Synergy between rapamycin and FLT3 ligand enhances plasmacytoid dendritic cell–dependent induction of CD4⁺CD25⁺FoxP3⁺ Treg

Moanaro Biswas,¹ Debalina Sarkar,¹ Sandeep R. P. Kumar,¹ Sushrusha Nayak,¹ Geoffrey L. Rogers,¹ David M. Markusic,¹ Gongxian Liao,² Cox Terhors,² and Roland W. Herzog¹

¹Department of Pediatrics, University of Florida, Gainesville, FL; and ²Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston MA

Key Points

- Rapamycin and Flt3L are synergistic in Treg induction when coadministered with antigen, resulting in improved tolerance induction.
- pDCs are required for efficient Treg induction and selectively expanded with Flt3L/rapamycin because of high mTOR activity.

CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Treg) are critical elements for maintaining immune tolerance, for instance to exogenous antigens that are introduced during therapeutic interventions such as cell/organ transplant or gene/protein replacement therapy. Co-administration of antigen with rapamycin simultaneously promotes deletion of conventional CD4⁺ T cells and induction of Treg. Here, we report that the cytokine FMS-like receptor tyrosine kinase ligand (Flt3L) enhances the in vivo effect of rapamycin. This occurs via selective expansion of plasmacytoid dendritic cells (pDCs), which further augments the number of Treg. Whereas in conventional DCs, rapamycin effectively blocks mammalian target of rapamycin (mTOR) 1 signaling induced by Flt3L, increased mTOR1 activity renders pDCs more resistant to inhibition by rapamycin. Consequently, Flt3L and rapamycin synergistically promote induction of antigen-specific Treg via selective expansion of pDCs. This concept is supported by the finding that Treg induction is abrogated upon pDC depletion. The combination with pDCs and rapamycin is requisite for Flt3L/antigen-induced Treg induction because Flt3L/antigen by itself fails to induce Treg. As coadministering Flt3L, rapamycin, and antigen blocked CD8⁺ T-cell and antibody responses in models of gene and protein therapy, we conclude that the differential effect of rapamycin on DC subsets can be exploited for improved tolerance induction. (Blood. 2015;125(19):2937-2947)

Introduction

Regulatory T cells (Treg) are critical in central and peripheral tolerance to self-antigens as well as exogenous antigens. Because of their ability to suppress immune responses, ex vivo expanded CD4⁺CD25⁺FoxP3⁺ Treg are used to prevent graft-versus-host disease in bone marrow transplants and are tested in clinical trials for autoimmune diseases. Treg can also be induced in vivo and play important roles in tolerance to cell and organ transplants, oral tolerance, and tolerance to therapeutic proteins in the treatment of genetic diseases.

One method of inducing antigen-specific CD4⁺CD25⁺FoxP3⁺ Treg is to introduce the antigen in the presence of rapamycin. The macroside immunosuppressant rapamycin (sirolimus) can inhibit intracellular signaling through mammalian target of rapamycin (mTOR; a serine/threonine kinase) complex 1 by binding to the immunophilin FK506 binding protein-12 (FKBP-12).¹ Thereby, rapamycin inhibits cycle progression of activated T cells, leading to T-cell anergy or deletion,¹ and inhibits the T-cell stimulatory activity of dendritic cells (DCs),²,³ resulting in impaired cytokine-driven cellular activation and selective depletion of T helper (Th) 1, Th2, and Th17 cells.⁴ This is associated with an increased expansion of CD4⁺CD25⁺FoxP3⁺ Treg in response to reduced mTOR signaling.⁵,⁹ Our previous studies have shown that Treg are induced by rapamycin, when coadministered with protein or peptide antigen, can suppress inhibitory antibody formation to factor (F) VIII and FIX in treatment of hemophilia A and B.¹⁰-¹² This approach was further improved by addition of the cytokine interleukin (IL) 10.¹¹,¹² Treg homeostasis is controlled by DCs, so that increased numbers of DCs lead to a corresponding accumulation of Treg.¹³ Hence, expansion of DCs, using the ligand for the FMS-like receptor tyrosine kinase Flt3 (CD135) indirectly leads to expansion of existing peripheral Treg.¹³,¹⁴,¹⁵ These observations prompted us to hypothesize that Treg induction with antigen/rapamycin combined with Treg expansion via Flt3L-induced DC proliferation should be synergistic and may represent an ideal strategy for effective in vivo Treg induction. Flt3 is a transmembrane glycoprotein expressed in stem and early hematopoietic precursor cells in the bone marrow, immature thymocytes, and steady-state DCs.¹⁶ Its cognate ligand (Flt3L) is a hematopoietic growth factor with essential functions in early progenitor and DC generation and is involved in the proliferation, differentiation, development, and mobilization of these cells in the bone marrow, peripheral blood, and lymphoid organs.¹⁶,¹⁷ Flt3/Flt3L signaling is critical to the generation and steady-state expansion of both the conventional (CD11c⁺, CD8⁺CD11c⁺) and plasmacytoid (CD11c⁺PDCA-1⁺) subsets of DCs.¹⁸,¹⁹ Flt3⁻/⁻ or Flt3L⁻/⁻ mice show deficient hematopoiesis and reduced DC numbers and, consequently, also reduced Treg numbers.¹⁶,²⁰
The molecular signaling pathways underlying Flt3L activity in DC development are only partially defined but include a role for signal transducer and activator of transcription (STAT) 3,21,22 However, a recent report has shown that Flt3L mediates its signaling through the phosphatidylinositol 3-kinase (PI3K)--mTOR pathway and is thus impaired by rapamycin,23 PI3K hyperactivation, through deletion of the negative regulator phosphatase and tensin homolog, causes increased DC proliferation.24 The serine/threonine kinase protein kinase B (PKB, also known as AKT) regulates multiple biological processes by binding various molecules, one of which is the lipid kinase PI3K.24 Importantly, mTOR is a pivotal downstream mediator of the PI3K/AKT pathway.25 Rapamycin-induced inhibition of mTOR signaling in DCS is associated with changes in DC generation, expansion, activation, and maturation.18,20-28 In particular, rapamycin inhibited the expansion of DCs in Flt3L-treated mice by 40% to 50% in 2 prior studies.2,23 These findings would argue against our hypothesis and instead predict that rapamycin should block Flt3L-induced DC and ultimately Treg expansion.

Here, we demonstrate that rapamycin and Flt3L can be used synergistically to substantially improve in vivo Treg induction. In this regimen, rapamycin blocks expansion of conventional DCs (cDCs) but not plasmacytoid DCs (pDCs), resulting in improved Treg induction, which is pDC dependent. Increased mTOR activity in pDCs makes their Flt3L signaling pathway more resistant to rapamycin.

Materials and methods

Mouse strains and experiments

All experimental animals were 6- to 10-week-old male mice and housed under special pathogen-free conditions. BDCA-2 (CLEC4C)-DTR transgenic C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). These mice have a simian diphtheria toxin receptor (DTR) under the transcriptional control of the pDC-specific BDCA2 promoter, so that administration of diphtheria toxin (DT) depletes pDCs.29 OT-II (B6.Cg-Tg(TcraTcrb)425Cbn; C57BL/6; Jackson Laboratories) are transgenic for a CD4 T-cell receptor (TCR) constitutively expressing part of its receptor31; (2) Flt3L has been shown to synergize with rapamycin in antigen-specific Treg induction and T effector cell deletion. To this end, DO11.10-tg x Rag-2−/− mice were injected 3 times per week for 4 weeks with rapamycin and the OVA-derived peptide OVA323-339, which is specific for DO11.10. Simultaneously, we coinjected one of the following recombinant proteins: IL-10, IL-2, Flt3L, Fc-glucocorticoid-induced tumor necrosis factor receptor-ligand (Fc-GITR-L), or a combination of Flt3L and Fc-GITR-L (Flt3L/Fc-GITR-L). These molecules were selected for the following reasons: (1) IL-2 is an important growth factor for Treg, which constitutively expresses part of its receptor1; (2) Flt3L has been shown to indirectly expand Treg through increasing DC numbers14; and (3) the Fc-GITR-L fusion protein dimer preferentially enhances proliferation of Treg, which naturally have highly upregulated expression of GITR.32 Each of these reagents was able to increase deletion of conventional CD4+ T cells (37% to 75% decrease in OVA-specific CD4+ T cells as compared with untreated animals; Figure 1A) and improve Treg induction (to 7% to 15% of CD4+ T cells; Figure 1B). Compared with IL-10 (the molecule we had originally used), Flt3L and Fc-GITR-L significantly further improved CD4+ T-cell deletion. A combination of Flt3L and Fc-GITR-L did not further increase the effect of either molecule alone. Surprisingly, coadministering antigen, rapamycin, and Flt3L most consistently increased the Treg population, even though Flt3-FcFlt3L signaling has been suggested to involve the mTOR pathway, which is directly impaired by rapamycin (Figure 1B-C).32 To better understand how Flt3L and rapamycin might work synergistically in Treg induction, we administered Flt3L alone, in combination with OVA323-339, or with both OVA323-339 and rapamycin. We found that Flt3L in combination with the OVA323-339 peptide was sufficient to deplete conventional CD4+ T cells by 35% to 45% (Figure 2A-B),
Figure 1. Multiple molecules can be used synergistically with rapamycin to decrease conventional CD4+ T cells and induce Treg. Percentage of CD4+ T cells (A) or CD205-FoxP3+ Treg/CD4+ T cells (B) in spleens of DO11.10-tg x Rag-2f/f BALB/c mice IP injected 3 times per week for 4 weeks with 100 µg of OVA323-339 plus rapamycin (4 mg/kg) and either Flt3L (80 µg/kg), Fc-GITR-L (8 mg/kg), a combination of Flt3L/Fc-GITR-L, IL-2 (50 ng/kg), or IL-10 (50 ng/kg). Untreated naïve animals serve as controls (n = 4-5 per group). Data are average ± standard deviation (SD). Statistically significant differences were determined by 2-way analysis of variance (ANOVA). (C) Examples of Treg induction with Flt3L/OVA323-339/rapamycin compared with OVA323-339/rapamycin and untreated control mouse.

Rapamycin is required for Treg induction

The induction of OVA323-339-specific CD4+ CD25+ FoxP3+ Treg was dependent on the presence of rapamycin in the treatment cocktail, even though Flt3L without rapamycin was sufficient to delete activated CD4+ T effector cells. Neither Flt3L alone nor Flt3L/OVA323-339 were sufficient in inducing Treg in DO11.10-tg x Rag-2f/f mice (Figure 3A-B and supplemental Figure 2A). Rapamycin by itself also failed to induce Treg. When a combination of Flt3L/OVA323-339/rapamycin was administered, Treg increased from undetectable to 5% to 9% (mean 6.2 ± 0.99) of CD4+ T cells. These results were confirmed in 2 independent experiments (Figure 3A-B and supplemental Figure 2A). Induced Treg expressed CD26L1, CD154/CTLA-4, GITR, and Helios (Figure 3C and supplemental Figure 2B).

Flt3L selectively expands pDCs in the presence of rapamycin

Upon administering Flt3L (80 µg/kg, 3 per week for 3 weeks) to DO11.10-tg x Rag-2f/f mice,14,38 we found a significant expansion of pDCs (CD11c+ PDCA-1+) in the spleen and bone marrow (Figure 4A, F). Whereas the number of cDCs (CD11c+) in the spleen had increased, Flt3L caused only a minor expansion of these cells in the bone marrow (Figure 4B, G). Coinjecting the OVA323-339 antigen together with Flt3L further expanded the number of cDCs in the spleen and bone marrow (Figure 4B, G), while limiting expansion of pDCs in bone marrow (Figure 4F). Surprisingly, treatment of DO11.10-tg x Rag-2f/f mice with Flt3L/OVA323-339/rapamycin abrogated cDC expansion (Figure 4B, G) while allowing expansion of pDCs to occur to a level similar to that in mice receiving Flt3L alone (Figure 4A,F). Thus, in response to Flt3L/OVA323-339/rapamycin, the DC population became enriched for pDCs with a corresponding proportional reduction of cDCs (Figure 4C-D, H-I).

whereas addition of rapamycin had no further effect on the T-cell depletion (Figure 2B and supplemental Figure 1). CD4+ T cells from both mice treated with OVA323-339 or Flt3L/OVA323-339 had an activated phenotype (decreased CD62L+, increased CD69+, CD44+ expression; Figure 2D-F). Addition of Flt3L increased CD69 expression and the proportion of AnnexinV+ CD4+ T cells to >20% (Figure 2C). We conclude that coadministering Flt3L and OVA323-339 caused activation and apoptosis of OVA323-339-specific conventional CD4+ T cells, consistent with activation-induced cell death.

The sum of results obtained thus far suggest that selective pDC expansion augments Treg induction.

Treg induction by Flt3L/antigen/rapamycin is pDC dependent

In order to determine directly whether Treg induction was indeed dependent on the number of pDCs, we depleted pDCs by intravenously injecting animals with a monoclonal antibody directed against PDCA-1 (clone 927; 2 500 µg doses separated by 2 weeks). Mice continued to receive Flt3L/OVA323-339/rapamycin during the course of anti-PDCA-1 injections for a complete treatment schedule of 3.5 weeks (Figure 5A). In mice that had received the PDCA-1 antibody, splenic pDCs were depleted by ∼40% (supplemental Figure 3A), as compared with mice that had received only the Flt3L/OVA323-339/rapamycin combination. Strikingly, the spleens of pDC-depleted mice contained sevenfold fewer Treg (Figure 5B), demonstrating that Treg induction with this protocol was indeed largely pDC dependent. We found the same effect in BALB/c mice in which pDCs had been depleted with a second antibody, namely the anti-PDCA-1 clone 120G8. Into these mice, 1 × 105 CD4+ CD25- cells purified from DO11.10-tg x Rag-2f/f mice had been adoptively transferred (Figure 5C), and during the course of the experiment, Flt3L/OVA323-339/rapamycin was administered (Figure 5C and supplemental Figure 4). When transplanted cells were identified with the clonotypic KJ1-26 antibody, the induction of OVA323-339-specific Treg (KJ1-26+ CD25+ FoxP3+) was significantly reduced in pDC-depleted recipient mice (∼76% depletion; supplemental Figure 3B), when compared with control recipients (Figure 5D).

In a third set of experiments, pDCs were depleted by injecting DT into BDCA-2-DTR mice, which caused up to 97% depletion of these cells (supplemental Figure 3C). In control mice, Flt3L/OVA323-339/rapamycin induced an OVA-specific Treg response as judged by analysis with MR9-4 antibody (which is specific for V beta 5.1/5.2, which is part of the I-Ab/OVA323-339-specific CD4+ TCR; Figure 5E-F and supplemental Figure 5B). Importantly, induction of MR9-4+ CD25+ FoxP3+ cells was abrogated upon pDC depletion (Figure 5F). By contrast, we did not find an effect of pDC depletion on Treg induction among CTV-labeled OT-II CD4+ CD25+ T cells adoptively transferred into these BDCA-2-DTR mice (supplemental Figure 5A, C). However, far fewer transferred OT-II cells were found at the end of the experiment when compared with DO11.10-tg CD4+ T cells transferred into BALB/c mice (supplemental Figure 3B-C), suggesting...
that the drug treatment more effectively deleted OT-II cells, which may have limited conversion to Treg.

Finally, in 2 of these experimental systems, pDC depletion further reduced OVA323-339-specific CD4+ T cells (supplemental Figure 3A, C), suggesting that pDCs to some extent may also limit deletion of effector T cells.

Rapamycin only partially blocks mTOR phosphorylation in pDCs

Recent reports have shown that rapamycin inhibits Flt3-Flt3L signaling in DCs by interfering with the PI3K-mTOR pathway. However, because substantially higher doses of rapamycin are required to block Flt3L-induced expansion of pDCs than of cDCs (Figure 6A), we examined potential underlying mechanisms causing this differential effect. First, higher expression of Flt3, the receptor for Flt3L, on the surface of pDCs compared with cDCs might be a contributing factor (supplemental Figure 6). Second, when purified pDCs and cDCs (5 × 10^5 cells per mL) were cultured ex vivo with Flt3L, p-mTORSer2448 expression was differentially upregulated (ie, to a more limited extent in cDCs than in pDCs; Figure 6B and supplemental Figure 7). Third, whereas rapamycin downregulated p-mTORSer2448 in pDCs or cDCs, rapamycin only partially blocked Flt3L-induced p-mTORSer2448 signaling in pDCs, whereas complete blockage was observed in cDCs.

These cytometry-based conclusions on p-mTORSer2448 expression were confirmed by western blotting (Figure 6D). Naive mice had low basal expression of p-mTORSer2448 in pDCs, which was substantially increased upon Flt3L treatment ex vivo (Figure 6D, top) and comparatively resistant to inhibition by rapamycin. In contrast, p-mTORSer2448 induction in cDCs was considerably lower upon Flt3L treatment and completely inhibited by rapamycin or Flt3L/rapamycin treatment. Densitometric analysis of mTORSer2448 expression relative to β-actin expression was used to quantitate western blot data (Figure 6D). In pDCs isolated from mice that had been injected daily for 10 days with 80 μg Flt3L/kg, a higher basal level of p-mTORSer2448 was observed in both pDCs and cDCs (Figure 6D, bottom). However, a similar pattern of differential p-mTORSer2448 inhibition was observed. Taken together, the data show that rapamycin less effectively inhibited the mTOR signaling pathway in pDCs as compared with cDCs.

Flt3L combined with rapamycin prevents immune responses in gene and protein therapy

To evaluate whether empowering Treg cells in vivo would block CD8+ T-cell responses, C57BL/6 mice were pretreated with combinations of Flt3L/OVA323-339, OVA323-339/rapamycin, or Flt3L/OVA323-339/rapamycin, followed by intramuscular injection of our scAAV1-CMV-OVA vector.34 When OVA-specific CD8+ T-cell responses were quantified by tetramer stain (Figure 7A), control mice (vector only treated) and Flt3L/OVA323-339-treated mice showed a substantial response at both the 2-week (11.2 ± 8% and 10.7 ± 8.2%) and 4-week time points (4.4 ± 2.6% and 8.4 ± 2.9%; Figure 7B). In contrast, tetramer positive CD8+ T-cell responses were low to undetectable in OVA323-339/rapamycin- and Flt3L/OVA323-339/rapamycin-treated
Subsequently, mice were challenged with weekly IV injections of FVIII received Flt3L/FVIII, rapamycin/FVIII, Flt3L/rapamycin, or nothing. Low-dose FVIII/rapamycin for 4 weeks. Four groups of control animals were pretreated with Flt3L/FVIII (Figure 7E) developed very high levels of rapamycin, Flt3L/antigen treatment immunized rather than tolerized the mice to FVIII.

Inhibitor titers were unaffected in mice that were pretreated with Flt3L/rapamycin without FVIII, followed by challenge with FVIII, indicating that the observed effects reflected tolerance induction to FVIII rather than nonspecific suppression that may have persisted after the regimen was stopped. Consistent with this conclusion was the observation that the regimen caused only transient changes in various immune cell frequencies in immune competent mice, which subsequently responded normally to an unrelated antigen (supplemental Figures 8 and 9). Anti-FVIII immunoglobulin (Ig) G1 titers displayed a similar pattern as inhibitor titers (Figure 7D). Interestingly, mice that were pretreated with Flt3L/FVIII (Figure 7E) developed very high-titer antibodies to FVIII (Figure 7F), indicating that in the absence of rapamycin, Flt3L/antigen treatment immunized rather than tolerated the mice to FVIII.

Discussion

Immune tolerance can be induced by tipping the balance from an effector to a Treg response, a strategy that can be applied to cell and organ transplantation, treatment of autoimmune diseases, replacement therapies for genetic diseases, and treatment of allergies. Rapamycin is particularly useful in this regard. The drug blocks the PI3K-mTOR signaling cascade via mTOR complex 1 and downstream activation of STAT4, STAT6, or STAT3 in conventional CD4+ T cells. Consequently, the activated T cell undergoes programmed cell death. At the same time, rapamycin increases transforming growth factor β levels in vivo and upregulates FoxP3 expression in CD4+ cells, thereby facilitating Treg induction. Because Treg have a downregulated mTOR pathway and preferentially use STAT5 and other signaling molecules, intracellular signaling can proceed in the presence of rapamycin.

In this study, we found that the tolerogenic effects of a rapamycin/antigen cocktail can be further enhanced by addition of several alternative third components, including cytokines or a ligand for the costimulatory molecule GITR, which is constitutively expressed by CD4+ CD25+ FoxP3+ Treg. Fc-GITR-L directly acts on Treg, stimulating their expansion in vitro and in vivo while maintaining their suppressive phenotype. The cytokine Flt3L is an attractive molecule that has already shown safety in clinical trials and is in clinical development for improved hematopoietic stem cell transplantation (registered at www.clinicaltrial.gov/ as NCT01465139). Our new study demonstrates that Flt3L can be used synergistically with rapamycin for improved Treg induction, thereby enhancing immune tolerance. Flt3L and rapamycin had a synergistic rather than additive effect on Treg induction because in the absence of rapamycin, no Treg were induced. Flt3L on its own promotes proliferation of already existing Treg indirectly by expanding DCs but does not promote de novo induction of Treg. The combination of Flt3L and antigen may enhance effector T-cell deletion but fails to induce tolerance, suggesting that Treg induction (through the addition of rapamycin) and/or limited cDC expansion is required.

Flt3L-induced signaling in pDCs is more resistant to the mTOR inhibitor rapamycin

Rapamycin blocked Flt3L-induced cDC expansion in bone marrow and spleen, resulting in selective expansion of pDCs, which suggested...
Figure 4. Flt3L expands pDCs and cDCs in the spleen and bone marrow. Rapamycin blocks cDC expansion (but not pDC expansion). Total numbers of pDC (CD11c<sup>+</sup> PDCA-1<sup>−</sup>) (A) and cDC (CD11c<sup>−</sup>PDCA-1<sup>+</sup>) (B) DC subsets in DO11.10-tg Rag-2<sup>−/−</sup> BALB/c mice per 10<sup>6</sup> splenocytes. Mice (n = 6-10) were treated 3 times per week for 3 weeks IP with Flt3L, Flt3L/OVA<sub>323-339</sub>, Flt3L/OVA<sub>323-339</sub>/rapamycin, or Flt3L/irrelevant peptide (FIX peptide). Enumeration of pDCs (C) and cDCs (D) as a percentage of total DCs (CD11c<sup>+</sup>). (E) Representative dot plot of naïve DO11.10-tg Rag-2<sup>−/−</sup> BALB/c mice splenocytes showing gating scheme for pDCs and cDCs. Total numbers of pDC (CD11c<sup>+</sup> PDCA-1<sup>−</sup>) (F) and cDC (CD11c<sup>−</sup>PDCA-1<sup>+</sup>) (G) DC subsets in DO11.10-tg Rag-2<sup>−/−</sup> BALB/c mice per 10<sup>6</sup> bone marrow cells. Mice (n = 6-10) were treated 3 times per week for 3 weeks IP with Flt3L, Flt3L/OVA<sub>323-339</sub>, Flt3L/OVA<sub>323-339</sub>/rapamycin, or Flt3L with an irrelevant peptide (FIX peptide). Enumeration of pDCs (H) and cDCs (I) as a percentage of total DCs (CD11c<sup>+</sup>). (J) Representative dot plot of naïve DO11.10-tg Rag-2<sup>−/−</sup> BALB/c mice bone marrow cells showing gating scheme for pDCs and cDCs. Plots are representative of data from 6 animals per experimental group. Statistical differences were determined by 2-way ANOVA with Bonferroni’s posttest comparisons.
Figure 5. Treg induction by Flt3L/antigen/rapamycin cocktail is pDC dependent. (A) Experimental timeline of DO11.10-tg Rag2<sup>−/−</sup> BALB/c mice that received 2 weekly injections of PDC-1 antibody (clone 927) to deplete pDCs. Mice were injected 3 times per week for 3.5 weeks with Flt3L/OVA<sub>323-339</sub>/rapamycin during the course of PDC-1 antibody administration. Flt3L/OVA<sub>323-339</sub>/rapamycin was continued for 2 more weeks after PDC-1 antibody treatment. (B) Treg induction was substantially lower in pDC-depleted mice after Flt3L/OVA<sub>323-339</sub>/rapamycin treatment. Data are average ± SD (n = 6 per group). Statistical differences were determined by Student's t-test. (C) Experimental timeline of BALB/c mice that received 5 IV injections of PDC-1 antibody (clone 120B2) over 3 weeks. Mice were infused with 1 × 10<sup>7</sup> CD4<sup>+</sup> CD25<sup>+</sup> effector T cells from DO11.10-tg x Rag2<sup>−/−</sup> mice 1 day after the first PDC-1 antibody injection. Mice continued to receive Flt3L/OVA<sub>323-339</sub>/rapamycin combination during the course of PDC-1 antibody administration. Control mice only received Flt3L/OVA<sub>323-339</sub>/rapamycin treatment. (D) Induction of OVA specific (KJ1-26<sup>+</sup>) Treg from transplanted donor (DO11.10) cells was significantly lower in pDC-depleted mice after Flt3L/OVA<sub>323-339</sub>/rapamycin treatment. Data are average ± SD (n = 8 per group). Statistical differences were determined by the Student t-test. (E) Experimental timeline of BDCA-2-DTR mice that received IV injections of DT (3 times per week for 3 weeks) to deplete pDCs. Mice continued to receive Flt3L/OVA<sub>323-339</sub>/rapamycin combination during the course of pDC depletion. Control mice only received Flt3L/OVA<sub>323-339</sub>/rapamycin treatment. (F) Increased OVA specific (MR9-4<sup>+</sup>) Treg in Flt3L/OVA<sub>323-339</sub>/rapamycin treated control mice as compared with naive animals, which is abrogated by pDC depletion. Data are average ± SD (n = 4 per group). Statistical differences were determined by 1-way ANOVA with Bonferroni’s multiple comparison posttest analysis.

Figure 6. Effect of pDC depletion on Treg induction. Data are average ± SEM (n = 4 per group). Statistical differences were determined by 1-way ANOVA with Bonferroni’s multiple comparison posttest analysis.

Robust Treg induction is pDC dependent

In 3 of 4 experimental models that we tested, depletion of pDCs substantially reduced Treg induction by the Flt3L/rapamycin/antigen regimen. In a breast cancer model, pDC depletion resulted in decreased Treg numbers (and thus increased effector T-cell responses), further supporting a critical role for pDCs in Treg induction. 56 Interestingly, we recently found that expansion of Treg using Flt3L is less effective in mice lacking GITR-L, a molecule that has been described in the literature to be expressed by pDCs. 51,52 It is therefore possible that pDCs aid in Treg induction via the GITR-L/GITR costimulatory pathway. One also needs to consider that rapamycin not only modulates T-cell but also DC function. For example, adoptively transferred rapamycin-conditioned DCs inhibit organ allograft rejection and graft-versus-host disease following hematopoietic cell transplantation. 1 In searching for a mechanism to explain the tolerogenic function in pDCs treated with our drug cocktail, we interrogated expression of several molecules that have been associated with a regulatory phenotype in DCs. 46,53-55 The chemokine receptor CCR9 is selectively expressed on pDCs of immature phenotype in vivo and has been implicated with inducing Treg in culture. 56 In our study, we observed a high surface expression to us that an increase in pDCs was responsible for the augmented Treg induction. This DC subset is able to produce large amounts of type I interferon and has important innate immune functions, such as in antiviral responses. However, pDCs can also be manipulated into assuming a nonactivated state (“tolerogenic pDCs”), characterized by incapacity to induce an effector T-cell response while promoting Treg expansion. 46,47 For example, pDCs have been implicated in regulating oral tolerance and self-tolerance. 48-50 The question arises why rapamycin blocked cDC but not pDC expansion. Given the previous evidence that intracellular signaling downstream of Flt3L-Flt3 occurs through the mTOR pathway, we compared the effects of Flt3L and rapamycin on purified cDCs and pDCs and found that pDCs had a more robust basal p-mTOR<sub>Ser2448</sub> signal, thus rendering this cell type more resistant to inhibition by rapamycin. This conclusion is further supported by a recent study by Agudo et al, who found that miR-126, a microRNA known to regulate angiogenesis in vascular endothelial cells, is within the immune system uniquely expressed in pDCs. 57 Within pDCs, miR-126 functions to suppress the translation of Tsc1, a negative regulator of mTOR, thereby upregulating mTOR activity. 57 Increased mTOR activity is critical for survival of pDCs (in part through interaction with vascular endothelial growth factor receptor 2 signaling) and also for their function in innate immunity by upregulating expression of several innate response genes. A remaining unresolved issue is that pDC development from bone marrow precursors was found to be particularly susceptible to inhibition by rapamycin. However, miR-126 expression gradually increases during pDC development, and it is thus possible that the expansion that we observed in the presence of rapamycin primarily resulted from proliferation of differentiated pDCs. In fact, we found that pDCs also had greater expression of the Flt3 receptor and may thus also be more receptive to Flt3L.
of CCR9 on naïve splenic pDCs (~85%) as compared with cDCs (~8%) (data not shown). Importantly, this high expression of CCR9 was retained in all treatment conditions, suggesting that pDCs maintain an immunoregulatory phenotype upon Flt3L/antigen/rapamycin treatment rather than maturing into immune response–promoting cells. However, we did not find evidence for the upregulation of the metabolic enzyme indoleamine-pyrole 2,3-dioxygenase or of the costimulatory molecule inducible T cell costimulator (ICOS) ligand on DCs (or of ICOS on Treg) upon Flt3L/antigen/rapamycin administration (data not shown). However, plasma samples showed increased transforming growth factor β, which is required for peripheral Treg induction (supplemental Figure 10).

Figure 6. Flt3 receptor expression and mTOR signaling in pDC and cDC subsets. (A) Effect of increasing rapamycin dose on pDC and cDC numbers. DO11.10-tg x Rag−/− BALB/c mice (n = 4 per group) were treated three times per week for 3 weeks IP with Flt3L/OVA323-339/increasing doses of rapamycin: 4 mg/kg (1× rapa), 8 mg/kg (2× rapa) and 16 mg/kg (3× rapa). Total numbers of pDCs and cDCs were enumerated and statistical differences compared by 1-way ANOVA with Dunnett’s multiple comparison posttest using naïve animals as the control group, against which all other treatment groups were tested. (B) Histogram overlays showing differential p-mTORSer2448 expression in splenic pDCs and cDCs of naïve DO11.10-tg x Rag−/− BALB/c mice on in vitro incubation for 60 minutes with Flt3L (red histogram), rapamycin (blue histogram), or a combination of Flt3L/rapamycin (purple histogram). Shown is 1 representative of 2 independent experiments. (C) Graphical representation of p-mTORSer2448 expression in pDC and cDC populations from panel B. Data show showing percent positive cells for each treatment, with histogram subtraction applied against the control group, which represents unstimulated cells. Histogram subtraction was applied using FCS express 4.0 software. The graphs represent data from 2 independent experiments. One-way ANOVA with Tukey’s multiple comparison test was used to calculate significance. (D) Representative western blot images of splenic pDCs and cDCs probed for p-mTORSer2448 (upper) and β-actin (lower). Flow-sorted pDCs and cDCs from spleens of naïve, non-pretreated DO11.10-tg x Rag−/− mice (top) or mice that received repeated Flt3L injections for 10 days (bottom), were serum starved for 2 hours and treated for 60 minutes with 2 μg/mL Flt3L, 100 nM rapamycin, or a combination of Flt3L/rapamycin. Images for pmTORSer2448 and β-actin were analyzed by the ImageJ densitometric software. Normalized relative density of pmTOR to β-actin is represented for both sets of western blot images.
Implications for immune tolerance therapy

The therapeutic relevance of our findings is illustrated by the results in gene and protein therapies. Because pretreatment of hemophilia A mice with Flt3L/rapamycin alone (i.e., without presence of FVIII antigen) failed to suppress inhibitor formation, the effect was not because of general immune suppression. Rather, FVIII-specific tolerance was established. The protocol led to only minor and highly transient decreases in lymphocyte populations, and cell numbers recovered within a month after stopping the regimen. Interestingly, others found that rapamycin and Flt3L could be combined to improve engraftment of cardiac allografts in mice.38 Consistent with our findings, these authors also noticed an increase in pDC expansion, which is further enhanced by selective pDC expansion when combined with Flt3L. Conversely, further increased doses of rapamycin limit pDC expansion and may thus negatively impact tolerance induction.

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Authorship


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Correspondence: Roland W. Herzog, University of Florida, Cancer and Genetics Research Complex, 2033 Mowry Rd, Gainesville, FL 32610; e-mail: rherzog@ufl.edu

References


Synergy between rapamycin and FLT3 ligand enhances plasmacytoid dendritic cell–dependent induction of CD4+CD25+FoxP3+ Treg

Moanaro Biswas, Debalina Sarkar, Sandeep R. P. Kumar, Sushrusha Nayak, Geoffrey L. Rogers, David M. Markusic, Gongxian Liao, Cox Terhorst and Roland W. Herzog