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Immune suppressive donor regulatory T cells (Tregs) can prevent graft-versus-host disease (GVHD) or solid organ allograft rejection. We previously demonstrated inhibiting STAT3 phosphorylation (pSTAT3) augments *FOXP3* expression, stabilizing induced Tregs (iTregs). Here we report human pSTAT3-inhibited iTregs prevent human skin graft rejection and xenogeneic GVHD yet spare donor anti-leukemia immunity. pSTAT3-inhibited iTregs express increased levels of skin-homing CLA antigen, immune suppressive GARP and PD-1, and IL-9 that supports tolerizing mast cells. Further, pSTAT3-inhibited iTregs significantly reduce alloreactive conventional T cells, Th1, and Th17 cells implicated in GVHD and tissue rejection, and impair infiltration by pathogenic Th2 cells. Mechanistically, pSTAT3 inhibition of iTregs provokes a shift in metabolism from oxidative phosphorylation (OxPhos) to glycolysis and reduced electron transport chain activity. Strikingly, co-treatment with coenzyme Q10 (coQ10) restores OxPhos in pSTAT3-inhibited iTregs and augments their suppressive potency. These findings support the rationale for clinically testing the safety and efficacy of metabolically tuned, human pSTAT3-inhibited iTregs to control alloreactive T cells.

Metabolic reprogramming augments potency of human pSTAT3-inhibited iTregs to suppress alloreactivity

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Summary: pSTAT3-inhibited human iTregs upregulate GARP and PD-1, control alloreactive T cells, and permit anti-leukemia immunity. CoQ10 restores OxPhos in pSTAT3-inhibited iTregs, augmenting their suppressive potency.

ABSTRACT

Immune suppressive donor regulatory T cells (Tregs) can prevent graft-versus-host disease (GVHD) or solid organ allograft rejection. We previously demonstrated inhibiting STAT3 phosphorylation (pSTAT3) augments *FOXP3* expression, stabilizing induced Tregs (iTregs). Here we report human pSTAT3-inhibited iTregs prevent human skin graft rejection and xenogeneic GVHD yet spare donor anti-leukemia immunity. pSTAT3-inhibited iTregs express increased levels of skin-homing CLA antigen, immune suppressive GARP and PD-1, and IL-9 that supports tolerizing mast cells. Further, pSTAT3-inhibited iTregs significantly reduce alloreactive conventional T cells, Th1, and Th17 cells implicated in GVHD and tissue rejection, and impair infiltration by pathogenic Th2 cells. Mechanistically, pSTAT3 inhibition of iTregs provokes a shift in metabolism from oxidative phosphorylation (OxPhos) to glycolysis and reduced electron transport chain activity. Strikingly, co-treatment with coenzyme Q10 (coQ10) restores OxPhos in pSTAT3-inhibited iTregs and augments their suppressive potency. These findings support the rationale for clinically testing the safety and efficacy of metabolically tuned, human pSTAT3-inhibited iTregs to control alloreactive T cells.

INTRODUCTION

Human Tregs can suppress alloreactive T cells responsible for GVHD and solid organ allograft rejection (1-5). However, broadly suppressive calcineurin inhibitors (CNI) remain standard of care for preventing GVHD and allograft rejection, yet protection offered by CNIs is incomplete (6-8). Moreover, CNIs impair donor immunity and disrupt Treg function and survival, thus opposing durable immune tolerance (9, 10). Further, CNIs also increase the risk for opportunistic infections and secondary malignancies among transplant recipients (11).

In contrast, adoptive transfer of human Tregs has been shown safe, and emerging data has shown efficacy in preventing GVHD after allogeneic hematopoietic cell transplantation (alloHCT) (5, 12). Modification of standard GVHD prophylaxis regimens, such as incorporating mTOR inhibitors and/or low-dose IL-2 improves Treg reconstitution after alloHCT (9, 10, 13-16) and also supports Treg persistence after adoptive transfer (12). Accordingly, contemporary phase I/II clinical trials are underway investigating the benefit of human Tregs in the prevention of GVHD and solid organ transplant rejection (2, 15, 17, 18).

Clinical scale production of human Tregs is complex, requires local cell therapy expertise, and current good manufacturing practices (cGMP)-compliant protocols for Treg purification and expansion. The use of peripheral or "natural" Tregs versus induced Tregs is a key translational consideration. Peripheral Tregs are rare in circulation but offer reliable suppressive potency and phenotypic stability compared to iTregs (12, 19). However, circulating peripheral Tregs are scarce and raise the risk of production failure when using typical starting material for product manufacturing, like apheresis mononuclear cells. Conversely, iTregs are generated from a large and readily available pool of conventional T cells (Tconv), yet these have a theoretical risk of reverting to inflammatory Tconv (20). On the other hand, the large numbers

of Tconv that can seed iTreg production mitigates hurdles regarding production. Thus, strategies to improve the phenotypic stability and potency of human iTregs are clearly warranted.

We have previously shown that pSTAT3 inhibition enhances the generation and phenotypic stability of human iTregs by provoking the demethylation of the Treg-specific demethylated region (TSDR) present in *FOXP3*, leading to increases in FOXP3 expression (21). As such, targeting pSTAT3 reduces the risk for Tconv reversion by the generated iTregs (21). Here we provide preclinical proof-of-concept evidence that adoptive transfer of human pSTAT3-inhibited iTregs have superior potency compared to control iTregs in suppressing alloreactive donor T cells, and in improving skin graft survival by limiting pathogenic T cell tissue invasion. Importantly, pSTAT3 inhibited iTregs reduce xenogeneic GVHD and preserve donor anti-leukemia immunity, a fundamental benefit of alloHCT.

Our human skin graft/NSG mouse xenotransplantation model is well suited to study human iTregs as the cells readily engraft in the immunodeficient mouse and skin is a clinically relevant organ in GVHD (22, 23). Skin is also a critical driver of alloreactivity and a well-established tissue to test experimental tolerance induction (24-26). Further, the model lends itself to the study of allospecific Tregs, as the Tregs are expanded with dendritic cells from the skin donor.

While targeting pSTAT3 significantly improves human iTreg potency, we demonstrate that pSTAT3 inhibition provokes a metabolic shift in iTregs from oxidative phosphorylation (OxPhos) toward glycolysis. We show co-treatment with coQ10 restores OxPhos in pSTAT3-inhibited iTregs and further enhances their suppressive potency against alloreactive T cells. These findings support testing the safety and efficacy of metabolically tuned, human pSTAT3-inhibited iTregs in transplantation tolerance.

RESULTS

Human pSTAT3-inhibited iTregs demonstrate superior suppressive potency.

Human iTregs were generated as described, using Treg-depleted $CD4^+$ T cells stimulated by allogeneic dendritic cells (DC) for 5 days (T:DC ratio 30:1) (21). The pSTAT3 inhibitor (27) S3i-201 (50 μM) or DMSO (<0.01%) were added once on day 0. The concentration of S3i-201 was previously optimized to potently suppress pSTAT3, while permitting pSTAT5 activity beneficial for Treg differentiation (21). Human recombinant IL-2 (10 IU/ml) was supplemented every other day. On day +5 of culture, the T cells were harvested and iTregs were purified by $CD25^+$ magnetic bead isolation. The final purity of the iTreg ($CD4^+$, $CD127^-$, $CD25^+$, $Foxp3^+$) (28, 29) product was >90% (Fig. 1 a). iTreg function was tested in standard suppression assays against DC-allostimulated T cells (22, 23, 30). S3I-201 or DMSO were not added to the 5-day suppression assay cultures. The pSTAT3-inhibited iTregs demonstrated significant suppressive potency against DC-allostimulated T cells compared to DMSO-treated iTregs (Fig. 1 b). This enhanced iTreg suppression was achieved when either DC or bead stimulators were used during iTreg generation (Fig. 1 c), supporting the notion that the STAT3 inhibitor acts primarily on iTregs and not DCs. Silencing of human STAT3 during iTreg differentiation using a validated siRNA confirmed that inhibiting STAT3 augments their suppressive activity against alloreactive T cells in vitro (Fig. 1 d).

Mechanistically, the superior suppressive activity of pSTAT3-inhibited iTregs was associated with an increased frequency of $GARP^+$ and $PD\text{-}1^+$ iTregs (Fig. 1 e-h). In contrast, the expression of other immune suppressive molecules on iTregs such as CD39, LAG3, and CTLA4 (Fig. 1 i-k), were not affected by pSTAT3 inhibition. Up-regulation of PD-1 and GARP in pSTAT3-inhibited iTregs was functionally relevant, as neutralization of PD-1 or LAP/TGFβ1,

the ligand for GARP (31, 32), with monoclonal antibodies significantly impaired the suppressive function of the pSTAT3-inhibited iTregs (Fig. 1 l). Conversely, inhibiting the CD39 ectonucleotidase with ARL67156 (30) had no effect on pSTAT3-inhibited iTreg potency (Fig. 1 l).

Human pSTAT3-inhibited iTregs significantly reduce skin graft rejection.

Skin is an important and clinically relevant GVHD-target organ (33, 34). To test the activity of pSTAT3-inhibited iTregs in vivo, we used our established human skin graft / NSG mouse xenogeneic model (22, 23). NSG mice received a 1cm^2, split thickness human skin graft. The mice rested for 30 days to permit skin graft healing and engraftment. During this time, human monocyte-derived DCs were generated from blood of the skin graft donor. These DCs were used to expand antigen-specific pSTAT3-inhibited iTregs or DMSO-treated controls from a healthy donor. The skin grafted mice were then transplanted with 5x10^6 human PBMCs to induce graft rejection; plus, either 1x10^5 pSTAT3-inhibited iTregs, DMSO-treated iTregs, or no iTregs. Thus, the iTregs were autologous to the PBMCs and allogeneic to the skin. The skin grafts were monitored daily for signs of rejection, including ulceration, necrosis, and scabbing (22, 23). Skin grafts that were >75% nonviable were considered rejected. Notably, human skin grafts from mice inoculated with pSTAT3-inhibited iTregs had significantly improved graft survival versus experimental groups treated with vehicle-treated iTregs or PBMCs alone (Fig. 2 a, b), and H&E sections from skin grafts at day +21 showed a trend toward reduced rejection pathology within the tissue at this early time point (Fig. 2 c, d). Ki-67 staining revealed normal proliferation of basal keratinocytes, but highly proliferative, tissue-invasive donor lymphocytes (35) in the dermis of skin grafts from mice receiving control PBMCs or untreated iTregs. In contrast, there

were significantly reduced numbers of dermal Ki-67$^+$ cells in the skin grafts from the pSTAT3-inhibited iTreg cohort (Fig. 2 e, f). Human pSTAT3-inhibited iTregs also significantly reduced xenogeneic GVHD of the lung, an important target organ in this model (30), while DMSO-treated iTregs were similar to PBMCs alone (Fig. 2 g, h). Importantly, human pSTAT3-inhibited iTregs engrafted, expanded in vivo, and clones were detectable by TCR-Vβ sequencing at day +21 (Supplemental Tab. 1).

Human pSTAT3-inhibited iTregs produce ample IL-9 and support cutaneous mast cells.

Cutaneous Lymphocyte Antigen (CLA) is critical for T cells to traffic to the skin, especially in the setting of inflammatory conditions (36). Additionally, IL-9 is an immune suppressive cytokine produced by Tregs and implicated in tissue tolerance. In particular, IL-9 supports mast cells that provide localized immune suppression and can prevent graft rejection in transplanted rodents (37). Compared to DMSO-treated iTregs, pSTAT3-inhibition significantly increased the frequency of IL-9$^+$, CLA$^+$ iTregs (Fig. 3 a, b). Moreover, NSG mice inoculated with pSTAT3-inhibited iTregs exhibited significantly greater numbers of human mast cells in their skin xenografts (Fig. 3 c, d).

Human pSTAT3-inhibited iTregs significantly reduce skin graft infiltration by pathogenic Th2 cells.

To assess effects of pSTAT3-inhibited human iTregs on T cells within the graft, NSG mice were transplanted with human skin (22, 23) and then inoculated with allogeneic human PBMCs alone or in combination with pSTAT3-inhibited or vehicle-treated iTregs. On day +21, the mice were humanely euthanized, and the skin grafts were harvested, preserved, and later analyzed by IHC

to determine the content of T cell subsets. Human $CD4^+$ T cells were significantly reduced in the grafts from mice treated with pSTAT3-inhibited iTregs, compared to PBMCs alone or PBMCs plus DMSO-treated iTregs (Fig. 4 a). The numbers of Treg ($CD4^+$, $Foxp3^+$) and Th1 ($CD4^+$, T-Bet^+) cells within the skin grafts were similar among mice inoculated with PBMCs alone or in combination with either Treg treatment (Fig. 4 b, c, e). In contrast, numbers of Th2 ($CD4^+$, $GATA3^+$) cells, which are implicated in T cell-mediated inflammatory skin syndromes (38), were significantly reduced in the skin grafts of transplanted mice treated with pSTAT3-inhibited iTregs cells, compared to mice inoculated with PBMCs alone or in combination with vehicle-treated iTregs (Fig. 4 d, e).

Human pSTAT3-inhibited iTregs significantly reduce pathogenic Th1 cells in the spleen. To determine the effects of pSTAT3-inhibited iTregs on peripheral T cells beyond the skin grafts, transplanted mice were humanely euthanized on day +21 and human T cells were isolated from the mouse spleens for phenotyping by flow cytometry. The spleens of mice treated with pSTAT3-inhibited iTregs were markedly smaller than mice inoculated with PBMCs alone or with DMSO-treated iTregs (Supplemental Fig. 1). Further, total numbers of human $CD4^+$ and $CD8^+$ T cells in the spleen were significantly decreased in mice treated with pSTAT3-inhibited iTregs (Fig. 5 a, b). This included proportional reductions in human central memory ($CD62L^+$, $CD45RO^+$), effector memory ($CD62L^-$, $CD45RO^+$), and naïve ($CD62L^+$, $CD45RO^-$) T cells residing in the spleen (Fig. 5 a, b) (39). Th1 cells ($CD4^+$, $IFN\gamma^+$) are implicated in GVHD and allograft rejection (40, 41). While Th1 cells were unchanged in the skin grafts, pathogenic Th1 cells were significantly decreased in the spleens of mice treated with pSTAT3-inhibited iTregs, compared to PBMCs alone (Fig. 5 c-e). Mice that received DMSO-treated iTregs demonstrated

a modest, not statistically significant reduction in Th1 cells, compared to PBMCs alone (Fig. 5 c-e).

Unlike the skin grafts, the amount of human Th2 cells ($CD4^+$, $IL\text{-}4^+$) in the spleen were similar among all experimental groups (Fig. 5 e-g). To gain insights into how pSTAT3-inhibited iTregs influence skin-homing of Th2 cells, we co-cultured iTregs with DC-allostimulated T cells. Interestingly, Th2 cells cultured with pSTAT3-inhibited iTregs expressed significantly less CLA (Fig. 5 h, i), which is critical for T cell homing to inflamed skin (36). Thus, pSTAT3-inhibited iTregs limit Th2 homing and infiltration of allogeneic skin tissue but permit their differentiation peripherally.

Human pSTAT3-inhibited iTregs reduce the amount of alloreactive Tconv and pathogenic Th17 cells in the periphery.

Next, we investigated whether adoptive transfer of pSTAT3-inhibited iTregs affected the amount of Treg or Tconv in the recipient spleens with further flow cytometry analyses. The frequency and absolute number of human splenic Tregs was similar across the experimental groups (Fig. 6 a-d). Though the frequency of human, activated ($CD4^+$, $CD127^+$, $CD25^+$) Tconv in the spleen was similar among the experimental groups, mice treated with pSTAT3-inhibited iTregs demonstrated a significant reduction in the absolute number of activated Tconv (Fig. 6 b, e, f). There was also a trend for increases in the ratio of Treg:Activated Tconv among mice treated with pSTAT3-inhibited iTregs (Fig. 6 g).

Th17 cells can develop from ex-Treg, that is $CD4^+$ T cells that lose *Foxp3* expression in vivo (42). Moreover, Th17 cells are sufficient to induce GVHD or allograft rejection (40, 43). Importantly, the frequency of human Th17 cells in the spleen was also significantly reduced in

mice treated with pSTAT3-inhibited iTregs, versus mice inoculated with PBMCs alone or vehicle-treated iTregs (Fig. 7 a-c).

Human pSTAT3-inhibited iTregs maintain anti-leukemia immunity by donor T cells.

Using our established method to generate human antitumor cytotoxic T lymphocytes (CTL) in vivo (22, 23), we tested the effects of pSTAT3-inhibited iTregs on the anti-leukemia immunity of donor T cells. CTLs were generated in human PBMC-xenotransplanted NSG mice injected with pSTAT3-inhibited iTregs or DMSO-treated iTregs, where an inoculum of irradiated U937 cells was administered on day 0 and day +7. Unvaccinated, xenotransplanted mice served as a negative control. As the iTregs were expanded with skin-donor dendritic cells, the pSTAT3-inhibited iTregs were designed to be antigen-specific and did not inhibit CTL generation or their lytic function against leukemia. Further, CTLs from mice injected with pSTAT3-inhibited or vehicle-treated iTregs were similar in their enhanced killing capacity against U937 targets in vitro, compared to unvaccinated controls (Fig. 8). Thus, although antigen-specific, pSTAT3-inhibited iTregs significantly suppress alloreactive T cells and skin graft rejection, they spare donor anti-leukemia immunity.

Metabolic reprogramming increases the potency of pSTAT3-inhibited iTregs.

STAT3 mediates proinflammatory IL-6 receptor signaling and is necessary for optimal electron transport chain (ETC) activity in the mitochondria (44, 45). Foxp3 directs oxidative phosphorylation (OxPhos) in iTregs (46), whereas Tconv prefer glycolysis (44, 47, 48). Others have demonstrated that Tregs that utilize glycolysis and exhibit reduced OxPhos are dysfunctional (49). Notably, pSTAT3 inhibition in iTregs compromised OxPhos (Fig. 9 a) and

induced a shift toward glycolysis (Fig. 9 b). To test if this detrimental effect on OxPhos by pSTAT3 inhibition could be overcome, we generated iTregs with S3i-201 and coQ10, as coQ10 can replace electron deficiencies by directly stimulating Complex II of the ETC (50). These studies revealed that coQ10 treatment elevated basal and restores the maximal spare capacity for OxPhos in pSTAT3-inhibited iTregs (Fig. 9 a), and significantly decreased glycolysis (Fig. 9 b). Notably, coQ10 treatment further augmented the suppressive potency of pSTAT3-inhibited iTregs in vitro (Fig. 9 c). Finally, in the human skin/NSG mouse xenograft model, pSTAT3-inhibited iTregs treated with coQ10 augmented protection from alloreactive T cells in vivo, where human iTregs generated with S3i-201 and coQ10 were superior at reducing skin graft rejection compared to pSTAT3-inhibition alone (Fig. 9 d, e). Indeed, rescuing OxPhos in pSTAT3-inhibited iTregs by ex vivo treatment with coQ10 optimized their suppressive potency, where 90% of the transplanted mice were free of graft rejection (Fig. 9 d).

DISCUSSION

Here we demonstrate that adoptive transfer of human pSTAT3-inhibited iTregs prevents skin graft rejection and xenogeneic GVHD by donor T cells. Therefore, adoptive transfer of pSTAT3-inhibited iTregs could benefit recipients of solid organ allografts or alloHCT alike, to prevent graft rejection or GVHD, respectively. Mechanistically, pSTAT3 inhibition in iTregs augments demethylation of the *FOXP3* TSDR and Foxp3 expression, stabilizing their suppressive phenotypic (21), and here we show this is associated with increased expression of the immune checkpoints GARP and PD-1 that are important for the enhanced potency of the pSTAT3-inhibited iTregs. Importantly, our current study offers preclinical proof-of-concept evidence that pSTAT3-inhibited iTregs: 1) have superior suppression over alloreactive human T cells in vivo; 2) limit tissue invasion by pathogenic Th2 cells into skin, a highly antigenic tissue and common GVHD target organ; 3) recruit immune suppressive mast cells via IL-9; 4) significantly reduce alloreactive Tconv, Th1, and Th17 cells in the periphery; and 5) preserve donor anti-leukemia activity, a key benefit of alloHCT. Moreover, the infused human pSTAT3-inhibited iTregs persist and expand in vivo.

Increased IL-6 responsiveness and STAT3 phosphorylation in $CD4^+$ T cells is associated with high rates of grade II-IV acute GVHD (16, 51). STAT3 competes with STAT5 for access to *FOXP3* promoters and antagonizes Treg development (52, 53). We previously attempted to enhance Treg expansion and peripheral induction early after alloHCT by polarizing pSTAT5 signaling in circulating $CD4^+$ T cells in our phase II GVHD prevention trial of low-dose IL-2 (16). In that study we achieved a significant, but temporary, increase in Treg reconstitution (16). Specifically, while IL-2 improved pSTAT5 activity in donor $CD4^+$ T cells, this regimen failed to mitigate aberrant pSTAT3 activation in the same cells and most patients that developed grade II-

IV acute GVHD exhibited high numbers of circulating pSTAT3$^+$ CD4$^+$ T cells (16). Systemic pSTAT3 inhibition is however translationally challenging, as current small molecule inhibitors are limited by toxicity (54). Thus, we hypothesized that directly inhibiting STAT3 phosphorylation during the generation of iTreg ex vivo, followed by their adoptive transfer to transplant recipients, could significantly suppress alloreactive T cells and reduce GVHD.

While several GVHD prevention trials using peripheral or "natural" Tregs have been conducted (2, 12, 18), very few have tested iTregs for the same indication (55). In solid organ transplantation, early phase trials are currently underway to investigate the safety and preliminary efficacy of human peripheral, but not induced, Tregs in allograft rejection prophylaxis (17). From a translational perspective, iTregs offer an advantage over peripheral Tregs as they come from a large and readily available pool of CD4$^+$ Tconv, compared to an otherwise rare population of peripheral Tregs. Thus, iTregs are amenable to efficient clinical scale production. However, iTregs suffer from phenotypic instability and can revert to potentially inflammatory Tconv (56). Likely given this plasticity, adoptive transfer of iTregs for GVHD prevention in preclinical models has shown mixed efficacy, where human iTregs showed efficacy in reducing xenogeneic GVHD (57), but murine iTregs failed to prevent GVHD (56). Further, in a phase I trial, human iTregs have safely been administered as GVHD prophylaxis, yet the trial was not powered to test efficacy and did not appear to significantly reduce GVHD (55). Similarly, in our preclinical xenogeneic NSG model, vehicle-treated iTregs failed to protect recipients against human skin graft rejection, yet importantly, they did not accelerate rejection or xenogeneic GVHD. In contrast and importantly, we have shown that inhibiting pSTAT3 optimizes iTreg suppressive functions to overcome tissue rejection by alloreactive human T cells.

Though pSTAT3 inhibition enhances iTreg potency and stabilizes its suppressive phenotype, mitochondrial STAT3 is necessary for optimal activity of the ETC (44, 45). In accord with these studies here we show that pSTAT3 inhibition compromises OxPhos in iTregs and provokes a shift toward glycolysis. Given these findings we reasoned that although pSTAT3-inhibited iTregs significantly reduce alloreactive T cells, impaired OxPhos might limit iTreg fitness and long-term durability. Indeed, we showed that treatment with coQ10, which is accepted by complex II of the ETC and facilitates OxPhos by shuttling electrons to complex III in the mitochondria (50), rescues OxPhos in pSTAT3-inhibited iTregs, and further augments their suppressive potency against alloreactive T cells both in vitro and in vivo. In contrast, adding coQ10 to vehicle-treated iTregs further improved OxPhos, but did not affect their suppressive function. We conclude that increased OxPhos alone is insufficient to enhance iTreg potency and that other targets affected by pSTAT3 inhibition also contribute to the superior suppressive responses of these iTregs (e.g., PD-1 and GARP). Our findings are consistent with those of the Chi Lab and others (49), in which Tregs that use glycolysis over OxPhos exhibit limited suppressive function. However, it is important to note that glycolysis is possibly beneficial in other aspects of Treg biology, such as migration (58) and differentiation (59). While we investigated the effects of pSTAT3 inhibition on iTreg metabolism, it is also possible that S3I-201 and/or coQ10 may have important biologic effects beyond those identified in the present study.

Adoptive transfer of metabolically tuned, pSTAT3-inhibited iTregs is a readily translatable strategy to prevent GVHD or graft rejection mediated by alloreactive human T cells. Moreover, pSTAT3-inhibited iTregs preserve donor anti-leukemia activity necessary for the beneficial graft-versus leukemia effect. Despite recent FDA approvals for agents to treat steroid-

refractory acute (60) and chronic GVHD (61), advances in GVHD prevention are needed. Additionally, innovative approaches are needed in solid organ allotransplantation to reduce dependence on broadly suppressive calcineurin-inhibitors and glucocorticoids. Thus, we are actively scaling up production of these novel iTregs to clinically test in GVHD prophylaxis. We predict that pSTAT3 inhibition combined with effective metabolic reprogramming by coQ10 will provide highly effective $CD4^+$ iTreg-based GVHD prophylaxis and submit that this strategy warrants full clinical investigation.

METHODS

Monoclonal Antibodies and Flow Cytometry

Fluorochrome-conjugated mouse anti-human monoclonal antibodies included anti-CD3, CD4, CD25, CD45RO, CD62L, CD127, GARP, PD-1, CD39, LAG3, CTLA4, IL-9, CLA, Foxp3, Ki-67, IFN-γ, IL-17A, and IL-4 (BD Biosciences, San Jose, CA. USA; eBioscience San Jose, CA. USA; Cell Signaling Technology, Boston, MA. USA) (Supplemental Tab. 2). LIVE/DEAD Fixable Yellow or Aqua Dead Cell Stain (Life Technologies, Grand Island, NY) was used to determine viability. Live events were acquired on a BD FACSCanto II or LSRII flow cytometer (FlowJo software, ver. 7.6.4; TreeStar, Ashland, OR, USA).

Treg Generation and Functional Experiments

Tregs were defined as $CD4^+$, $CD127^-$, $CD25^+$, $Foxp3^+$ cells(28, 29). Induced Tregs were generated by stimulating purified $CD4^+$, $CD25^-$ T cells with allogeneic monocyte-derived dendritic cells (T cell to DC ratio = 30:1) for 5 days in the presence of pSTAT3 inhibitor, S3i-201 (50 μM), or DMSO (0.1%) for 5 days in medium supplemented with recombinant human IL-2 (20 IU/ml) as published(21). Where indicated, iTregs were generated with CD3/CD28 stimulator beads. In select experiments, STAT3 siRNA (Dharmacon Accell) was used to knock down STAT3 molecularly. Treg potency was determined using our standard suppression assay (26). S3i-201 or DMSO was only added to the initial culture to expand the Tregs. No drug was added to the suppression assay medium. Conventional, alloreactive T cell (Tconv) proliferation was measured by Ki-67 expression using flow cytometry. Where indicated, anti-human LAP/TGFβ1 (ligand of GARP)(31, 32) monoclonal antibody (mAb), -PD-1 (10 μg/ml) mAb, CD39 ectonucleotidase inhibitor (ARL67156, 125 μM)(30), or control (PBS plus isotype) was

added to the suppression assay medium to neutralize GARP, PD-1, or CD39 activity, respectively.

Xenograft Model and in vivo CTL Generation

NSG mice received a 1-cm^2, split thickness human skin graft(22, 23). The skin grafts were allowed to heal and facilitate engraftment. During this time, human monocyte derived DCs (moDC) were generated from blood autologous to the skin graft donor using GmCSF and IL-4, then matured with a standardized combination of inflammatory cytokines and prostaglandin E2 (PGE2) as described(21, 62). These moDCs were then used to generate and expand allogeneic pSTAT3-inhibited iTregs or vehicle-treated iTregs from a healthy donor (OneBlood or Memorial Blood Centers), using S3i-201(21, 27, 51) or DMSO respectively. In select experiments, the iTregs were supplemented with CoQ10 (10 ng/ml) during culture. The skin grafted mice were then transplanted with $5x10^6$ human PBMCs to induce graft rejection, along with either $1x10^5$ pSTAT3-inhibited iTregs, vehicle-treated iTregs, or no iTregs (note that the iTregs were autologous to the PBMCs, and allogeneic to the skin). The skin grafts were then monitored daily for signs of rejection, including ulceration, necrosis, and scabbing(22, 23). Skin grafts that were >75% nonviable were considered rejected.

In select experiments, mice were humanely euthanized on day +21; skin grafts and host spleens were harvested for analysis. Skin rejection was performed blinded according to standard criteria (26, 33, 38). Processed spleens cells were phenotyped by flow cytometry for Tregs, Tconv, Th1, Th2, and Th17 cells(22, 23, 30). IHC was performed on the skin grafts to identify Tregs (CD4 and Foxp3), Th1 (CD4 and T-bet), and Th2 (CD4 and GATA3) and scanned by use of

ScanScope XT (Aperio Technologies, Vista, CA, USA) with a 200×/0.75 NA objective lens at a rate of 3 min/slide via Basler Tri-linear array as described(51). In addition, Ki-67 staining was conducted to identify proliferative, infiltrative lymphocytes in the dermis and tryptase staining was performed to characterize mast cell content in the grafts.

To test the impact of pSTAT3-inhibited iTreg effects on the donor T cell-mediated response against U937 leukemia cells, mice were transplanted with $5x10^6$ human PBMCs, with or without pSTAT3-inhibited or DMSO-treated iTregs ($1x10^5$), and also received an inoculum of irradiated U937 cells ($2x10^6$) on day 0 and +7(22, 23). Control mice received PBMCs alone without tumor. Mice did not receive skin grafts for these experiments. On day +12, the mice were humanely euthanized, and the spleens were harvested. Human $CD3^+$ T cells within the spleens were purified by magnetic beads. Tregs were specifically not removed from the harvested T cells. The human T cells were co-cultured with fresh U937 cells at varying T cell to Target ratios for 4 hours. Tumor lysis assays were performed in vitro using a colorimetric assay(22, 23).

Metabolism Experiments

Pretreated, purified pSTAT3-inhibited or DMSO-treated iTregs ($2x10^5$) were washed once and then plated in XFe96 microplates in unbuffered DMEM containing 10 mM Glucose, 1 mM Sodium Pyruvate, and 2 mM L-Glutamine for mitochondrial stress test assays or unbuffered DMEM only for glycolytic stress test assays. The concentration of the compounds used were 1 μM Oligomycin A, 1 μM FCCP, 500 nM Rotenone, 500 nM Antimycin A, 10 mM Glucose, and 1 mM 2-DG. Data normalized using Calcein AM.

Statistical Analysis

ANOVA was used for group comparisons, including a Sidak's or Dunn's post-test for correction of multiple comparisons. A paired t test was used for paired comparisons. For comparison of survival curves, a Log-rank test was used. The statistical analysis was conducted using Prism software version 5.04 (GraphPad). Statistical significance was defined by a two-tailed $P < 0.05$ (two-tailed).

Study Approval

Skin and peripheral blood mononuclear cells were acquired from consented mastectomy patients using an IRB-approved protocol at Moffitt Cancer Center and the University of Minnesota Masonic Cancer Center. NSG mice (male or female, age 6-24 weeks old) were purchased from Jackson Laboratory and housed within American Association for Laboratory Animal Care-accredited Animal Resource Centers at Moffitt Cancer Center or the University of Minnesota. All mice were treated in adherence with the NIH Guide for the Care and Use of Laboratory Animals and the protocols used were approved by local institutional animal care and use committees.

Author Contributions: K.W. performed experiments, analyzed and interpreted data, and edited the manuscript. M.R.F. performed and analyzed experiments and edited the manuscript. J.R. performed experiments. J.K. designed experiments and provided statistical expertise. M.C.L, J.V.K., and J.H. consented patients and acquired human skin grafts. E.M.S., M.A.L., M.H. and C.F. performed histologic and pathologic analyses of xenograft data and edited the manuscript.

N.J.L., H.R.L., and S.M.S. synthesized S3i-201 and edited the manuscript. J.P., S.Z.P., D.M. Jr., J.L.C., B.R.B., and C.A. assisted in the design of experiments and edited the manuscript. B.C.B. designed, led, and performed experiments, analyzed and interpreted data, and wrote the manuscript.

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Competing Interests: B.C.B. holds a patent related to CD4+ T cell pSTAT3 as a marker and therapeutic target of acute GVHD. N.J.L., H.R.L., and S.M.S hold a patent related to the composition and use of S3I-201. Neither the inventors nor their institutions have received payment related to claims described in the patents. All other authors have no competing financial interests to declare.

Supplemental Material:

Supplemental Table 1: Human pSTAT3-inhibited iTregs expand and are detectable in vivo.

Supplemental Figure 1: Receipt spleen size is reduced after adoptive transfer of human pSTAT3-inhibited iTregs.

Supplemental Table 2: Key reagents.

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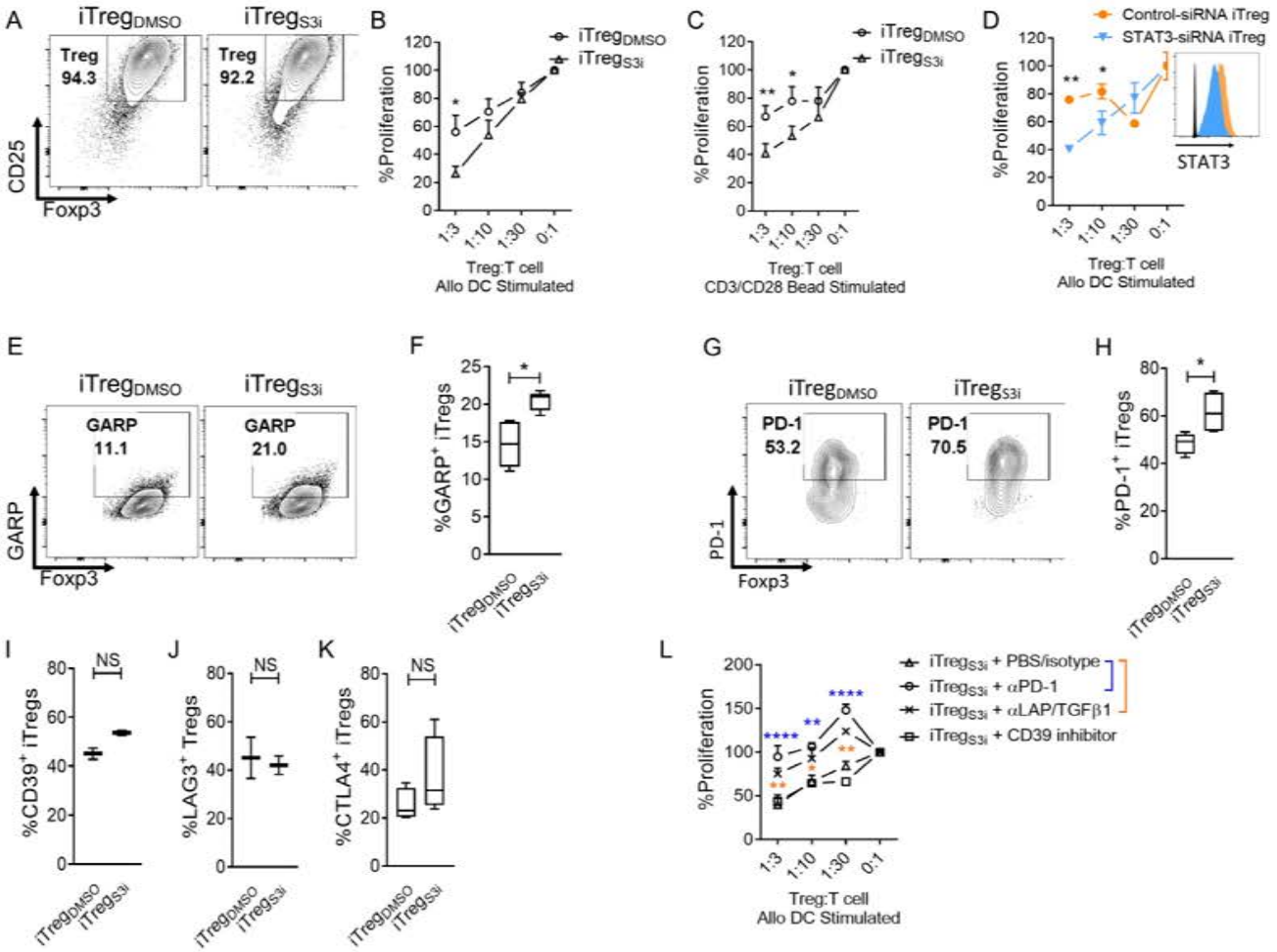

Figure 1. Human pSTAT3-inhibited iTregs demonstrate superior suppressive potency. A) Contour plots show the purity of the generated CD4$^+$ iTregs after CD25 selection using magnetic bead isolation. The suppressive potency of purified, iTregs generated over 5 days with B) allogeneic DC stimulators or C) CD3/CD28 beads, while exposed to S3i-201 (50μM) or DMSO was tested at different ratios of iTreg to T cell responders in alloMLRs. No S3i-201 or DMSO was added to the suppression assay. Graphs show mean ± SEM. n=4 independent experiments. D) The suppressive potency of iTregs generated with STAT3 or non-targeted siRNA (mean ± SEM) is shown. Histograms shows STAT3 expression in the non-targeted siRNA treated iTregs (orange, gMFI 2870) and STAT3 siRNA-treated iTregs (blue, gMFI 1705). 1 of 2 independent experiments is shown. Contour plots and box and whisker plots show the frequency (max, min, median) of (E,F) GARP$^+$, (G,H) PD-1$^+$, and (I-K) CD39$^+$, LAG3$^+$, or CTLA4$^+$ iTregs (CD4+, CD127neg, CD25+, Foxp3+) after expansion with S3I-201 or DMSO from up to 5 independent experiments. L) Graph shows the suppressive potency (mean ± SEM) of pSTAT3-inhibited iTregs treated with anti-human PD-1, LAP/TGFβ mAb, CD39 inhibitor ARL67156, or control (PBS plus isotype) from 1 of 2 independent experiments. ANOVA (A, C, D, L) or paired t-test (F, H, I, J, K). *P<0.05 or **P=0.001-0.01.

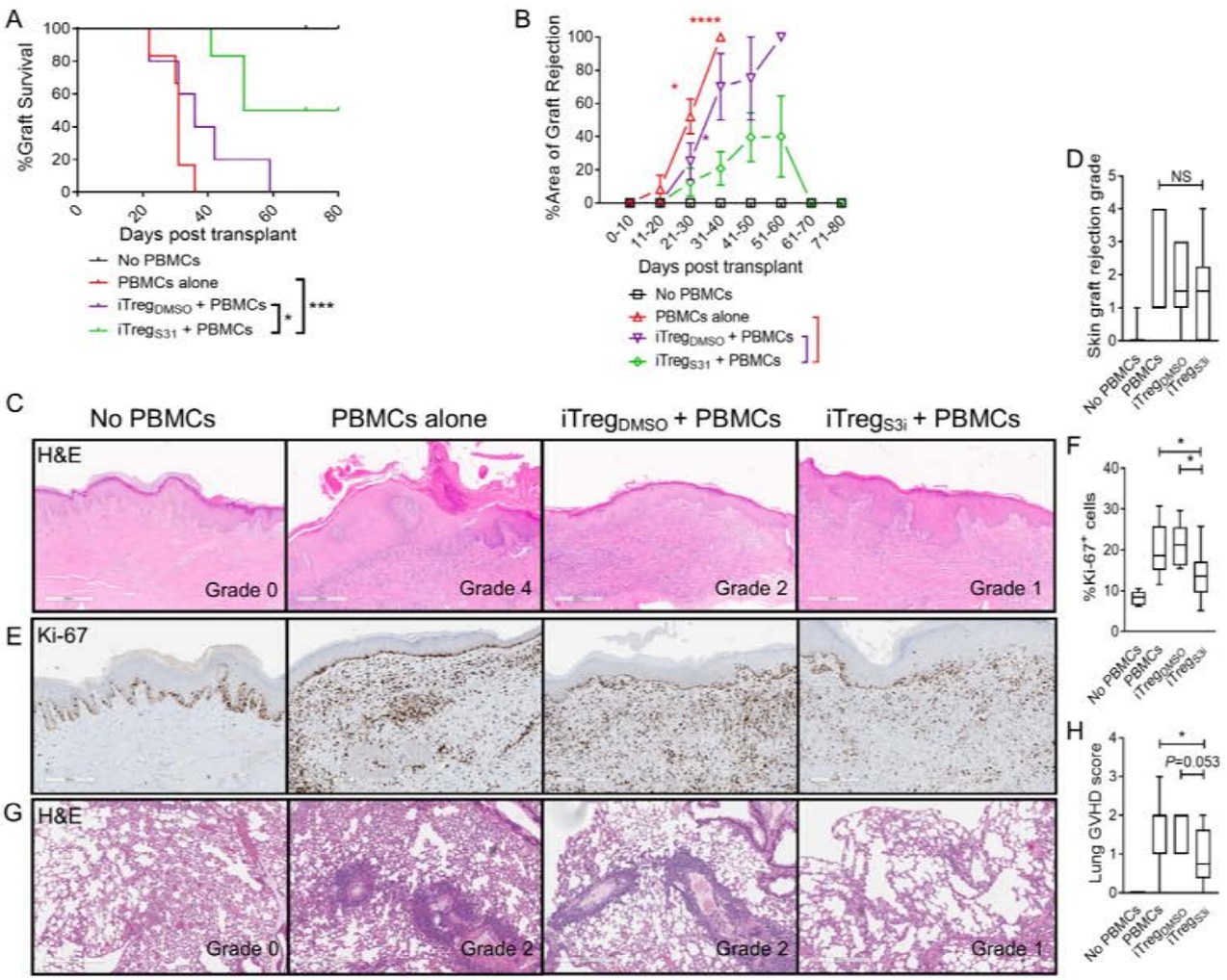

Figure 2. Human pSTAT3-inhibited iTregs significantly reduce skin graft rejection. A) NSG mice received a 1cm^2 human skin graft. Allogeneic pSTAT3-inhibited (S3i) or DMSO-treated iTregs were generated using DCs from the skin donor. After 30 day, the mice received 5x10^6 human PBMCs (autologous to the iTregs and allogeneic to the skin) plus 1x10^5 pSTAT3-inhibited or DMSO-treated iTregs. Graphs shows skin graft A) survival and B) % area of graft rejection (mean ± SEM). C,D) Representative H&E images and graph shows skin graft rejection scores (max, min, median) determined at day +21. E,F) Ki-67 expression identifies normal proliferating basal keratinocytes and expanding tissue-invasive donor lymphocytes in the dermis of the grafts. Representative IHC images and graph shows the amount (max, min, median) of infiltrating, proliferative, dermal Ki-67$^+$ cells in the graft at day +21. G,H) Representative H&E images and graph (max, min, median) shows the amount of xenogeneic GVHD within the lungs of transplanted NSG mice. n=3 independent experiments with 6-11 mice/group. Log-rank (A) or ANOVA (B, D, F, H). *P<0.05, ***P=0.0001-0.001, ****P<0.0001. NS = not significant.

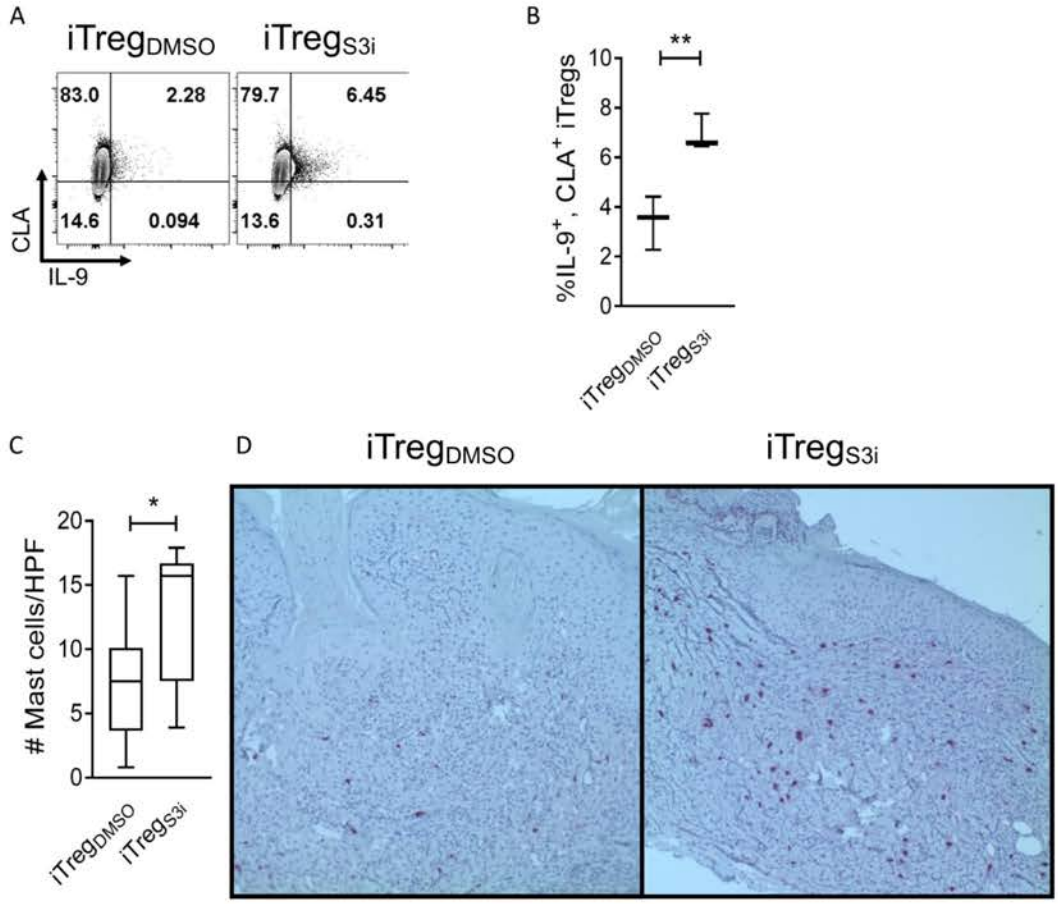

Figure 3. Human pSTAT3-inhibited iTregs produce ample IL-9 and support cutaneous mast cells. pSTAT3-inhibited or DMSO-treated human iTregs were generated as described. A) Contour plots and (B) graph shows the frequency (max, min, median) of IL9$^+$, CLA$^+$ CD4$^+$ iTregs after expansion with S3i-201 or DMSO for 5 days from 3 independent experiments. NSG mice received a human skin graft, allogeneic PBMCs, and pSTAT3-inhibited (S3i) or control iTregs. On day +21, the skin grafts were analyzed for mast cell content by tryptase expression using immunohistochemistry. (C,D) Graph (max, min, median) and representative images show skin grafts from mice treated with pSTAT3-inhibited iTregs demonstrated significantly more mast cells (tryptase = red). n=3 independent experiments with 9 mice/group. Paired t-test (B, C). *P<0.05 or **P=0.001-0.01.

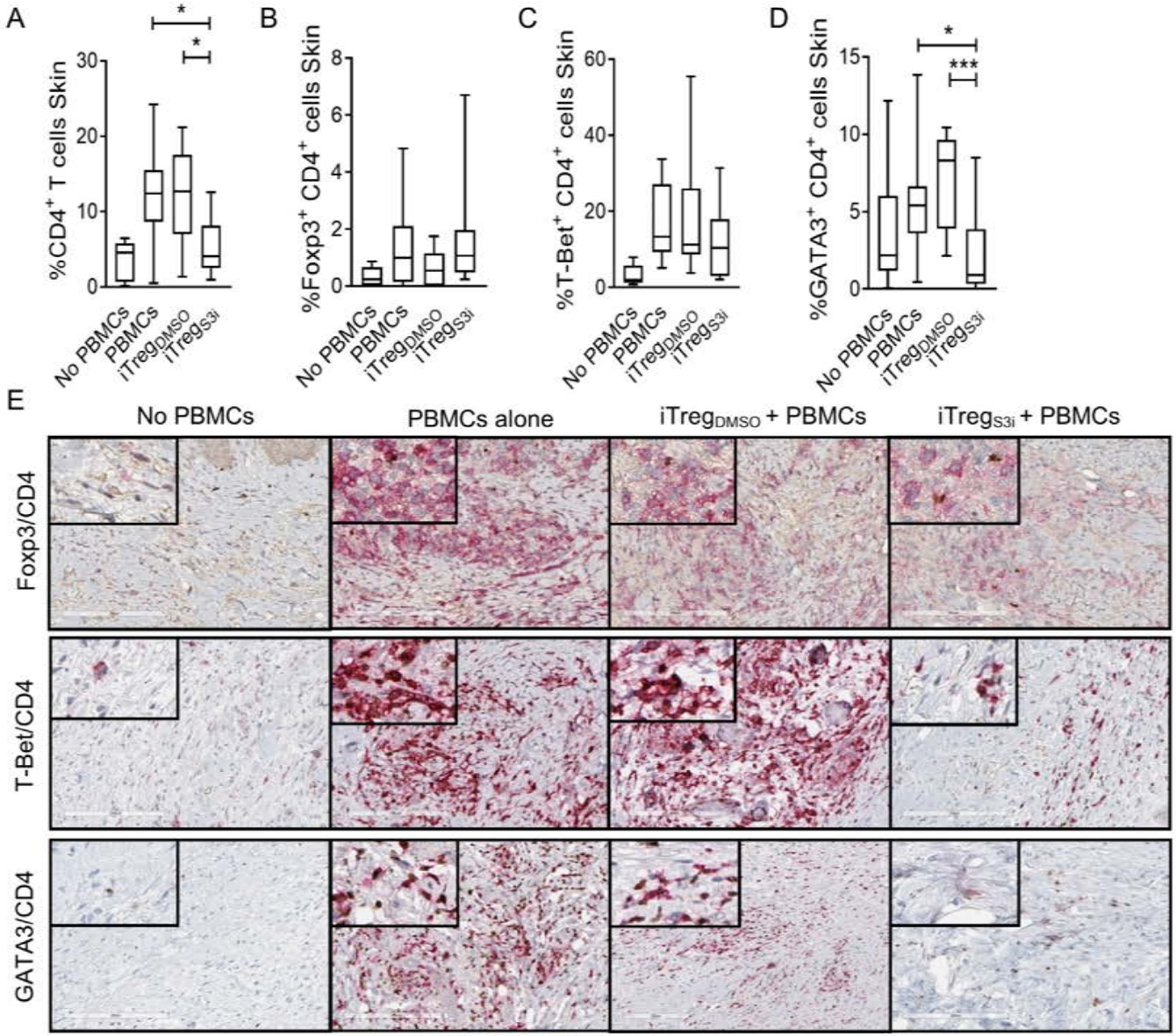

Figure 4. Human pSTAT3-inhibited iTregs significantly reduce skin graft infiltration by pathogenic Th2 cells. NSG mice received a human skin graft, allogeneic PBMCs, and pSTAT3-inhibited (S3i) or control iTregs. On day +21, the skin grafts were analyzed for (A, E) CD4+ (red), (B, E) Treg (CD4 red, Foxp3 brown), (C, E) Th1 (CD4 red, T-Bet brown), and (D, E) Th2 cells (CD4 red, GATA3 brown). Box and whisker plots show max, min, and median. n=3 experiments, up to 11 mice/group. ANOVA (A-D). **P*<0.05, ****P*=0.0001-0.001.

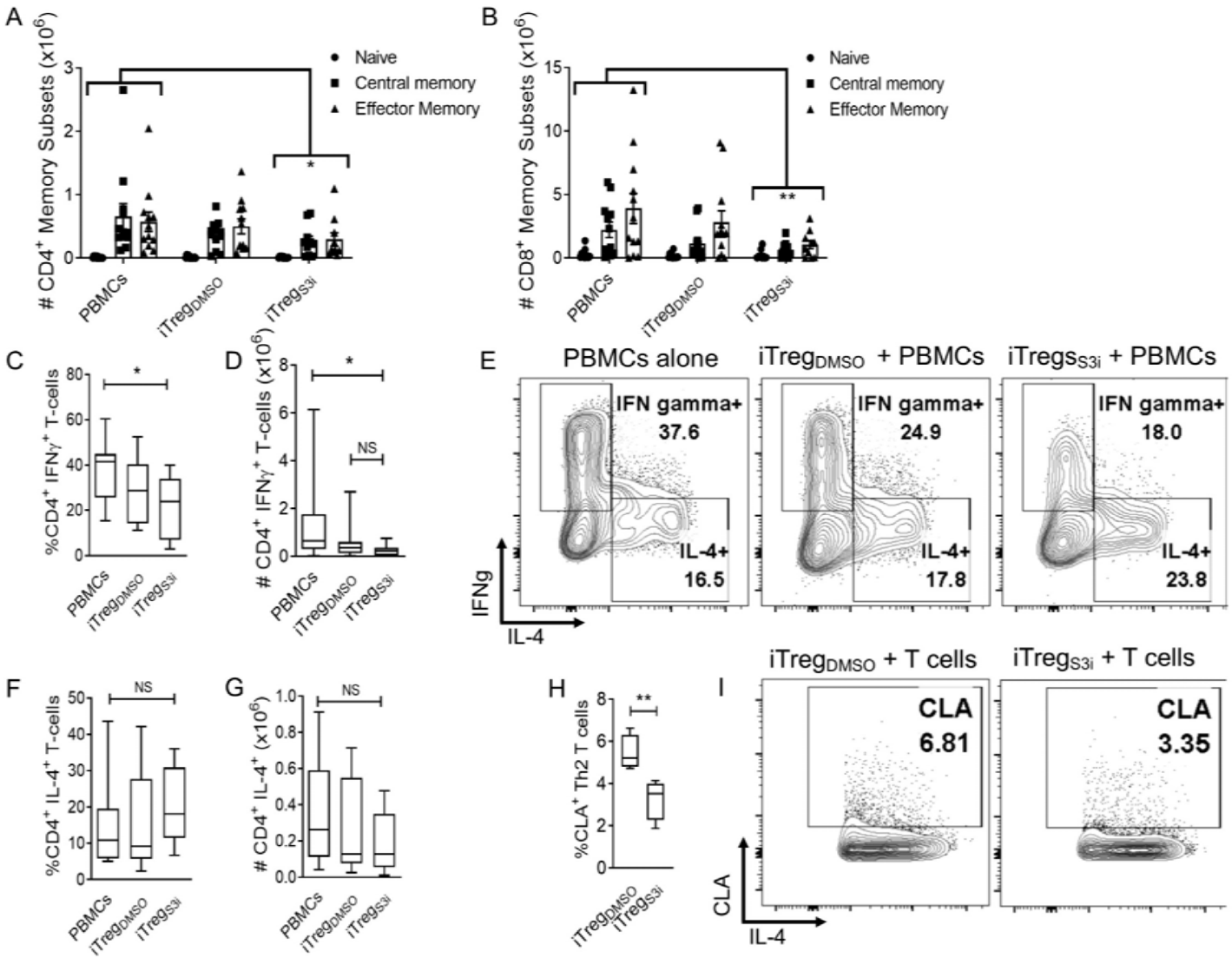

Figure 5. Human pSTAT3-inhibited iTregs significantly reduce pathogenic Th1 cells in the spleen. NSG mice received a human skin graft, allogeneic PBMCs, and pSTAT3-inhibited (S3i) or control iTregs. On day +21, the recipient spleens were harvested and analyzed for human T cell numbers and effector subsets. Graphs show the absolute numbers of A) $CD4^+$ and B) $CD8^+$ central memory ($CD62L^+$, $CD45RO^+$), effector memory ($CD62L^-$, $CD45RO^+$), and naïve T cells ($CD62L^+$, $CD45RO^-$) ± SEM. (C-G) Box and whisker plots (max, min, median) show the frequency and absolute number of human Th1 ($CD4^+$, $IFN\gamma^+$) and Th2 cells ($CD4^+$, $IL\text{-}4^+$), with representative contour plots showing the T cell populations. n=3 experiments, up to 11 mice/group. Human pSTAT3-inhibited or DMSO-treated iTregs were cultures with autologous CD4+ T cells with allogeneic dendritic cells (T cell to DC ratio 30:1) for 5 days. The frequency of skin-homing CLA expression (H) on the $CD4^+$, $IL\text{-}4^+$ (max, min, median) was determined by flow cytometry. I) Representative contour plots are shown. n=4 independent experiments. ANOVA (A-D, F, G) or paired t-test (H). *P<0.05 or **P=0.001-0.01.

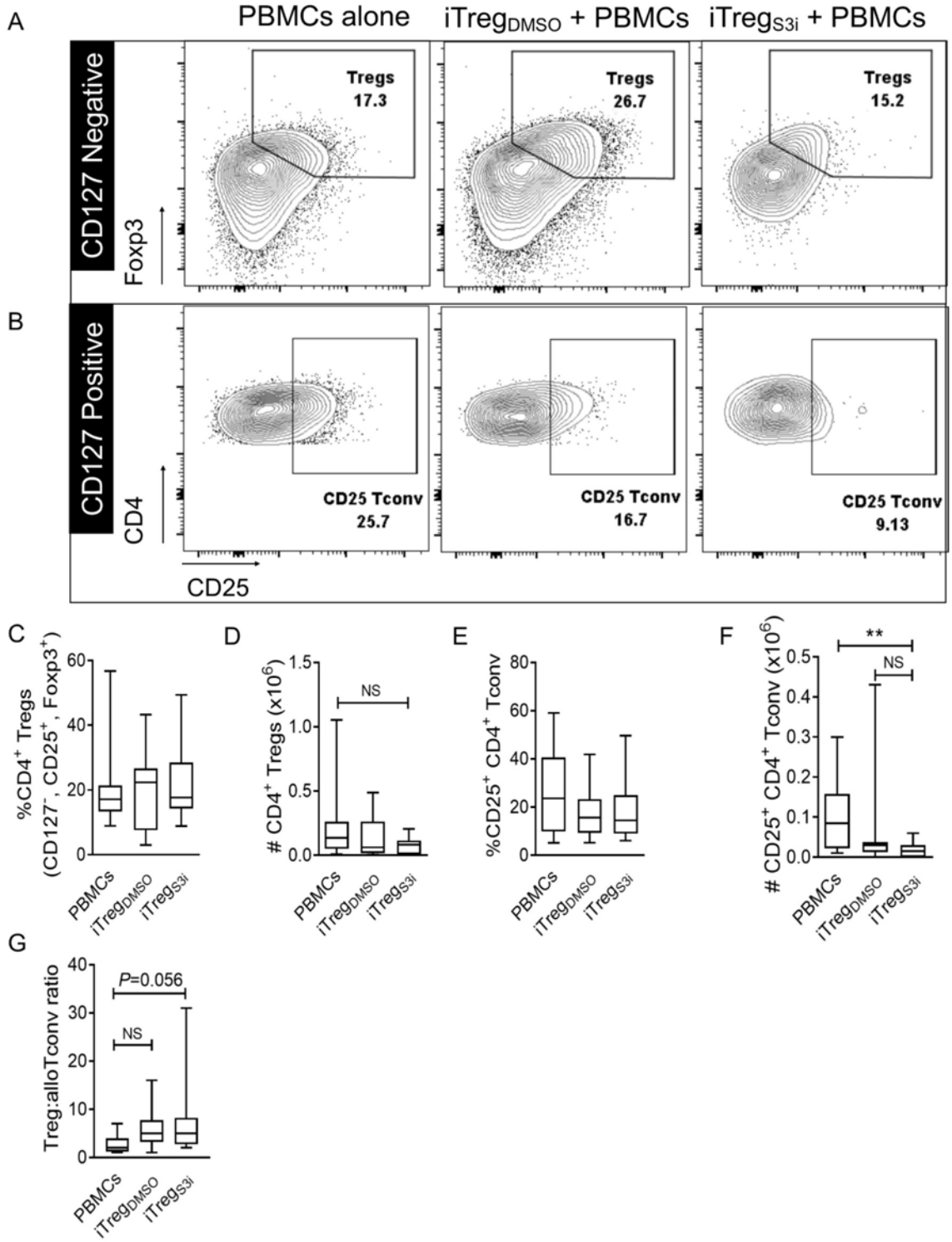

Figure 6. Human pSTAT3-inhibited iTregs reduce the amount of alloreactive Tconv. NSG mice received a human skin graft, allogeneic PBMCs, and pSTAT3-inhibited (S3i) or control iTregs. On day +21, the recipient spleens were harvested and analyzed for human Treg ($CD4^+$, $CD127^-$, $CD25^+$, $Foxp3^+$) and activated Tconv ($CD4^+$, $CD127^+$, $CD25^+$). (A,B) Representative contour plots are shown. Box and whisker plots (max, min, median) demonstrate the frequency and absolute numbers of human Tregs (C,D) and activated Tconv (E,F). (G) Graph (max, min, median) shows the ratio of Treg to activated Tconv within the recipient spleens at day +21. n=3 experiments, up to 11 mice/group. ANOVA (C-G). *P<0.05 or **P=0.001-0.01. NS = not significant.

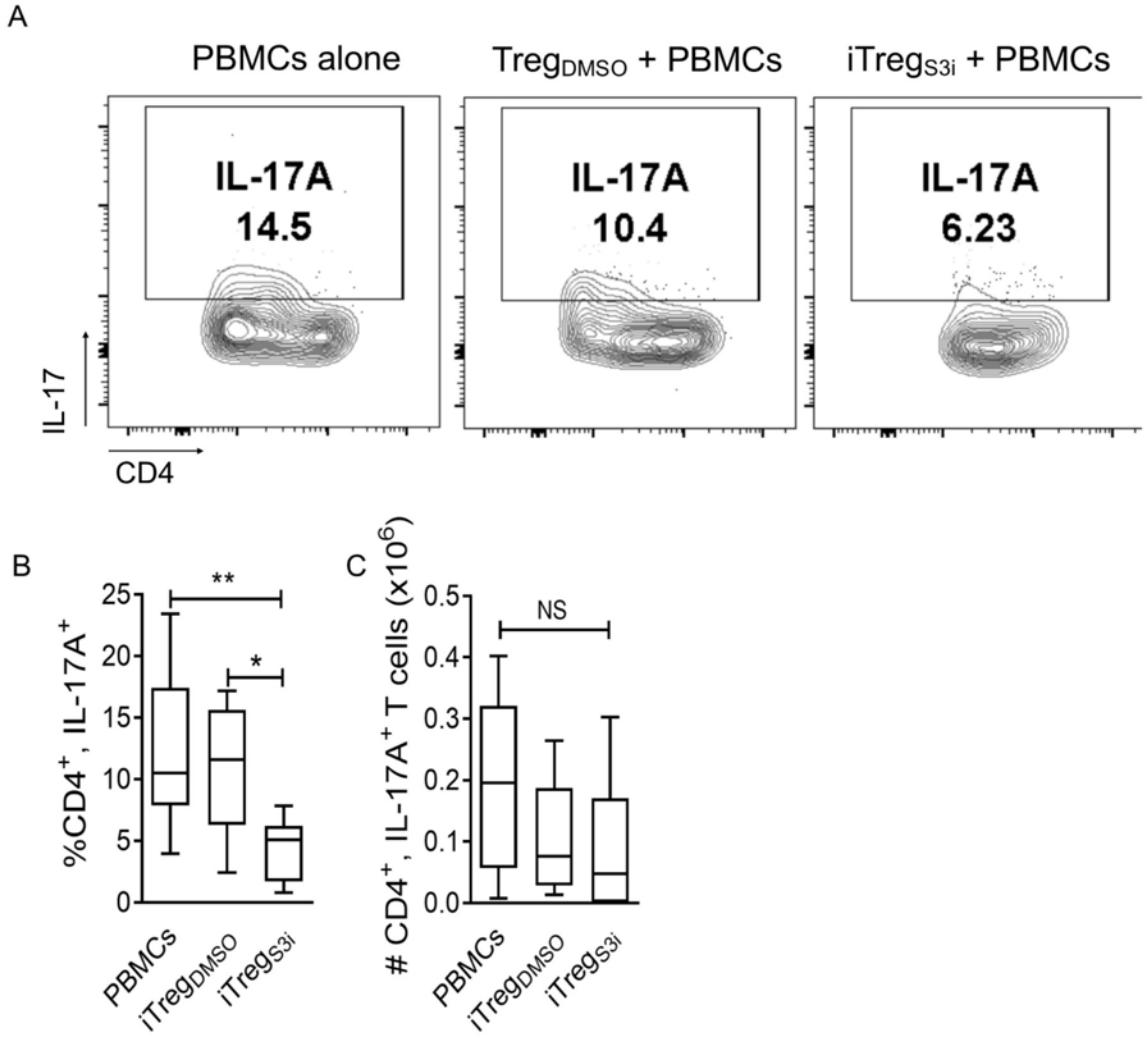

Figure 7. The amount of human Th17 cells are reduced in mice treated with pSTAT3-inhibited iTregs. NSG mice received a human skin graft, allogeneic PBMCs, and pSTAT3-inhibited (S3i) or control iTregs. On day +21, the recipient spleens were harvested and analyzed for human Th17 cells ($CD4^+$, $IL\text{-}17A^+$). Representative contour plots (A) and graphs (max, min, median) show the frequency (B) and absolute number (C) of human Th17 cells within the recipient spleens at day +21. n=3 experiments, up to 11 mice/group. ANOVA (B, C). *P<0.05 or **P=0.001-0.01. NS = not significant.

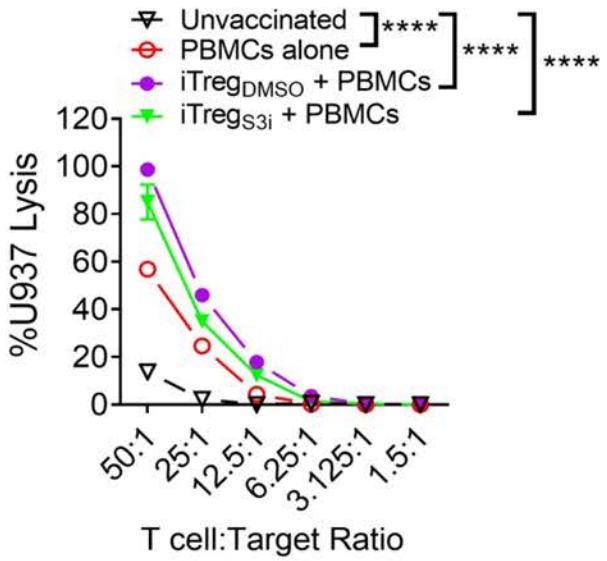

Figure 8. Human pSTAT3-inhibited iTregs maintain anti-leukemia immunity by donor T cells. Graph depicts mean lysis ± SEM by U937-specific, human T cells generated in vivo using NSG mice transplanted with human PBMCs, pSTAT3-inhibited (S3i) or control iTregs, and then vaccinated with irradiated U937cells ($2x10^6$, ATCC) on days 0 and +7. Human T cells from non-vaccinated mice containing PBMCs alone group served as a negative control. On day +12, the mice were euthanized, and human T cells were harvested and purified from the spleen using magnetic bead separation. Tregs were specifically not removed from the harvested T cells. The purified T cells were cultured with fresh U937 targets at varying ratios. U937 lysis was measured using a colorimetric assay after 4 hours of culture. Results shown are from one of two independent experiments, using 4 mice per group. ANOVA. ****P<0.0001.

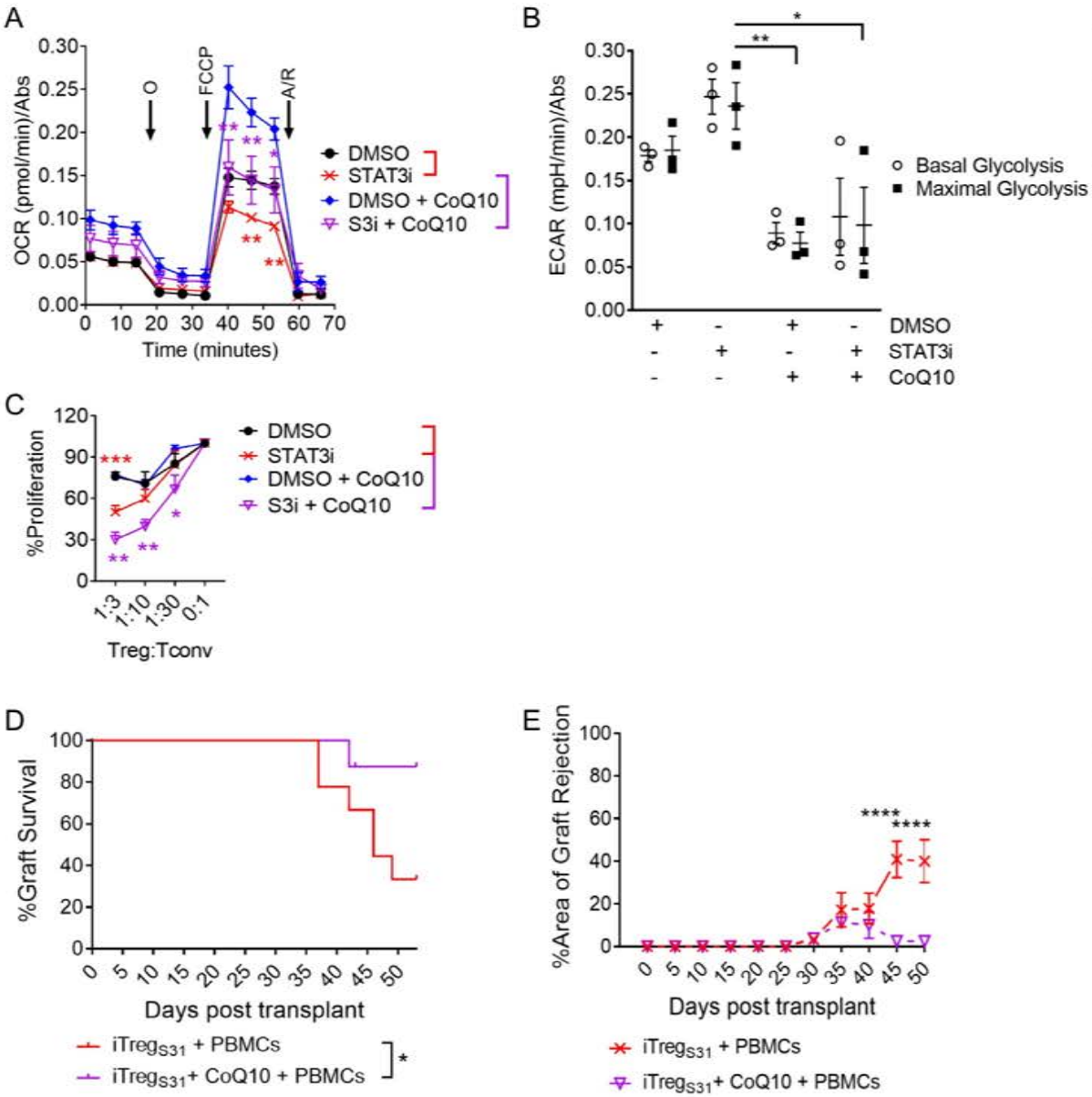

Figure 9. pSTAT3-inhibited iTregs increase their potency after metabolic reprogramming. Human iTregs were generated with allogeneic DCs while exposed to S3i-201 or DMSO for 5 days, purified by CD25 magnetic bead isolation, and then the A) oxygen consumption rate (OCR) and B) extracellular acidification rate (ECAR) were evaluated using a Seahorse XF Analyzer. Where indicated coQ10 (10ng/ml) or PBS was added to the iTreg culture to rescue OxPhos. In all cases, the medium was supplemented with IL-2 during iTreg expansion. As OxPhos was improved by adding coQ10 to pSTAT3-inhibited iTregs, we then tested its effect on iTreg function. C) Graph shows the suppressive activity (mean ± SEM) of pSTAT3-inhibited or control iTregs, treated with or without coQ10, against alloreactive T cells. n=4 independent experiments. NSG mice received a human skin graft, $5x10^6$ allogeneic PBMCs, and $1x10^5$ pSTAT3-inhibited (S3i) treated with or without coQ10 during culture. Graphs show skin graft D) survival and E) % area of graft rejection (mean ± SEM). n=2 independent experiments with up to 9 mice/group. ANOVA (A-C, E) or Log-rank (D). *P<0.05, **P=0.001-0.01, ***P=0.0001-0.001, and ****P<0.0001.