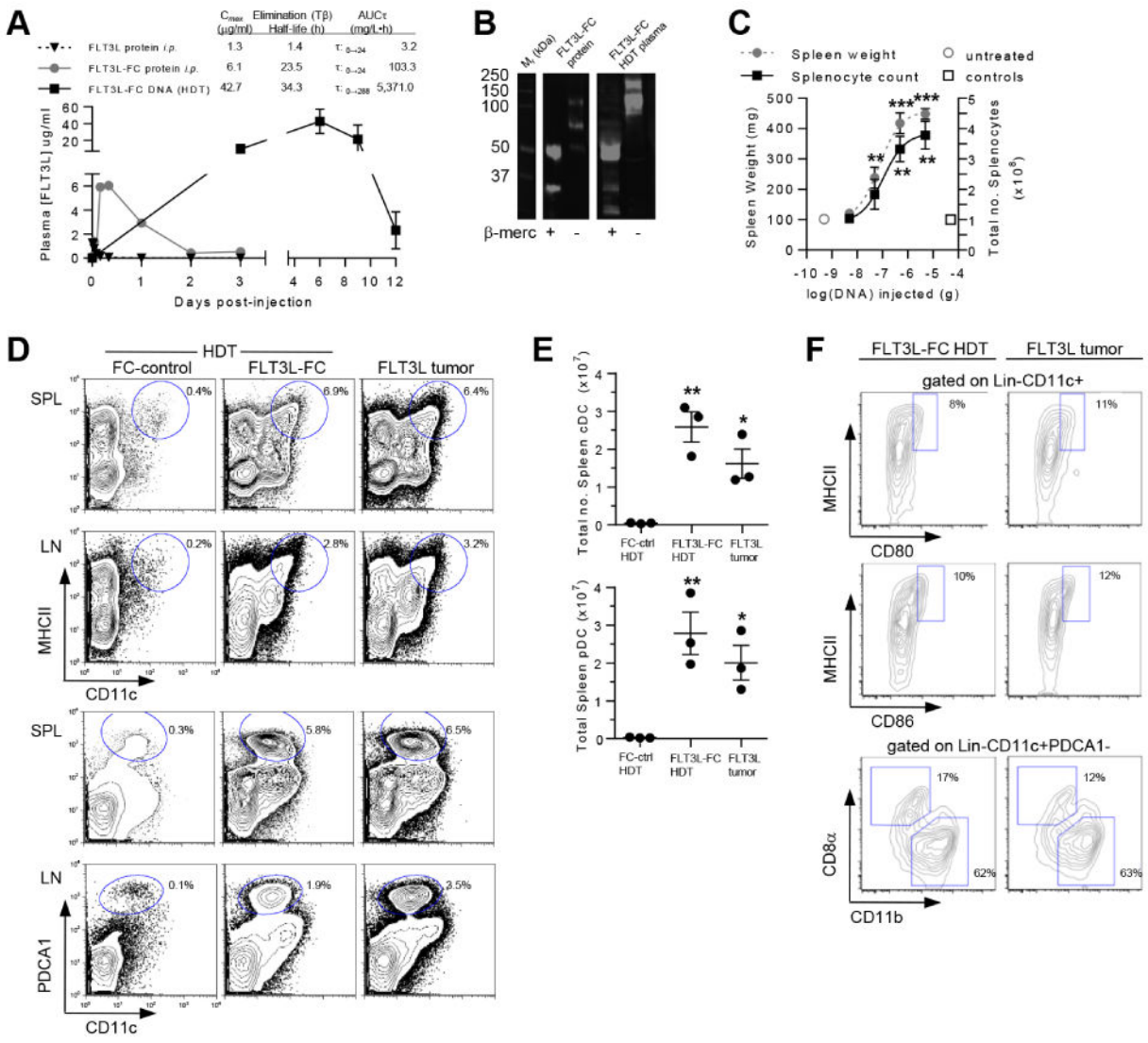

Fig. 1.

Robust expansion of dendritic cells in vivo by FLT3L-FC HDT. (A) Pharmacokinetics. Mice were injected with recombinant FLT3L or FLT3L-FC protein (10μg i.p.) or FLT3L-FC DNA by HDT (10μg i.v.). Plasma was collected at the indicated timepoints and FLT3L levels determined by ELISA. n=3 mice per group, mean ±SEM. (B) FLT3L-FC dimerization in vivo. Western analysis of plasma from FLT3L-FC HDT mice taken 12 days post-injection using anti-FLT3L antibody (reducing (β-mercaptoethanol) or non-reducing conditions). Purified recombinant FLT3L-FC used as a control: two FLT3L-specific bands (monomers) were detected under reducing conditions (the upper band is a more-glycosylated form), whereas homodimers were detected under non-reducing conditions. Similar FLT3L-specific bands (monomers in reducing conditions and homodimers in non-reducing conditions) were detected in plasma following FLT3L-FC HDT. (C) Pharmacodynamics. Mice were injected with the indicated gram quantities of FLT3L-FC by HDT and spleen weight/splenocyte numbers determined 12 days later. n=3 mice mice per group, mean ±SEM, ** p<0.01, ***p<0.001 by t-test compared to untreated controls. (D) Spleen (SPL) and inguinal lymph

node (LN) cells from mice injected with FC-control or FLT3L-FC by HDT, or s.c. with 5×10^6 FLT3L-secreting B16 tumor cells were harvested 12 days post-injection, stained for cDC (Lin-CD11c+MHCII+, upper plots) and pDC (Lin-CD11c+PDCA1+, lower plots). (E) Absolute numbers of cDC and pDC in the spleen. n=3 mice/group, mean±SEM, *$p<0.05$, **$p<0.01$ by t-test compared to FC-control (FC-ctrl). (F) DC immunophenotype. Spleen DC (Lin-CD11c+) from FLT3LFC HDT or FLT3L tumor mice were stained for MHC class II and CD80 or CD86 (upper plots). Spleen cDC (Lin-CD11c+PDCA1-) were stained for CD8α and CD11b (lower plots). Flow cytometry data are of one mouse representative of three mice with similar phenotype.

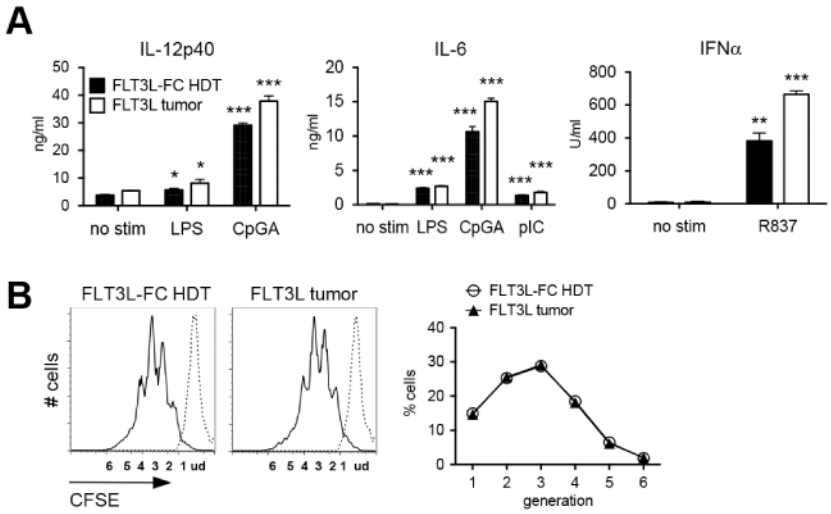

Fig. 2.

Functional analysis of dendritic cells expanded by FLT3L-FC HDT.

(A) Spleen and PLN from mice injected with FLT3L-FC by HDT or s.c. with 5×10^6 FLT3L secreting B16 tumor cells were harvested 13 days post injection, pooled and cDC were sorted by flow cytometry (Lin-CD11c+MHCII+PDCA-) and stimulated in vitro with LPS (5 μg/ml), CpGA (5 μg/ml), or pIC (5 μg/ml) for IL-6 and IL-12p40 cytokine secretion analysis. Magnetic bead-purified pDC were isolated and stimulated with R837 (0.5 μg/ml) for IFNα cytokine secretion analysis. Cytokine secretion was determined 20 h later by ELISA of culture supernatants. n=3-4 mice per group. Graphs show the mean ± SEM of TLR induced cytokine secretion in triplicate cultures (n=3), *p<0.05,**p<0.01,***p<0.001 by *t*-test compared to unstimulated control. (B) cDC obtained as in (A) were co-cultured with spleen-derived CFSE-labeled CD4+ T cells in the presence of anti-CD3 (1 μg/ml). On day 3 of culture, T cells were analyzed by flow cytometry for proliferation. The intensity of CFSE was determined among gated CD4+ T cells. Histograms are from one of four replicate cultures with similar cell distributions. Graphs show the mean ± SEM of replicate cultures (n= 4). ud, undivided cells (CFSE-labeled CD4+ T cells incubated with anti-CD3 without DC).