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## Targeting PI3K $\delta$ Function For Amelioration of Murine Chronic Graft-Versus-Host Disease

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### Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. S.T. is an employee of Gilead Sciences, Inc. B.V. is a consultant to Karus Therapeutics (Oxford, UK). The other authors have no conflicts of interest to disclose.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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## Abstract

Chronic graft-versus-host disease is a leading cause of morbidity and mortality following allotransplant. Activated donor effector T-cells can differentiate into pathogenic T helper (Th)-17 cells and germinal center-promoting Tfollicular helper cells, resulting in cGVHD.

Phosphoinositide-3-kinase- $\delta$ , a lipid kinase, is critical for activated T-cell survival, proliferation, differentiation, and metabolism. We demonstrate PI3K $\delta$  activity in donor T-cells that become Tfh is required for cGVHD in a non-sclerodermatous multi-organ system disease model that includes bronchiolitis obliterans, dependent upon GC B-cells, Tfh, and counterbalanced by Tfollicular regulatory cells, each requiring PI3K $\delta$  signaling for function and survival. Although B-cells rely on PI3K $\delta$  pathway signaling and GC formation is disrupted resulting in a substantial decrease in Ig production, PI3K $\delta$  kinase-dead mutant donor bone marrow derived GC B-cells still supported BO cGVHD generation. A PI3K $\delta$ -specific inhibitor, compound GS-649443 that has superior potency to idelalisib while maintaining selectivity, reduced cGVHD in mice with active disease. In a Th1-dependent and Th17-associated scleroderma model, GS-649443 effectively treated mice with active cGVHD. These data provide a foundation for clinical trials of FDA-approved PI3K $\delta$  inhibitors for cGVHD therapy in patients.

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## Introduction

Graft-versus-host disease (GVHD) is a major obstacle for allogeneic hematopoietic stem cell transplant patients, greatly impacting their quality of life. GVHD is a primary cause of mortality, second only to primary disease relapse. Chronic GVHD (cGVHD) is a leading cause of morbidity, occurring in 20–70% of aHSCT patients<sup>1,2</sup>. CGVHD clinical presentations are varied and virtually every organ in the body can be affected; amongst the more severe outcomes are cGVHD of the lung, manifesting as bronchiolitis obliterans (BO)

and skin as scleroderma<sup>3</sup>. Due to this broad and varied pathogenesis, multiple murine models have been developed to recapitulate a larger portion of the disease spectrum<sup>4-6</sup>. A common feature among models and in patients is the driving role of chronically stimulated alloreactive Tregs in disease pathogenesis<sup>3,7</sup>. Activated alloreactive donor CD4<sup>+</sup> T-cells differentiate into Tfollicular helper (Tfh) and IL-17-producing helper T-cells (Th17s) that have known pathogenic roles in cGVHD<sup>4,8-10</sup>.

Tfh cells are a specialized CD4<sup>+</sup> Th cell subset that provide essential signals to support germinal center (GC) B-cell, memory B-cell or antibody-producing plasma cell (PC) development<sup>11-13</sup>. A subpopulation of T regulatory (Treg), Tfollicular regulatory (Tfr) cells, suppress Tfh and GC B-cells to regulate the GC reaction<sup>14</sup>. Immunoglobulin (Ig) produced by PCs and deposited in target tissues, such as the lung, liver, and colon contributes to organ damage in BO cGVHD and skin in the scleroderma model<sup>15</sup>. We previously reported that Tfh and GC B-cells are required for the development of murine BO cGVHD, a model that recapitulates many aspects of human cGVHD pathology, with the predominant exception of scleroderma<sup>15-19</sup>. In this BO cGVHD model, weight loss and mortality are low (around or less than 20%). Th17 cells, a source of the pro-inflammatory cytokine IL-17 that contributes to autoimmunity<sup>20</sup>, are also involved in BO as well as our sclerodermatous model of cGVHD<sup>21,22</sup>.

Phosphoinositide-3-kinases (PI3Ks) are a family of lipid kinases that regulate numerous signaling cascades via the phosphorylation of 3-hydroxyl group of phosphatidylinositol lipid substrates<sup>23</sup>. Structural and substrate preferences divide the PI3Ks into three classes (I, II, III)<sup>24</sup>. Within the class I PI3Ks, present in all cell types, there are several isoforms, each comprised of regulatory and catalytic subunit heterodimers<sup>23</sup>. The p110 $\delta$  catalytic subunit, referred to as PI3K $\delta$ , is an isoform preferentially expressed in leukocytes, regulating immune cell signalling<sup>25,26</sup>. PI3K $\delta$  is activated upon T-cell receptor engagement, CD28 costimulation, and cytokine receptor signaling to sustain an activated Treg phenotype and promote the function of these cells, including regulation of survival, cell cycle progression, differentiation and metabolism<sup>27-30</sup>. Loss of PI3K $\delta$  diminishes Treg activity<sup>31,32</sup>. Relevant to our models of cGVHD, PI3K $\delta$  signaling has been found to be necessary for both murine and human IL-17 production<sup>32-34</sup>. Recent work has demonstrated that PI3K $\delta$  mutant T-cells have impaired alloimmune activity and that PI3K $\delta$  inhibition was able to effectively suppress alloreactive Tregs to prevent solid organ heart transplant rejection<sup>35</sup>. In non-chronic models of GVHD, PI3K $\delta$  inhibition ameliorated lethality and reduced severity of clinical signs and organ damage<sup>36,37</sup>.

Similar to its role in immune cells, PI3K signaling controls proliferation, survival and metabolism of cancer cells. Certain hematological malignancies have been found to have upregulated PI3K $\delta$  activity<sup>38,39</sup>. Idelalisib is a PI3K $\delta$  specific inhibitor that has been approved to treat hematological malignancies, such as chronic lymphocytic leukemia, follicular lymphoma (that can be of GC B- or T-cell origin) and small lymphocytic lymphoma<sup>40,41</sup>. While demonstrating therapeutic benefit, there are also concerning toxicities associated with Idelalisib, including hepatotoxicity, diarrhea/colitis, pneumonitis and intestinal perforation. Due to these off target effects, efforts are being made to develop new drugs. One such compound utilized here is GS-649443, a PI3K $\delta$  isoform-specific inhibitor

that has demonstrated superior potency to idelalisib while maintaining selectivity<sup>42,43</sup>. In vitro and in vivo studies demonstrated that this inhibitor reduces inflammatory cytokines, including IFN $\gamma$  and IL-17<sup>43,44</sup>.

The role of PI3K $\delta$  in the pathophysiology of cGVHD is unknown and deserves investigation in order to develop new therapeutics to treat steroid-resistant or refractory cGVHD. In this study, we sought to determine the requirement of PI3K $\delta$  function in cGVHD pathogenesis. We show that donor T-cells deficient for PI3K $\delta$  activity are unable to induce cGVHD. Further, we demonstrate that the PI3K $\delta$  specific inhibitor, GS-649443, used for treatment of ongoing cGVHD, diminished the GC reaction and antibody production in BO cGVHD. GS-649443 was also efficacious in sclerodermatous cGVHD model, reducing pro-inflammatory IL-17 production. Together, these results provide basic mechanistic insights regarding cGVHD pathophysiology and pre-clinical support for testing of PI3K $\delta$  inhibitors as a therapeutic strategy for steroid-refractory or resistant cGVHD.

## Materials and Methods

### Mice

C57Bl/6 (B6, H2<sup>b</sup>) and Balb/c (H2<sup>d</sup>) mice were purchased from the National Cancer Institute. B10.BR (H2<sup>k</sup>) and B10.D2 (H2<sup>d</sup>) mice were purchased from Jackson Laboratory. Mice were housed in a specific-pathogen-free facility used with the approval of the University of Minnesota's animal care committee. To explore the effects of PI3K $\delta$  loss in donor cells in cGVHD, we used bone marrow (BM) and/or splenocytes from catalytically inactive p110 $\delta$ <sup>D910A/D910A</sup> (further referred to as p110 $\delta$ <sup>D910</sup>) homozygous mutant<sup>45</sup> and p110 $\delta$ <sup>D910A/WT</sup> (wildtype) heterozygous mutant mice, shipped overnight from Drs. Amy Johnson, Klaus Okkenhaug, Anne-Katrien Stark, and Bart Vanhaesebroeck.

### Bone Marrow Transplantation

For the BO cGVHD, B10.BR recipients were conditioned with cyclophosphamide (Sigma St. Louis, M) 120mg/kg/day intraperitoneally, on days -3 and -2, and TBI 8.3 Gy, day -1. Recipients then received  $10 \times 10^6$  B6 T-cell-depleted (TCD) BM only or with  $7.5 \times 10^4$  purified splenic T-cells (cGVHD). For the B10.D2 $\rightarrow$ Balb/c scleroderma model, Balb/c recipients were conditioned with TBI, 7 Gy, day -1 and then received  $10 \times 10^6$  B10.D2 TCD BM only or with  $1.8 \times 10^6$  CD4 and  $0.9 \times 10^6$  CD8 T-cells on day 0<sup>22,46,47</sup>. Mice were monitored daily for survival and weighed twice weekly. In the scleroderma model, mice were assessed twice weekly for clinical and cutaneous GVHD, as previously described<sup>48</sup>.

### Pulmonary Function Tests

Pulmonary function tests (PFTs) were performed as previously described<sup>49</sup>. Briefly, mice were anesthetized with Nembutal, intubated and ventilated using the Flexivent system (Scireq Montreal, QC). Pulmonary resistance, elastance and compliance were reported using Flexivent software version 7. We observe that cGVHD controls have increased pulmonary resistance and elastance along with decreased compliance as compared to BM only controls in our BO cGVHD model<sup>15</sup>.

## PI3K $\delta$ Inhibition

GS-649443<sup>42</sup>, provided by Gilead, was delivered in a vehicle consisting of 10% Ethanol, 20% cremophor EL and 70% normal saline. Mice were given GS-649443 (10mg/kg) twice daily (BID) by oral gavage from days 28–56 (BO model) or days 21–50 (scleroderma model). Mice in the vehicle control group were treated with the same volume of vehicle.

## Histopathology and Immunostaining

Tissue sections were embedded in Optimal Cutting Temperature (OCT) compound, snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Lungs were inflated by 75% OCT before harvest and freezing. For Trichrome staining, 6- $\mu\text{m}$  cryosections were fixed overnight in Bouin's solution and stained with Masson's Trichrome staining kit (Sigma HT15). Collagen deposition was quantified as a ratio of blue area to total area using ImageJ. For Histopathology, acetone-fixed 6- $\mu\text{m}$  cryosections were hematoxylin and eosin stained and evaluated<sup>50</sup> without knowledge of treatment by APM. For immunoglobulin deposition immunostaining, acetone-fixed 6 $\mu\text{m}$  cryosections were stained with goat anti-mouse IgG (BD55401). Confocal images were acquired on Olympus Confocal Laser Scanning Microscope at 20X and quantified by ImageJ.

## Statistical Analysis

GraphPad Prism 7 was used to conduct statistical analysis. One-way ANOVA with Bonferroni correction and Student's t-test were used for statistical analysis as indicated. Error bars indicate mean  $\pm$  standard deviation (SD). Significance: \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ ; \*\*\*\* $P < .0001$ .

## Results

### Fully intact donor T-cell PI3K $\delta$ activity is essential for BO cGVHD generation

The prominent contribution of PI3K $\delta$  activity to T-cell survival and function prompted us to determine whether donor T-cells with decreased or absent PI3K $\delta$  kinase activity would fail to cause cGVHD in the BO model. T cells from p110 $\delta^{\text{D910A/wt}}$  mice that have a knock-in mutation in one allele leading to heterozygote levels of catalytically inactive, mutant PI3K $\delta$  were given to a cohort of mice and compared to BM only and cGVHD controls. Mice receiving WT BM and either heterozygous p110 $\delta^{\text{D910A/wt}}$  or WT T-cells had 90% survival and 5% weight loss compared to day 0 body weights (not shown). BO cGVHD pulmonary dysfunction was comparable to WT T cell controls (Figure S1).

Next, we asked if PI3K $\delta$  activity in donor BM was required for cGVHD. Homozygous p110 $\delta^{\text{D910A}}$  BM with WT T-cells still resulted in pulmonary dysfunction consistent with cGVHD (Figure 1A). As compared to cGVHD only controls, mice receiving p110 $\delta^{\text{D910A}}$  BM with WT T-cells had significantly lower Treg and Tfr frequencies (Figure 1B–C). Tfh frequencies in mice that received p110 $\delta^{\text{D910A}}$  BM with WT T-cells were reduced from that of the cGVHD but still increased from their BM only control. An unfavorable Tfr:Tfh ratio, similar to that of the cGVHD control (Figure 1D–E), was observed. Since the magnitude of antibody responses, that originate in the GC, can be functionally predicted by the Tfr/Tfh ratio in a wide range of diseases in both mice and humans<sup>14</sup>, the low Tfr:Tfh ratio associated

with an increased GC B-cell frequency (Figure 1F) was anticipated. Lung pathology scores correlated with pulmonary function tests, with WT BM only compared to WT BM plus supplemental WT T-cells ( $0.1 \pm 0.1$  vs  $2 \pm 0.1581$ ,  $p < 0.001$ ) and p110 $\delta^{D910A}$  BM compared to p110 $\delta^{D910A}$  BM WT T-cells ( $0.2 \pm 0.1225$  vs  $0.8 \pm 0.255$ ,  $p = 0.067$ )(data not shown). Whereas the statistical difference between the first two groups was significant, statistical comparison in the histopathology scores between the recipients receiving p110 $\delta^{D910A}$  BM only reached a statistical trend. These latter data suggest either a modest effect of the KO BM on altering cGVHD severity or sample size limitations. Infusion of p110 $\delta^{D910A/wt}$  T-cells with p110 $\delta^{D910A/wt}$  BM cells did not avert cGVHD pulmonary dysfunction (Figure S1).

Since haploinsufficient T-cells and BM cells did not have evidence of reduced cGVHD, we proceeded to studies using homozygous p110 $\delta^{D910A}$  T-cells. We hypothesized that donor T-cells lacking all PI3K $\delta$  kinase activity would be inferior in inducing and sustaining cGVHD as compared to their WT counterparts. We observed no significant changes in weight or survival between cGVHD controls and mice that received p110 $\delta^{D910A}$  donor T-cells (Figure S2A–B). Mice that received p110 $\delta^{D910A}$  donor T-cells did not develop pulmonary dysfunction associated with BO cGVHD (Figure 2A). Loss of PI3K $\delta$  activity resulted in a significant decrease in the frequency of splenic Tfh cells (Figure 2B) with unaltered Treg (Figure S2C) and Tfr frequencies (Figure 2C). We observed an increased Tfr:Tfh ratio (Figure 2D) and decreased GC B cell frequencies (Figure 2E) in mice that received p110 $\delta^{D910A}$  versus WT donor T cells, consistent with studies demonstrating that the ratio of Tfr:Tfh controls the GC reaction<sup>51</sup>. As expected by the significant improvement in pulmonary function parameters, recipients of p110 $\delta^{D910A}$  donor T cells had significantly reduced histopathology scores (Figure 2F). T cells and BM cells that had haploinsufficient PI3K $\delta$  expression did not provide adequate protection from cGVHD, suggesting that high level PI3K $\delta$  inhibition will be required to treat cGVHD in the clinic.

### **Therapeutic administration of GS-649443 ameliorates cGVHD in a non-sclerodermatous, BO model**

To validate if PI3K $\delta$  can be targeted as a novel therapeutic strategy, we tested the novel PI3K $\delta$  inhibitor, GS-649443, in our BO model of cGVHD. GS-649443 given at 10mg/kg, PO, BID beginning on day 28, the time of established cGVHD<sup>15</sup>, was well-tolerated as shown by weight and survival curves (Figure S3A, B). Treatment at a lower dose of 5mg/kg, PO, BID did not improve pulmonary function (Figure S4). Vehicle treatment alone had no significant effect on cGVHD outcome for any parameters tested. GS-649443 improved PFTs (Figure 3A), reduced the lung pathology associated with cGVHD (Figure 3B) and decreased Tfh (Figure 3C) frequencies. Both the Treg (Figure S3C) as well as Tfr (Figure 3D) frequencies were decreased by GS-649443 treatment. Although the Tfr:Tfh ratio was similar to that of the vehicle controls (Figure 3E), the GC B-cell frequency in GS-649443 treated mice was significantly decreased (Figure 3F). Together, these data point to either to a direct effect of GS-649443 on GC B-cells and/or reduction of Tfh frequency below threshold limits to cause a GC response.

### Reduced Ig and collagen lung deposition in GS-649443-treated mice phenocopies findings in recipients given p110 $\delta$ <sup>D910A</sup> donor T-cells

cGVHD has several autoimmune-like features, including but not limited to the deposition of antibodies and fibrosis of target organs, including the lung<sup>52</sup>. In accordance with improved PFTs and immune analysis, we demonstrated that lung IgG (Figure 4A) and collagen deposition (Figure 4B) was decreased in mice that received WT BM plus p110 $\delta$ <sup>D910A</sup> donor T-cells. Mice that received GS-649443 treatment also had reduced lung IgG and collagen deposition (Figure 4).

### Therapeutic administration of the PI3K $\delta$ -specific inhibitor GS-649443 ameliorates sclerodermatous cGVHD

A major clinical and histopathological manifestation absent from the multi-organ system BO cGVHD model is scleroderma<sup>53</sup>. We utilized a multiple minor histocompatibility mismatch model (B10.D2 $\rightarrow$ BALB/c) that presents with a cutaneous cGVHD and associated increased Th17 Teffs and systemic inflammatory response<sup>22</sup>. GS-649443 treatment significantly improved skin and clinical scores of mice (Figure 5A–B). GS-649443 treatment decreased IL-17<sup>+</sup> T-cell frequency (Figure 5C), characteristic of cGVHD in this model and IL-17<sup>+</sup>IFN $\gamma$ <sup>+</sup> double positive cells (Figure S5A), which can contribute to autoimmunity<sup>22,54</sup>. IFN $\gamma$ <sup>+</sup> T-cells remained increased in mice treated with GS-649443 (Figure S5B) indicating potentially only a partial amelioration of disease. Nonetheless, decreased IL-17-producing T-cells resulted in correspondingly lower, although not quite significant, IgG deposition in the skin of scleroderma mice (Figure S5C).

## Discussion

PI3K $\delta$  is a key regulator of Teff function, found here to be required for cGVHD development. Here, we have demonstrated that cGVHD generated in distinct murine models that simulate several, but not all, cGVHD manifestations, are dependent upon PI3K $\delta$  activity. We demonstrated that PI3K $\delta$  activity in donor T-cells but not B-cells is necessary to initiate and/or sustain the GC response critical for cGVHD in the BO model. We utilized the PI3K $\delta$  isoform-specific inhibitor GS-649443 to show that PI3K $\delta$  inhibition is effective in treating ongoing, established cGVHD in both the BO and sclerodermatous models. Overall, our data show that the PI3K $\delta$  signaling pathway is required to generate and maintain murine cGVHD in two, independent models with distinct pathophysiology and few overlapping cGVHD manifestations.

PI3K $\delta$  has roles in Teffs and other immune cell types, notably B-cells, Tregs and macrophages. Mice lacking functional PI3K $\delta$  exhibit B-cell defects. Such mice have fewer mature B-cells, reduced B-cell receptor-induced proliferation, decreased B-cell differentiation into antibody-producing cells, substantially reduced Ig production and disrupted GCs in response to antigen challenge<sup>45,55,56</sup>. Interestingly, p110 $\delta$ <sup>D910A</sup> BM with WT T-cells still induced pulmonary dysfunction that was significantly worse than their p110 $\delta$ <sup>D910A</sup> BM only counterpart. The magnitude of the GC B-cells was sufficient to induce pulmonary dysfunction. Because Tregs also reside in the BM, p110 $\delta$ <sup>D910A</sup> BM would produce Tregs or Tfrs defective in suppressing Tfh that may have contributed to GC B-cell



driven pulmonary dysfunction. Related to this possibility, PI3K $\delta$  signaling supports Treg development and function. We previously showed Tregs and Tfrs are critical in controlling GC reactions and cGVHD<sup>57</sup> and that PI3K $\delta$  inhibition results in diminished *in vitro* and *in vivo* suppressor function and Treg survival<sup>31,35</sup>. Indeed, both the Treg and Tfr populations were decreased in mice that received p110 $\delta$ <sup>D910A</sup> BM alone or with T-cells. The resulting overall unfavorable Tfr:Tfh ratio creates an environment in the B-cell follicle permissive for an increased GC B-cell frequency<sup>14</sup>. In this study, we observed decreased Tfr and GC B-cell frequencies associated with the therapeutic benefit of GS-649443 treatment.

Macrophages are known to be key mediators of several types of inflammatory immune responses, including those culminating in fibrosis. Indeed, macrophages were proven to be a source of Transforming Growth Factor-beta (TGF- $\beta$ ), a mediator of tissue fibrosis<sup>21</sup>. Macrophage depletion<sup>21</sup> or inhibition of macrophage migratory capacity<sup>58</sup> precluded the generation of cGVHD in both the BO and scleroderma models. Optimal macrophage function has been associated with various PI3K isoforms, including PI3K $\beta$ , PI3K $\delta$  and PI3K $\gamma$ <sup>40</sup> and in particular PI3K $\delta$  has been shown to inhibit macrophage migration<sup>59</sup>. Although the improvement in cGVHD outcome with GS-649443 correlated with a reduction in GC reaction, decreased macrophage migration may have contributed to disease amelioration. Such may occur by a direct effect by PI3K $\delta$  inhibition on donor macrophage function or indirectly inhibit macrophage migration as a consequence of low GCs, Ig deposition in cGVHD organs and subsequently lower levels of macrophage chemoattractants. Further studies will be required to determine how PI3K $\delta$  affects macrophage migration and function in the context of cGVHD. Additional studies are needed to determine whether altered Tfr/Tfh, reduced Th17 cell as seen in the scleroderma model, or impaired macrophage migration are the dominant or critical mechanism(s) of by which PI3K $\delta$  inhibition ameliorates cGVHD BO.

Increased PI3K $\delta$  signaling has been found in autoimmune diseases<sup>60</sup> and has been of interest for therapeutics in autoimmune and inflammatory disease mouse models. In models of experimental autoimmune encephalitis (EAE), PI3K $\delta$  mutant mice were noted to have a defective Th17 response and reduced disease severity<sup>34</sup>. PI3K $\delta$  inhibition slowed disease progression and organ damage in a murine model of systemic lupus erythematosus, an autoimmune disease with T- and B-cell involvement similar to several immunological abnormalities associated with cGVHD<sup>61</sup>. Loss of PI3K $\delta$  activity improved outcomes in multiple sclerosis, rheumatoid arthritis, psoriasis and autoimmune (type 1) diabetes models<sup>40</sup>. We observed similar results with PI3K $\delta$  inhibition in cGVHD models studied here, including decreased damage to the lung, Ig deposition and IL-17. Of note, prior *in vitro* assays have shown that pharmacologic pan-PI3K inhibition was more effective than more selective inhibition of p110 $\delta$  alone for preventing differentiation of Th1 cells, as determined by IFN $\gamma$  production; in contrast, IL-17 was completely blocked by both inhibitor types<sup>34</sup>. Moreover, p110 $\delta$ <sup>D910A</sup> mice had greater reduction in Th17 compared to Th1 responses in an EAE model<sup>34</sup>. While cytokines were not directly measured in our BO cGVHD, previously we have reported that IL-17 contributes to cGVHD in the BO model, as demonstrated by the lack of cGVHD using RORC deficient T cells and reversal of established disease using small molecule ROR $\gamma$ t inhibitors or neutralizing anti-IL-17 mAb treatment<sup>10</sup>.

In addition to regulation of IL-17 production, sustained PI3K $\delta$  activation has been found to be necessary for optimal IFN $\gamma$  production<sup>32</sup>. In the scleroderma model, inhibition of the  $\delta$  isoform with GS-649443 did not impact the frequencies of IFN $\gamma$  expressing donor T-cells. These data are however consistent with the reduced efficacy in IFN $\gamma$  suppression seen in CD8+ T cell later after TCR stimulation. Importantly, our data indicate that IFN $\gamma$  inhibition alone is not essential for reducing disease severity. In the cGVHD BO model, the role of IFN $\gamma$  in mediating disease has not been elucidated. However, in acute GVHD models, the lack of donor IFN $\gamma$  production increased pulmonary GVHD and GVL responses, while reducing GI GVHD<sup>62</sup>. Thus, we do not favor the explanation that reduced IFN $\gamma$  production by PI3K $\delta$  inhibition is fundamentally important for cGVHD with BO.

A sizable population of allo-BMT patients have a hematological malignancy, many of whom will develop cGVHD and hence are potential candidates for PI3K $\delta$  treatment for post-BMT relapse and/or cGVHD. Because donor T-cells are principal protectors against relapse providing the beneficial graft-versus-leukemia (GVL) response<sup>63</sup>, the GVL response could be diminished by PI3K $\delta$  inhibition in cGVHD patients in whom PI3K $\delta$  activity is not a driving force in malignancy. However, for many cGVHD patients, especially those with long-standing disease, the GVL effect already may have eliminated residual malignant cells by the time that therapy would begin and for patients with steroid-resistant or refractory cGVHD, profound immune suppression may subvert existing GVL responses. Future studies will need to be conducted to determine how inhibition of PI3K $\delta$  will impact on GVL and other immune function in the context of cGVHD treatment.

Several important issues remain to be addressed. For example, PI3K signaling is involved in many different aspects of immunity and therefore inhibition could impact immune reconstitution. The impact of this therapy on cells of the immune system will be an important consideration going forward. GS-649443 ameliorated cGVHD in both the BO and scleroderma models, treatment was initiated at early times after disease establishment. The efficacy of PI3K $\delta$  inhibition in patients with steroid-refractory or advanced cGVHD remains to be determined. While the toxicities associated with PI3K $\delta$  inhibitors are of concern for future therapeutic applications, structural modifications, such as the one utilized in this study, offer the promise to decrease off target effects related with treatment and improve the historically poor outcome of cGVHD patients failing to respond to steroids. Nonetheless, careful pharmacological toxicology studies must be performed given the potential broader implications of PI3K $\delta$  inhibition on systems beyond immunity and inflammation. Lastly, the potential broader off target effects of GS-649443 on other PI3K isoforms and other kinases for those drugs destined for clinical applications deserve thorough exploration.

In conclusion, these results demonstrate that PI3K $\delta$  activity is necessary for the development of cGVHD in murine models. We have demonstrated that targeting PI3K $\delta$  can result in a decreased GC reaction. Inhibiting PI3K $\delta$  improved cGVHD disease outcome by reducing pathogenic Tfh/GC B-cells resulting in decreased antibody and collagen deposition in the lungs. PI3K $\delta$  inhibition is also able to decrease inflammatory cytokines associated with cGVHD. These studies add to current knowledge of application of PI3K $\delta$  inhibition for disease treatment and present support for targeting PI3K $\delta$  for cGVHD therapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations list

<b>BID</b>	Twice daily
<b>BO</b>	Bronchiolitis obliterans
<b>cGVHD</b>	chronic graft versus host disease
<b>EAE</b>	Experimental autoimmune encephalitis
<b>GC</b>	Germinal center
<b>GVL</b>	Graft versus Leukemia
<b>Ig</b>	Immunoglobulin
<b>KO</b>	Knock out
<b>IL</b>	Interleukin
<b>OCT</b>	Optimum cutting temperature
<b>PC</b>	Plasma cell
<b>PFT</b>	Pulmonary function test
<b>PI3K</b>	Phosphoinositide-3-kinase
<b>SD</b>	Standard deviation
<b>Teff</b>	Teffector cells
<b>TCD</b>	T cell depleted
<b>TGF-<math>\beta</math></b>	Transforming growth factor-beta
<b>Th</b>	T helper
<b>Tfh</b>	T follicular helper
<b>Treg</b>	T regulatory
<b>Tfr</b>	T follicular regulatory
<b>WT</b>	Wild type

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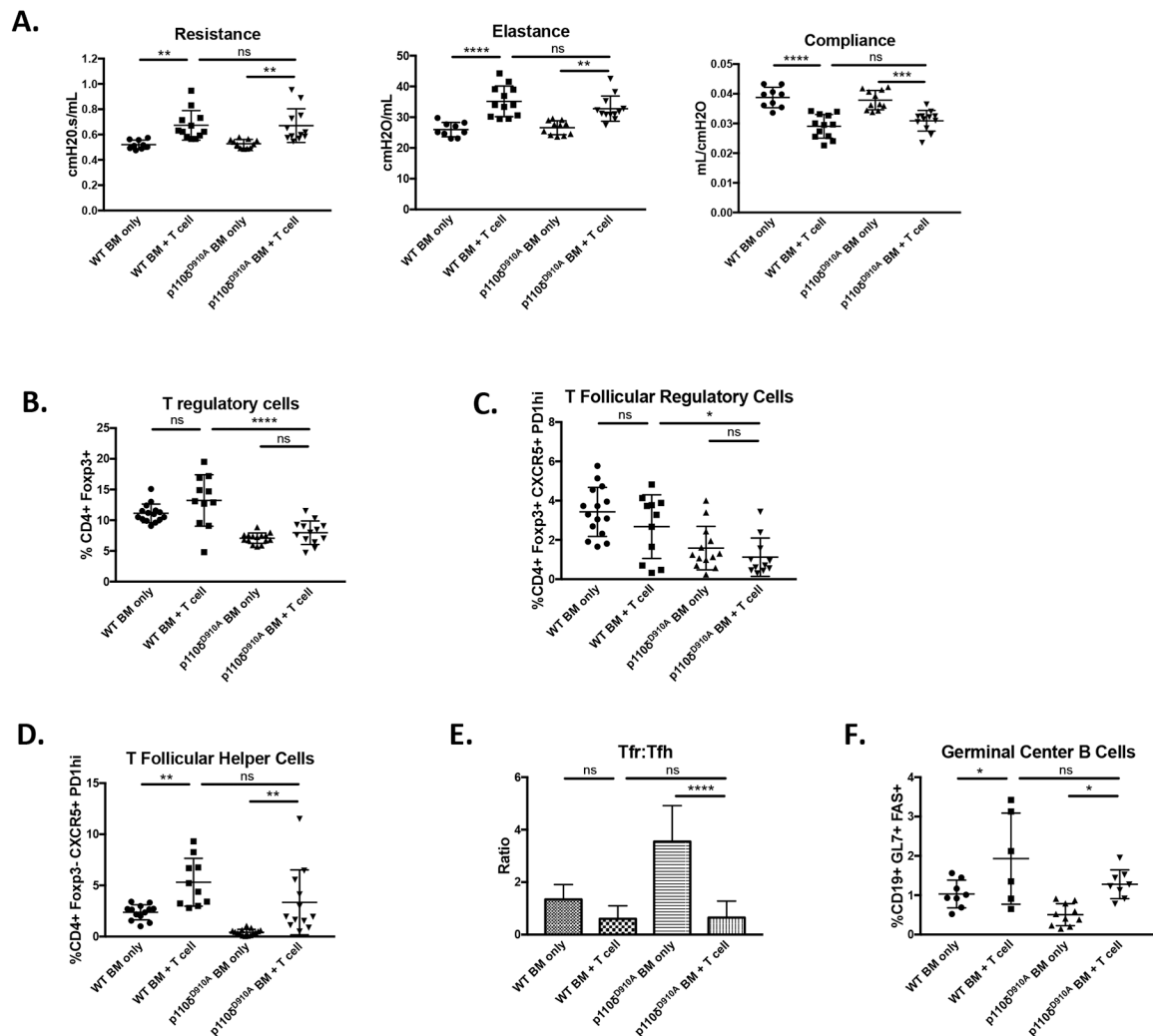
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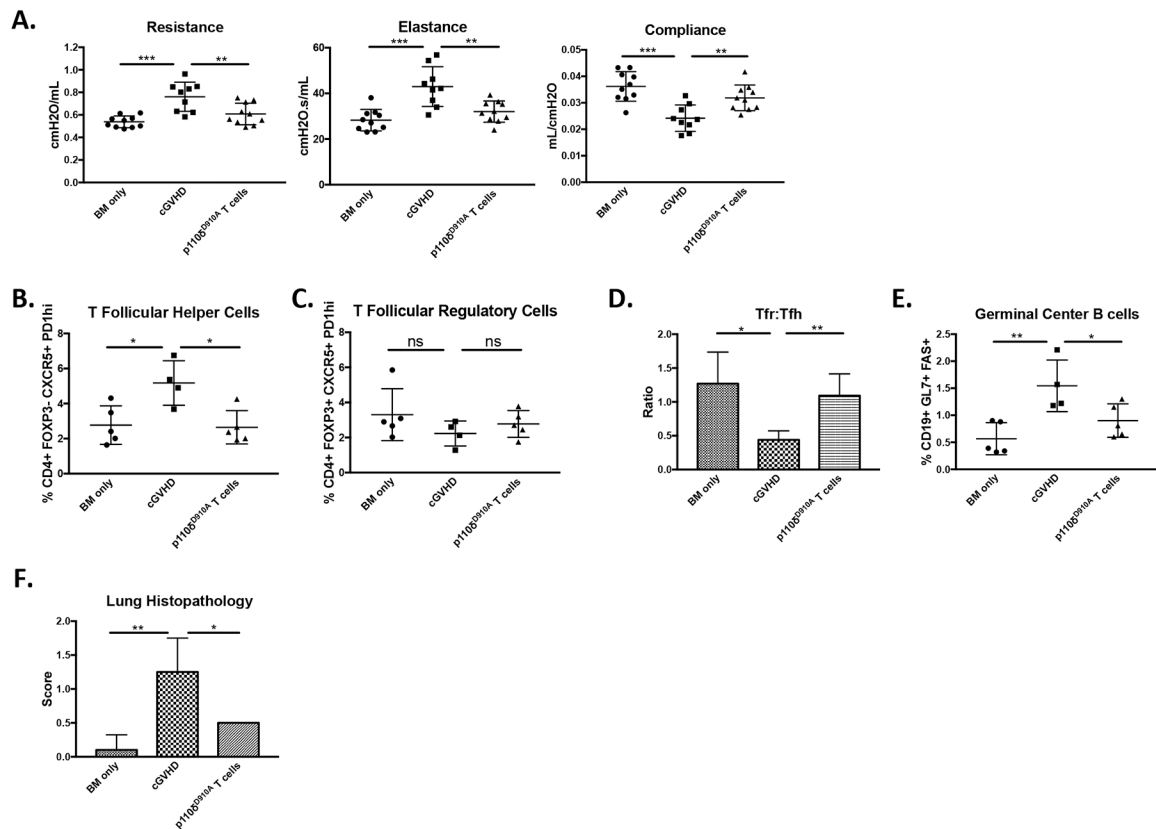
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### Figure 1. Mice receiving p110δ<sup>D910A</sup> BM develop cGVHD

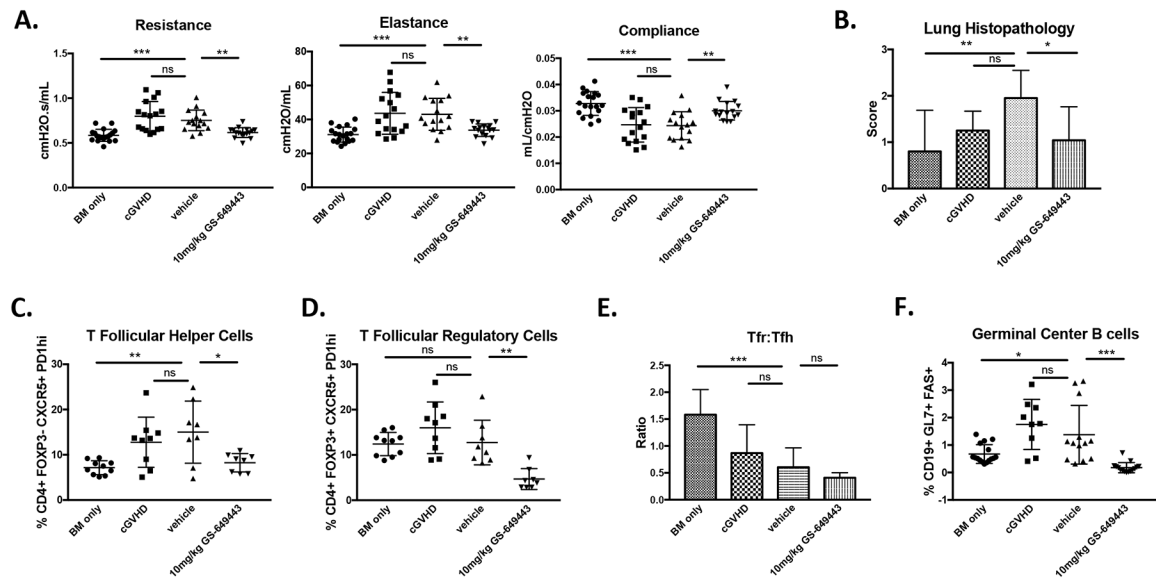
B10.BR mice were conditioned with Cytoxan and TBI and infused with BM alone or with WT purified splenic T-cells (cGVHD) along with mice receiving p110δ<sup>D910A</sup> BM alone or with WT T-cells. (A) Day 56PFTs show that mice that received p110δ<sup>D910A</sup> BM with WT T cells still developed BO comparable to cGVHD controls. (B-C) The frequency of splenic Tregs and Tfr demonstrate that these populations are reduced in Tfh both groups that received p110δ<sup>D910A</sup> BM. (D) The splenic Tfh frequency was decreased in p110δ<sup>D910A</sup> BM supplemented with T-cell group compared to the cGVHD control. (E) The Tfh frequency was still increased from the p110δ<sup>D910A</sup> BM resulting in a Tfr:Tfh ratio similar to that of the cGVHD control. (F) The frequency of splenic GC B-cells were decreased in mice that received the p110δ<sup>D910A</sup> T cells compared to cGVHD control but still increased from p110δ<sup>D910A</sup> BM. A-E Data are from 2 pooled, independent experiments, with 5–7 mice per group per experiment. In F data are representative from 1 experiment. Data shown with mean ± SD. One-way ANOVA with Bonferroni correction for multiple comparisons used with significance: \* $P > .05$ ; \*\* $P > .01$ ; \*\*\* $P > .001$ .





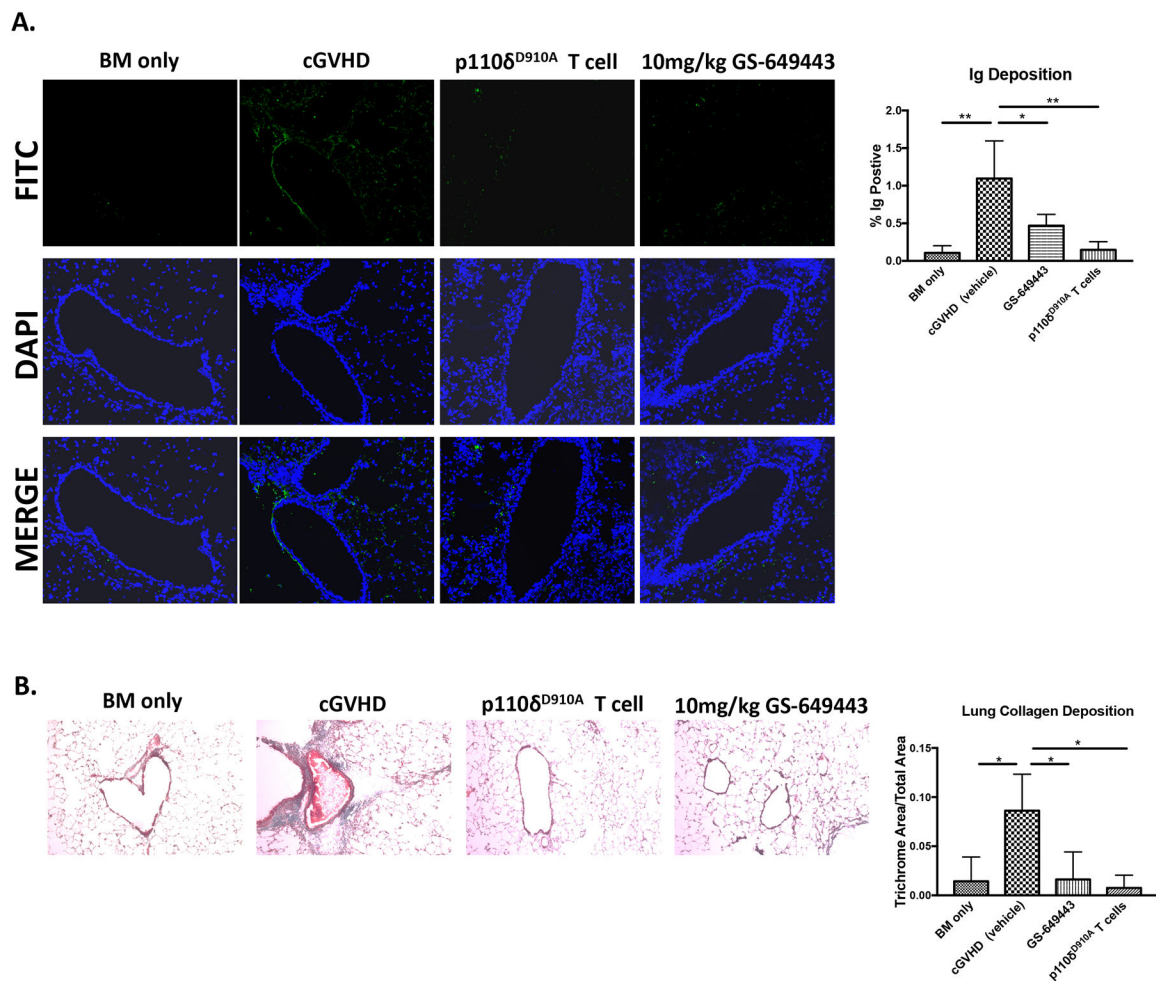
**Figure 2. PI3K $\delta$  is necessary in donor T-cells for cGVHD development**

B10.BR mice were conditioned with Cytoxan and TBI and infused with BM alone or with WT purified splenic T-cells (cGVHD) or catalytically inactive T-cells. (A) Pulmonary function tests performed on day 56 show that the p110 $\delta$ <sup>D910A</sup> T cells did not induce BO cGVHD. (B) The frequency of splenic Tfh was decreased in mice that received the p110 $\delta$ <sup>D910A</sup> T cells. The Tfr frequency was not changed among any of the groups (C), however the Tfr:Tfh ratio was significantly improved (D). (E) The frequency of splenic GC B-cells was also decreased in mice that received p110 $\delta$ <sup>D910A</sup> T cells (F) Hemotoxylin and eosin staining of lungs show that mice receiving p110 $\delta$ <sup>D910A</sup> T-cells had had improved histopathology. Data are representative of 2 independent experiments with similar result with 4–5 mice per group, shown with mean  $\pm$  SD. Student's t-test was used when comparing two groups with significance: \* $P > .05$ ; \*\* $P > .01$ ; \*\*\* $P > .001$ .



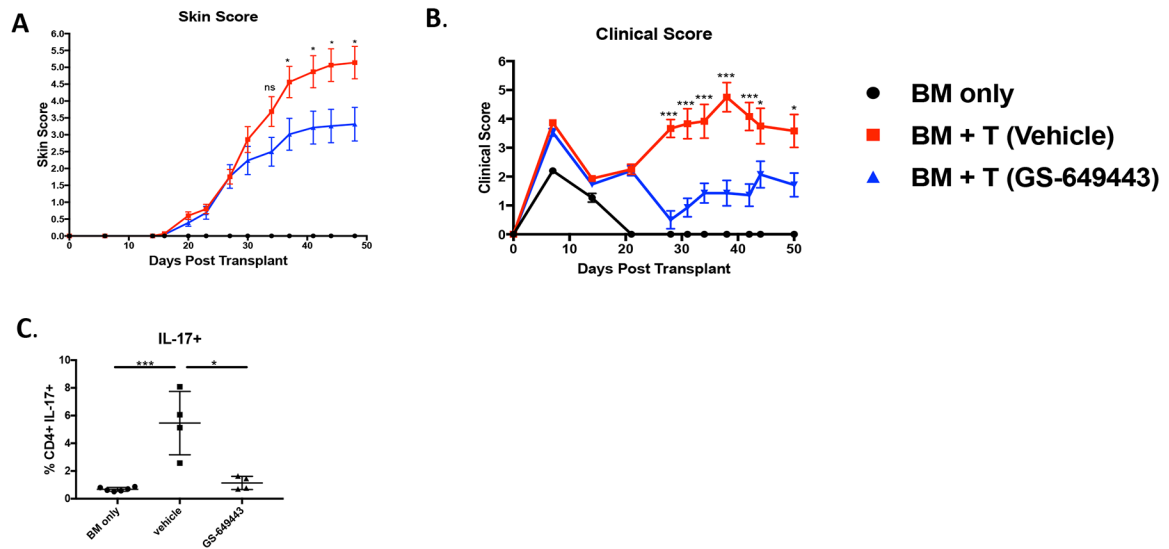
**Figure 3. Therapeutic administration of PI3K $\delta$  specific inhibitor GS-649443 ameliorates disease in a non-sclerodermatous, BO model of cGVHD**

B10.BR mice were conditioned with Cytoxan and TBI received BM alone or with B6 purified splenic T-cells (cGVHD) treated mice received vehicle or PI3K $\delta$  specific inhibitor GS-649443 (10mg/kg/BID) beginning on day 28 after transplant. (A) Day 56 PFTs show that GS-649443 improved lung function of cGVHD mice. (B) Hemotoxylin and eosin staining of lungs show that mice treated with the inhibitor had improved histopathology. (C) The frequency of splenic Tfh was significantly decreased in mice treated with GS-649443. (D) These mice still had reduced frequency of Tfr cells and the ratio of Tfr:Tfh was not improved (E). (F) The frequency of splenic GC B cells was significantly reduced in mice treated with GS-649443. A and F are pooled from 3 independent experiments. B-E are pooled from 2 independent experiments, with 4–6 mice per group per experiment. Data are shown with mean  $\pm$  SD. One-way ANOVA with Bonferroni correction for multiple comparisons used with significance: \* $P > .05$ ; \*\* $P > .01$ ; \*\*\* $P > .001$ .



**Figure 4. Histopathology and immunoglobulin (Ig) deposition of GS-649443 treated mice phenocopies mice that received p110δ<sup>D910A</sup> donor T-cells**

Transplant set up was the same as figures 2 and 3. (A) Representative images of Ig deposition staining. Ig deposition was quantified in ImageJ. (B) Representative images of Masson’s Trichrome staining. Collagen was identified as area stained blue and quantified using ImageJ indicating decreased collagen deposited in the lungs of mice that received p110δ<sup>D910A</sup> T-cells and mice treated with GS-649443. Data are from one experiment with 3–5 mice per group, shown with mean ± SD. One-way ANOVA with Bonferroni correction for multiple comparisons used with significance: \**P* > .05; \*\**P* > .01; \*\*\**P* > .001.



**Figure 5. Therapeutic administration of the PI3K $\delta$  specific inhibitor GS-649443 ameliorates sclerodermatous cGVHD**

Balb/c mice received TBI and received WT B10.D2 BM alone (BM only) or with  $1.8 \times 10^6$  CD4 $^+$  and  $0.9 \times 10^6$  CD8 $^+$  T-cells. Treatment groups received PI3K $\delta$  specific inhibitor GS-649443 (10mg/kg/BID) starting at day 21. (A) Mice treated with GS-649443 had improved skin scores. (B) GS-649443 improved clinical scores in treated mice. Analysis of lymph nodes taken at day 50 post-transplant, each sample is pooled from 2 mice, with 8–12 mice per group (C) Mice treated with GS-649443 had reduced IL-17 frequency. (D) IL-17 and IFN $\gamma$  double positive population frequency were also decreased. (E) IFN $\gamma$  positive population frequency was not decreased with treatment. (F) Representative images of Ig deposition in the skin of mice treated with GS-649443 quantified using ImageJ (G). Data in (A) is pooled data from two independent experiments, (B-C) are representative from 2 independent experiments. Data are shown with mean  $\pm$  SD. Student's t-test was used with significance: \* $P > .05$ ; \*\* $P > .01$ ; \*\*\* $P > .001$ .