PLOS ONE

RESEARCH ARTICLE

Interleukin-35 mitigates the function of murine transplanted islet cells via regulation of Treg/Th17 ratio

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Citation: Zongyi Y, Funian Z, Hao L, Xin W, Ying C, Jialin Z, et al. (2017) Interleukin-35 mitigates the function of murine transplanted islet cells via regulation of Treg/Th17 ratio. PLoS ONE 12(12): e0189617. https://doi.org/10.1371/journal.pone.0189617

Editor: Paolo Fiorina, Children's Hospital Boston, UNITED STATES

Received: May 2, 2017

Accepted: November 29, 2017

Published: December 13, 2017

Data Availability Statement: All relevant data are within the paper.

Funding: This work was funded by the National Nature and Science Funding of China [grant numbers: 81370882] http://www.nsfc.gov.cn/ and the Nature and Science Funding of Liaoning Province (grant numbers: 2013021058) to LB, http://kjjh.lninfo.gov.cn/.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Pancreatic islet transplantation is a promising treatment for type 1 diabetes (T1D). Interleukin-35 (IL-35) is a recently discovered cytokine that exhibits potent immunosuppressive functions. However, the role of IL-35 in islet transplant rejection remains to be elucidated. In this study, we isolated islet cells of BALB/c mouse and purified CD4+ T cell subsets of a C57BL/6 mouse. The model for islet transplantation was established *in vitro* by co-culture of the islet cells and CD4+ T cells. IL-35 (20 ng/ml) was administered every other day. Following co-culture, the islet function and Treg/Th17 ratio were analyzed on days 1, 3, and 5. Furthermore, the Th17/Treg ratio was modulated (1:0–2), and the function of islet cells as well as proliferation of Th17 cells were analyzed. T cell sorting was performed using the magnetic bead sorting method; Treg and Th17 count using flow cytometry; cell proliferation detection using the carboxyfluorescein diacetate succinimidyl ester (CFSE) method, and islet function test using the sugar stimulation test. Results showed that Th17 counts increased in the co-culture system. However, after administration of IL-35, the number of Treg cells increased significantly compared to that in the control group (50.7% of total CD4+ T cells on day 5 in IL-35 group vs. 9.5% in control group) whereas the proliferation rate of Th17 cells was significantly inhibited (0.3% in IL-35 group vs. 7.2% in control group on day 5). Reducing the Th17/Treg ratio significantly improved the function of transplanted islets. Treg inhibited Th17 proliferation and IL-35 enhanced this inhibitory effect. IL-35 mitigates the function of murine transplanted islet cells via regulation of the Treg/Th17 ratio. This might serve as a potential therapeutic strategy for in-vivo islet transplant rejection and T1D.

Introduction

Pancreatic islet transplantation (PIT), a treatment for type 1 diabetes (T1D), is a minimally invasive procedure that can restore normoglycemia and insulin independence without surgical

Abbreviations: CBD, common bile duct; CFSE, carboxyfluorescein diacetate succinimidyl ester; Ebi3, Epstein-Barr-virus-induced gene 3; ELISA, enzyme-linked immunosorbent assay; FoxP3, Forkhead box P3; GSIS, glucose-stimulated insulin secretion; IL-35, Interleukin-35; PIT, pancreatic islet transplantation; RORγt, retinoic acid receptor-related orphan receptor γt; STAT5, signal transducer and activator of transcription 5; T1D, type 1 diabetes; Th1, T helper 1 cell; Th17, T helper 17 cell; Th2, T helper 2 cell; Treg, regulatory CD4 +CD25+Foxp3+ T cell.

complications [1, 2]. Current immunosuppression strategies poses several risks (such as infection and cancer) to transplant recipients [3–7]. Although a recent report showed that in most experienced institutions, the 5-year survival rate of transplanted islets reached up to 50% [8], overall long-term results remain unsatisfactory [9].

Emerging evidences suggest that T helper 17 (Th17) and regulatory CD4+CD25+Foxp3+ T (Treg) cells have a distinct differentiation pathway, which are different from that of T helper 2 (Th2) cells or T helper 1 (Th1) cells [10–12]. Tregs play an anti-inflammatory role mainly by releasing inhibitory cytokines such as TGF-β and IL-10 or contact-dependent suppression on other immune cells, including CD8+, CD4+ T cells and B cells [12]. Increase in Tregs have been reported to be involved in the development of immune tolerance [13] and solid organ transplantation (e.g. kidney transplant [14–17], liver transplant [18–22] and heart transplant [23, 24]). In contrast, Th17 cells, mainly expressed by factors such as retinoic acid receptor-related orphan receptor γt (RORγt), have been reported to play a potent pro-inflammatory role by producing the signature cytokine IL-17A [25–29]. A series of studies have reported that Th17 cells widely contribute to autoimmune diseases and transplant rejection [26, 27, 30–34]. Recent studies found that the balance between Tregs and Th17 plays an important role in the above diseases, by regulation of the immunologic homeostasis through the secretion of anti- or pro-inflammatory cytokines, depending on the activation of Forkhead box P3 (FoxP3) and signal transducer and activator of transcription 5 (STAT5) or RORγt and STAT3, respectively [30, 31, 33, 35, 36].

IL-35, consisting of IL-12α subunits and Epstein-Barr-virus-induced gene 3 (Ebi3), is a recently discovered cytokine exhibiting potent immunosuppressive functions [37–40]. It is secreted by and contributes to the proliferation of Tregs. It not only promotes differentiation of conventional CD4+T cells into Tregs but also converts Tregs into induced regulatory T cells (iTr35); the latter lack FoxP3 expression, release IL-35 but not IL-10 or TGFβ, and possess stronger immunosuppressive properties than Tregs [35, 37–39, 41–45]. Numerous studies have concentrated on the functions of IL-35 in autoimmune and inflammatory diseases, such as psoriasis [30], T1D [41], arthritis [42], asthma [44, 46] and leukemia [47].

However, the role of the balance of Treg/Th17 and the therapeutic potential and effects of IL-35 in islet transplantation has been unclear so far. Hence, here, we aimed to clarify and examine the role of Treg/Th17 and the kinetic effects of IL-35 in an *in vitro* mouse islet transplantation model.

Materials and methods

Animals

All animal experiments were approved by the local animal ethics committee at the First Hospital of China Medical University. Male BALB/c and C57BL/6 mice aged 8–12 weeks and weighing 23–28 g were used for the study. The mice were supplied by the laboratory animal center of China Medical University (Shenyang, China) and raised carefully in accordance with international guidelines (National Institutes of Health 85–23) as well as the current version of the China Law on the Protection of Animals. The mice were raised in pathogen-free cages and kept at a relative humidity of 50–70% and temperature of 20–25˚C. Mice was sacrificed using exsanguination method under anesthesia (1.5% sevoflurane).

Isolation and purification of islets

Pancreatic islets were prepared by the collagenase P (Roche Diagnostics Scandinavia, Bromma, Sweden) method from overnight fasted BALB/c mice. In brief, a mouse was anesthetized with 1.5% sevoflurane and fixed in the supine position. The skin was disinfected with 75% ethanol

followed by sterile laparotomy. The common bile duct (CBD) close to the duodenum was ligated for the retrograde puncture of CBD, followed by a slow perfusion of 3 mL collagenase-P (pre-chilled at 4˚C) to fully expand the pancreatic body and tail. The heart was excised to drain the blood and the pancreas was recovered by blunt isolation. The isolating solution (composed of 500 mL Hanks solution containing 10 mM of HBSS and 15 mM of $CaCl_2$) was filter-sterilized through a 0.22-μm filter and adjusted to pH 7.2–7.4 prior to storage at 4˚C until use. The digestive solution, with a final concentration of 1 mg/mL collagenase-P, was freshly prepared before using the afore-mentioned isolating solution (pH 7.2–7.4). Ficoll-400 density gradient centrifugation was employed to purify the islets as per a previous study [48].

Lymphocyte isolation and CD4+CD25-/+, IL-17A+ T cell sorting

Single splenic lymphocytes were isolated from C57BL/6 mice as previously described [39, 42, 49]. CD4+, CD4+CD25+, IL-17A+, and CD4+CD25- T cells were sorted by using the Stemcell magnetic sorter (cat: 18000), the human/mouse CD4 T cell negative selection kit (cat: 19852), the human/mouse Treg positive selection kit (cat: 18782), following the manufacturer's instructions. All these instruments and kits were from Stemcell Technologies Inc, Shanghai, China.

Flow cytometry staining

Flow cytometry analysis of CD4+, CD4+CD25+, CD4+IL-17A+ and CD4+CD25+FoxP3+ T cells were performed according to the Intracellular Cytokine Staining protocol or the Cell Surface Immunofluorescence Staining Protocol described in the T Cell Staining Kit (Biolegend, San Diego, CA, USA). All flow antibodies and relative reagents were from Biolegend: FITC-anti-human/mouse CD4 (cat: 100406), PE/Cy5-anti-human/mouse CD25 (cat: 102010), PE-anti-mouse/rat/human FoxP3 (cat: 320008), and APC-anti-human/mouse IL-17A (cat: 506916). For intracellular staining, single T cells were stimulated for 6 hours with Cell Activation Cocktail (with Brefeldin A) (2 μl/ml; cat: 423304, Biolegend, San Diego, USA). The stained cells were counted using the BD FACSCanto II. The data obtained were analyzed using Flow Jo 7.6 software (Tree Star, Inc., Oregon, USA). Gating strategies were performed following the manufacturer's instructions (BD).

Cell proliferation assays

We detected the proliferation of Th17, CD4+CD25- T cells (effector cells) using CFSE labeling as previously described [50]. Freshly purified T cells were re-suspended in phosphate buffer saline (0.1% BSA) at 2 x 10^6 cells/ml and incubated with CFSE (1 μl/ml; Abcam, Cambridge, UK) for 15 min at 37˚C. These cells were then washed and re-suspended in 1640 Medium for 10 min to stabilize the CFSE staining. Cells were re-suspended in the culture medium after a final wash step.

Viability and functional assays of islet cell clumps

The glucose-stimulated insulin secretion (GSIS) assay was employed to detect the function of co-cultured islets as previously described [51]. Krebs–Ringer bicarbonate was used as the base media. The basal glucose level used was 2.5 mM, following which a glucose level of 16.7 mM was used to stimulate the islet cells. The insulin concentration of supernatant was analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Alpha Diagnostic Intl. Inc., USA) following the product manual. The insulin staining was performed as previously described [52].

In vitro IL-35 and Treg treatment

For *in vitro* experiments, the cells were categorized into three groups: IL-35 group, Treg group, and control group. In the IL-35 group, approximately 50 islets and 2 x 10^5 CD4+ T cells were co-cultured in 24-well plates. IL-35 (20 ng/ml) (cat: RPC008Mu, CLOUD-CLONE CORP., USA) was added to the plate and replenished every time the culture medium was changed (every other day). In the Treg group, we regulated the ratio of Treg and Th17 cells as 0:1, 0.5:1, 1:1, and 2:1 by increasing the amount of Tregs and co-cultured these T cells (totally 2 x 10^5 cells) and 50 islets in 24-well plates, separately. IL-35 (20 ng/ml) was added to each replicate of these plates. In the control group, we only co-cultured 50 islets and 2 x 10^5 CD4+ T cells without any treatment. All T cells were stimulated with plate-bound anti-CD28 (2 μg/ml) and anti-CD3 (5 μg/ml) as previously described [42]. IL-2 (500 U/ml) were added to each plate for cell growth and replenished every time the culture medium was changed (every other day). The culture medium used was RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich), streptomycin (0.1 mg/ml; Sigma-Aldrich), L-glutamine and benzylpenicillin (100 U/ml, Roche Diagnostics Scandinavia, Bromma, Sweden). The culture medium was changed every second day. The function and survival state of islet cells were analyzed and the amount of Treg and Th17 in each group were quantified on days 1, 3 and 5 after co-culture. In the plates of the Treg group, we labeled the Th17 cells with CFSE before co-culture, and then detected the proliferation of these cells on day 5 after co-culture. Every experiment described above was repeated at least three times.

Statistical analysis

Statistical analysis was performed using the GraphPad Software 6.0 (CA, USA). Comparisons between two groups were performed using unpaired t-tests. Mann-Whitney Rank Sum Tests were used for nonparametric observations. A p-value below 0.05 was considered statistically significant.

Results

IL-35 down-regulated the Th17/Treg ratio in the co-culture system

To determine the role of IL-35 in the CD4+ T cell subset, we purified CD4+CD25+, IL-17A+, and CD4+CD25- T cells from the spleen of C57BL/6 mice, and cultured these cells *in vitro* with plate-bound anti-CD3/CD28 antibodies and appropriate amount of IL-2. Results showed that in comparison with the control group, IL-35 markedly enhanced the proliferation of Tregs (50.7% in IL-35 group vs. 9.5% in control group on day 5, $P<0.01$) under these conditions with time (Fig 1). In contrast, although the absolute counts of Th17 showed only a slight increase, their ratio in CD4+ T cells was significantly decreased in the IL-35 group compared to that in the control group (0.3% vs. 7.2% on day 5, $P<0.01$) (Fig 2). Thus, a remarkable difference in the ratio of Th17/Treg in CD4+ T cells was observed under these conditions, particularly on day 5 after co-culture (1.4% in control group vs. 0.1% in IL-35 group, $P<0.01$) (Fig 3). Taken together, IL-35 down-regulated the Th17/Treg ratio in the co-culture system.

IL-35 ameliorated the function of islet cells

We next analyzed the function of islet cells under these conditions. Results showed that at a low glucose level (2.5 mM), insulin release of islet cells in the IL-35 group was markedly higher than that in the control group on day 5 (5.3 ng/15 islets*h in IL-35 group vs. 0.7 ng/15 islets*h in control group, $P<0.01$) (Fig 4a); similar results were observed at a high glucose level

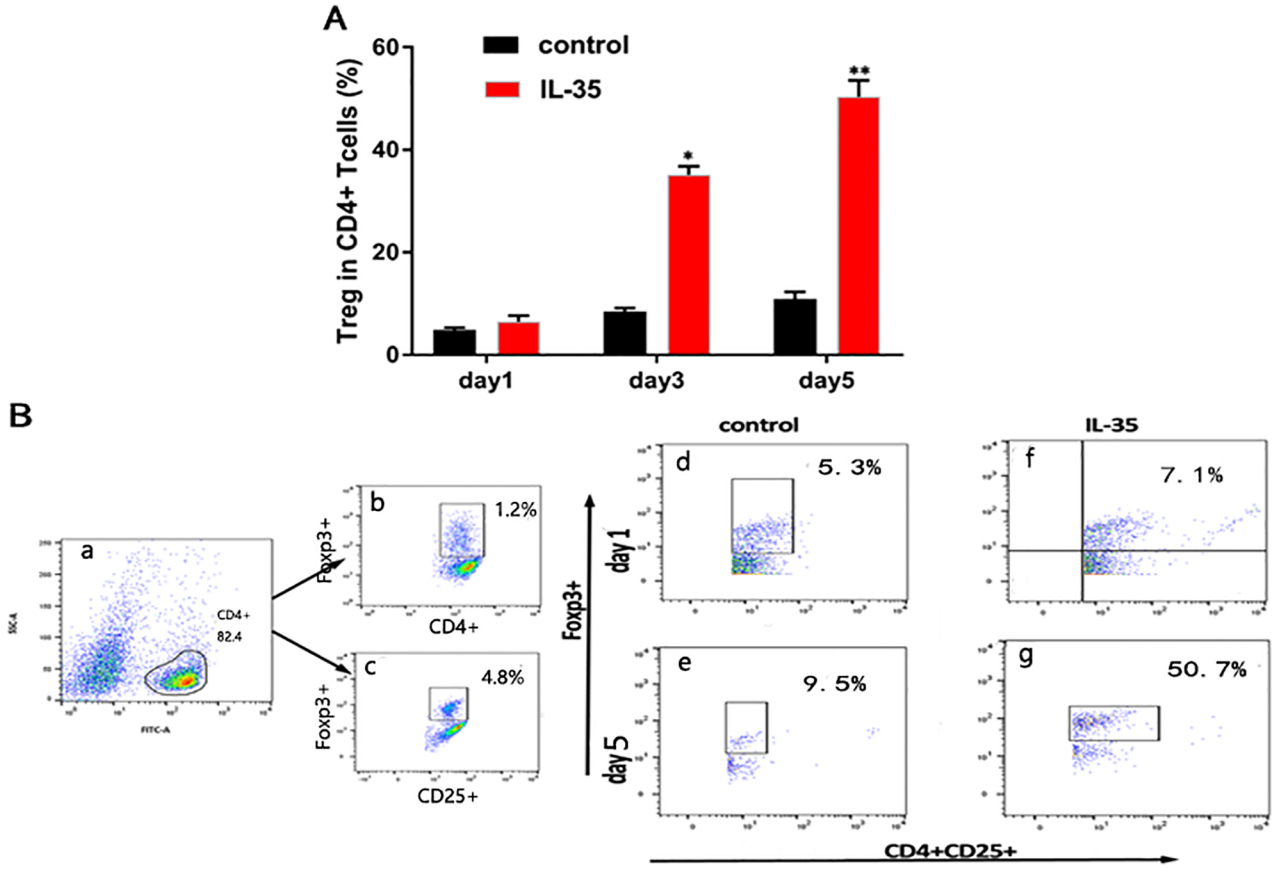

Fig 1. IL-35 increased the number of Treg in CD4+ T cells. (A)The ratio of Treg in CD4+ T cells. (B) Representative diagram showing in comparison with the control group, IL-35 markedly enhanced the proliferation of Tregs (50.7% in IL-35 group vs. 9.5% in control group on day 5, P<0.01) under these conditions with time. Results are expressed as means ± SEM, from two experiments (n = 3 times/group/experiment). Unpaired t-tests were performed for comparisons between control- and IL-35- groups on corresponding days. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

https://doi.org/10.1371/journal.pone.0189617.g001

(16.7 mM) on day 5 (46.2 ng/15 islets*h in IL-35 group vs. 9.8 ng/15 islets*h in control group, P<0.01) (Fig 4b). Results of acridine orange (AO)/ethidium bromide (EB) staining also showed that IL-35 delayed the survival of co-cultured islet cells (Fig 5). Altogether, IL-35 treatment evidently ameliorated the insulin secretory function of islet cells.

Increasing Treg ratio in CD4+ T cells ameliorated the function of islet cells

Next, we decided to determine whether the improved function of islet cells was affected by the IL-35-mediated regulation of Th17/Treg ratio. For this, we modulated the Th17/Treg ratio (1:0, 1:0.5, 1:1, 1:2) in the co-culture system. We found that, on day 5, with an increase of Treg ratio, the proliferation of the CFSE-labeled Th17 cells was suppressed markedly (suppression ratio rising from 7.82% in 1:0 group to 48.2% in 1:2 group); besides, stronger suppression was observed when IL-35 was added, compared to that in the control group (Fig 6). Additionally, we analyzed the function of islet cells in Th17:Treg = 1:0 group and Th17:Treg = 1:2 group and found that regardless of the glucose level, islet cells in the Th17/Treg = 1:2 group had better insulin secretory function and survival rate than that in the Th17/Treg = 1:0 group (Fig 7).

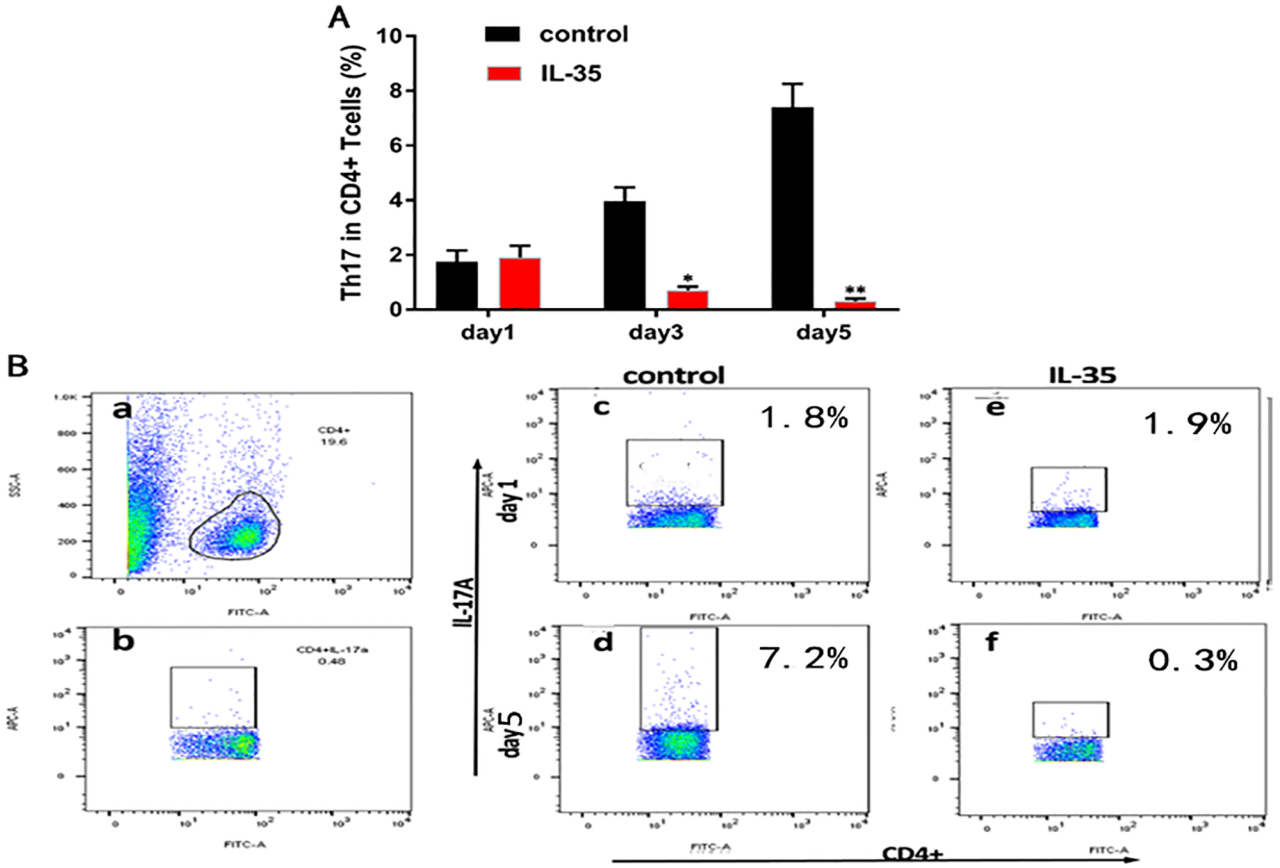

Fig 2. IL-35 decreased the prevalence of Th17 in CD4+ T cells. (A)The ratio of Th17 in CD4+ T cells. (B) Representative diagram showing Th17's ratio in CD4+ T cells was significantly decreased in the IL-35 group compared to that in the control group (0.3% vs. 7.2% on day 5, P<0.01). Results are expressed as means ± SEM, from two experiments (n = 3 times/group/experiment). Unpaired t-tests were performed for comparisons between control- and IL-35- groups on corresponding days. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

https://doi.org/10.1371/journal.pone.0189617.g002

Discussion

Despite decades of investigation, mitigation of transplant immune rejection with less severe complications remains a challenge. Traditional clinical anti-rejection drugs, such as cyclosporin A and tacrolimus, comprehensively inhibit T cell activity by mainly binding to calcineurin of the cells and suppressing IL-2 release, which leads to numerous severe adverse effects [53]. Thus, it is important to find new drugs that can be specifically directed against specific T sub-populations on anti-transplanted rejection and result in less adverse effects [3].

Recently, CD4+ T cell sub-populations, Treg and Th17 cells, have drawn increased attention, and emerging evidence shows that the novel cytokine IL-35 and regulation of the ratio of these cell types play an important role in the development of autoimmunity and immune tolerance [11, 14, 45, 54–57]. Therefore, in the present study, we aimed to explore whether this mechanism exists in the development of mouse islet transplant rejection.

We found that the cell counts of Th17 and Treg cells were increased in the co-culture model and an increasing percentage of Tregs could inhibit the proliferation of Th17 cells. Similar results have been found in other studies, such as those for acute lung injury [31], *M. neoaurum* infection [33] and inflammation [58]. On one hand, the proliferation and growth of

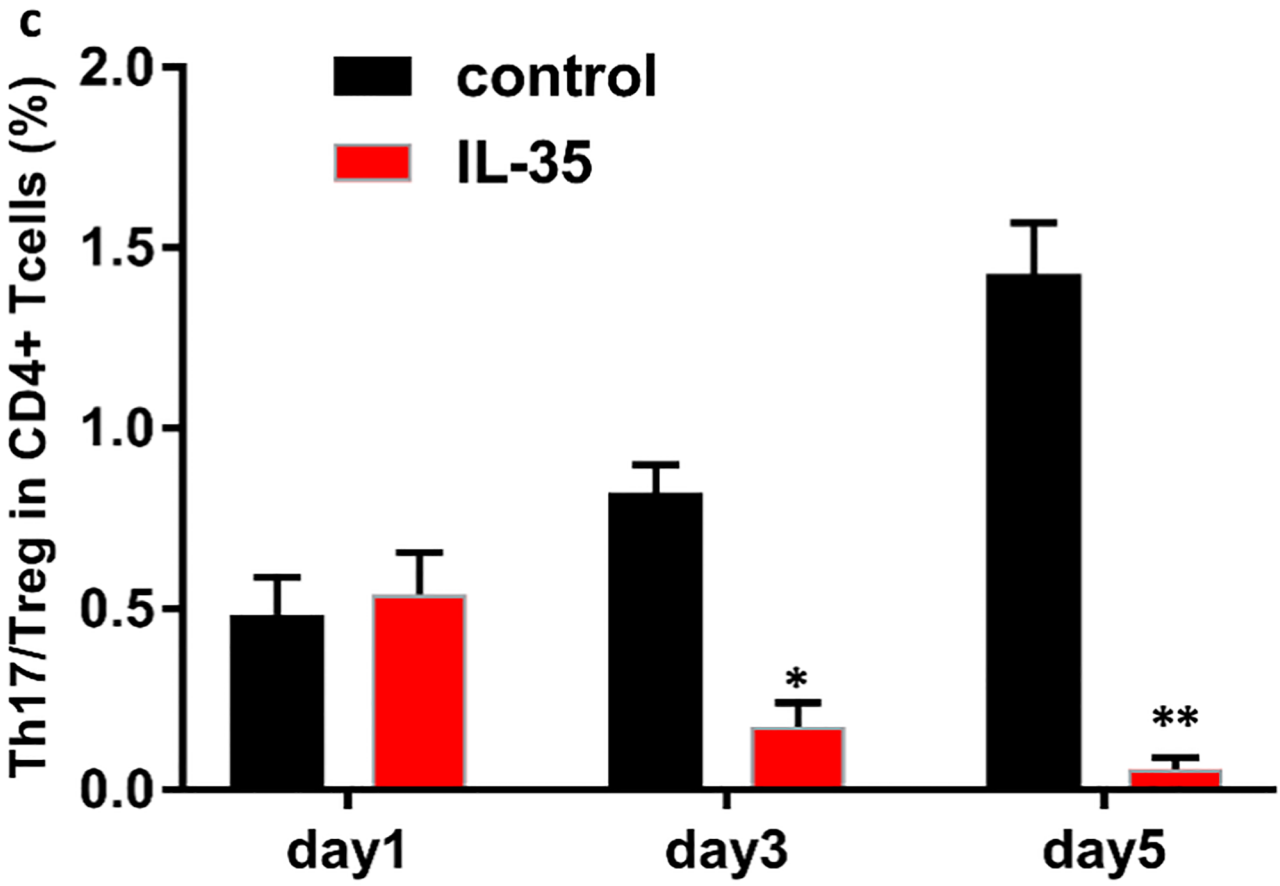

Fig 3. IL-35 down-regulated the ratio of Th17/Treg in CD4+ T cells. The ratio of Th17/Treg in CD4+ T cells in IL-35 group or control group on day 1, 3, 5 after co-culturing. A remarkable difference in the ratio of Th17/Treg in CD4+ T cells was observed under these conditions, particularly on day 5 after co-culture (1.4% in control group vs. 0.1% in IL-35 group, P<0.01). Results are expressed as means ± SEM, from two experiments (n = 3 times/group/experiment). Unpaired t-tests were performed for comparisons between control- and IL-35- groups on corresponding days. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

https://doi.org/10.1371/journal.pone.0189617.g003

Tregs consume limiting T-cell growth factors such as IL-2 and release inhibitory cytokines such as IL-10, IL-35, and TGF-β on other immune cells, including Th17. On the other hand, differentiation of naïve CD4+ T cells towards each subset depends on the local cytokine environment. TGF-β is essential for the development of Treg and Th17 and IL-2 inhibits the polarization of Th17 cells. Co-availability of both TGF-β and IL-6 leads to the differentiation of naïve CD4+ T cells towards Th17; only TGF-β overdose (derived from added Tregs) favors the differentiation of Th17 into Tregs (Fig 8), thus decreasing the ratio of Th17 in CD4+ T cells. Furthermore, we found that a decrease in Th17/Treg ratio improved the function of transplanted islets, which was consistent with previous reports. Wu et al. [4] found that *ex vivo* expanded human Tregs in a humanized mouse model could improve the survival status of an islet allograft. A study from Canada showed that Treg cells could be recruited to transplanted islets, to suppress the activation of effector T-cells, and furthermore to induce alloantigen-specific tolerance [59]. Some results from an international co-operation group (named the ONE study) supported that Treg therapy can prevent immunological rejection of transplanted organs without the need for long-term use of pharmacological immunosuppression agents [60]. However, a study from Korea showed that in the peri-transplantation period, autologous Tregs infusion failed to induce transplanted immune tolerance in islet xenotransplantation

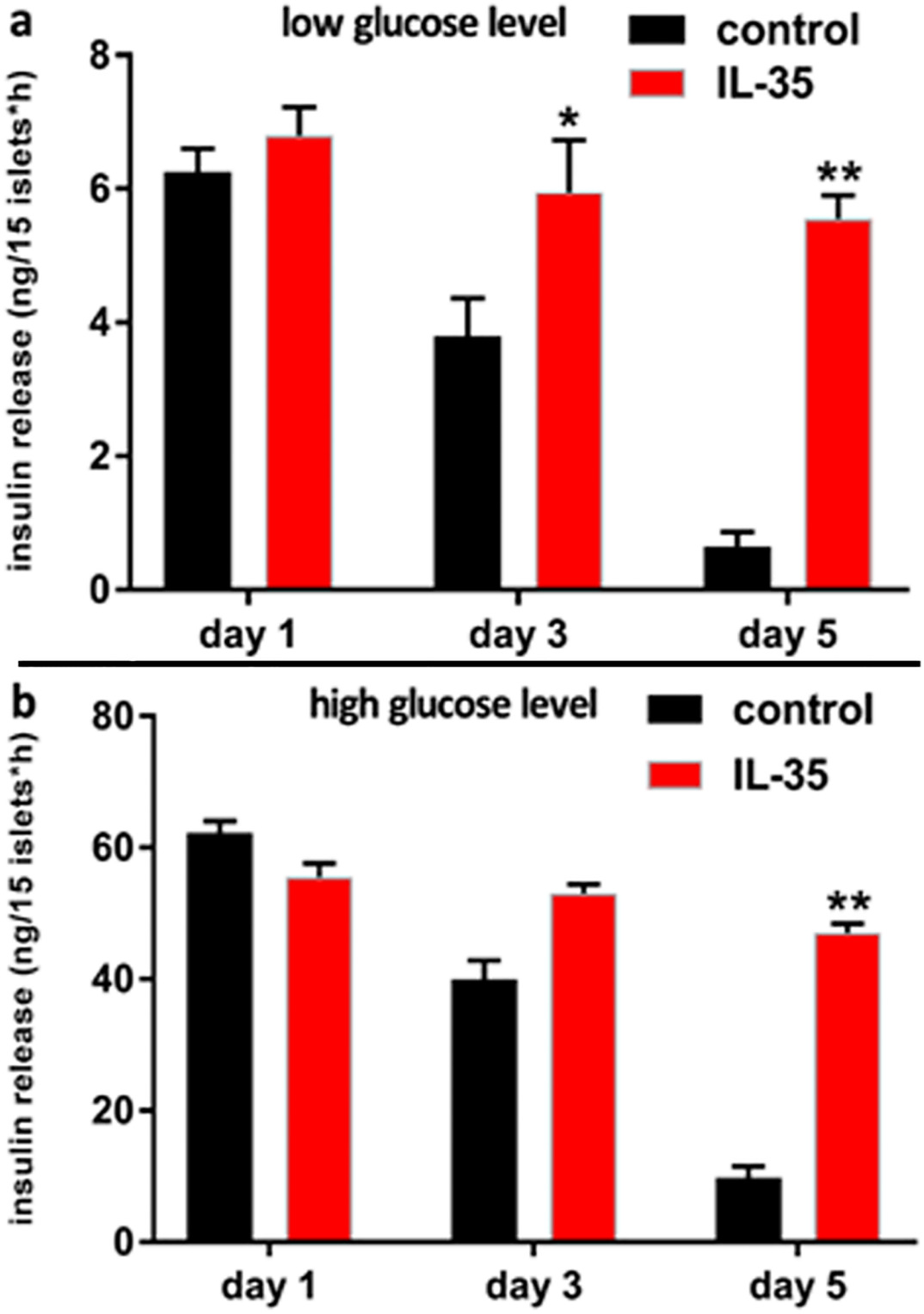

Fig 4. IL-35 ameliorated the function of islets in co-culture system. The amount of insulin release of 15 co-cultured islets in low glucose level (a) and in high glucose level (b). Results are expressed as means ± SEM, from two experiments (n = 3 times/group/experiment). Unpaired t-tests were performed for comparisons between control- and IL-35- groups on corresponding days. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

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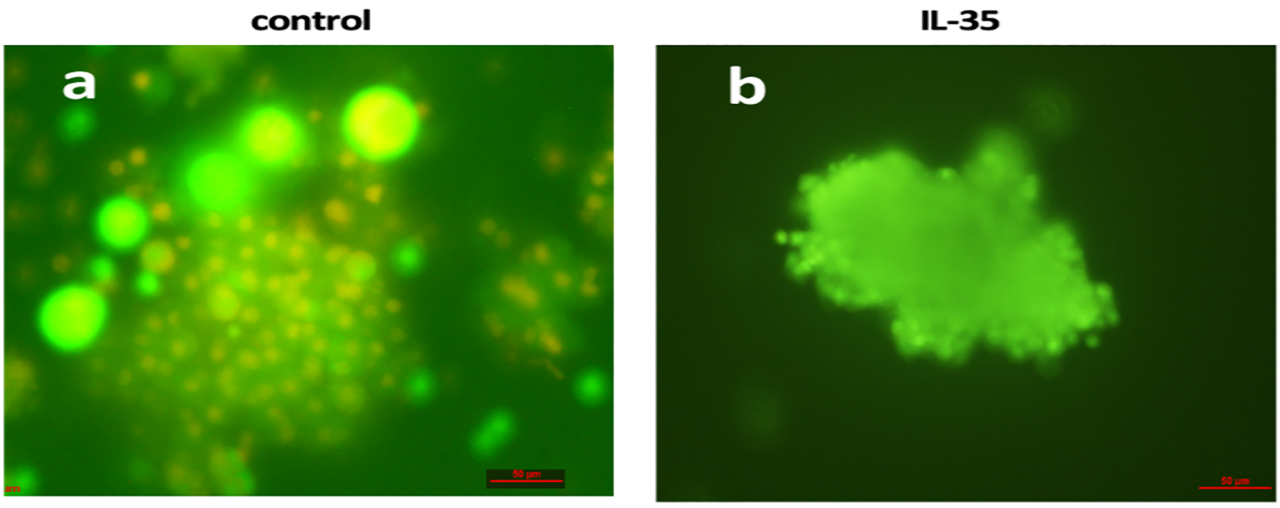

Fig 5. IL-35 delayed the survival of co-cultured islets. The islet cells were stained using AO/EB staining kit. The dead islet cells were labeled with yellow fluorescence, while the living islet cells were labeled with green fluorescence. In control group, most of islet cells were dead (a); however, the islet cells in IL-35 group were still living (b).

https://doi.org/10.1371/journal.pone.0189617.g005

settings (pig to non-human primates) [9]. The study suggested that there might be certain limitations on Tregs in inducing islet-transplanted tolerance and further study is needed in this context.

Moreover, we found that IL-35 *in vitro* treatment could down-regulate the ratio of Th17/Treg and prevent islet allograft failure. The same trend could be found in stem cell transplantation

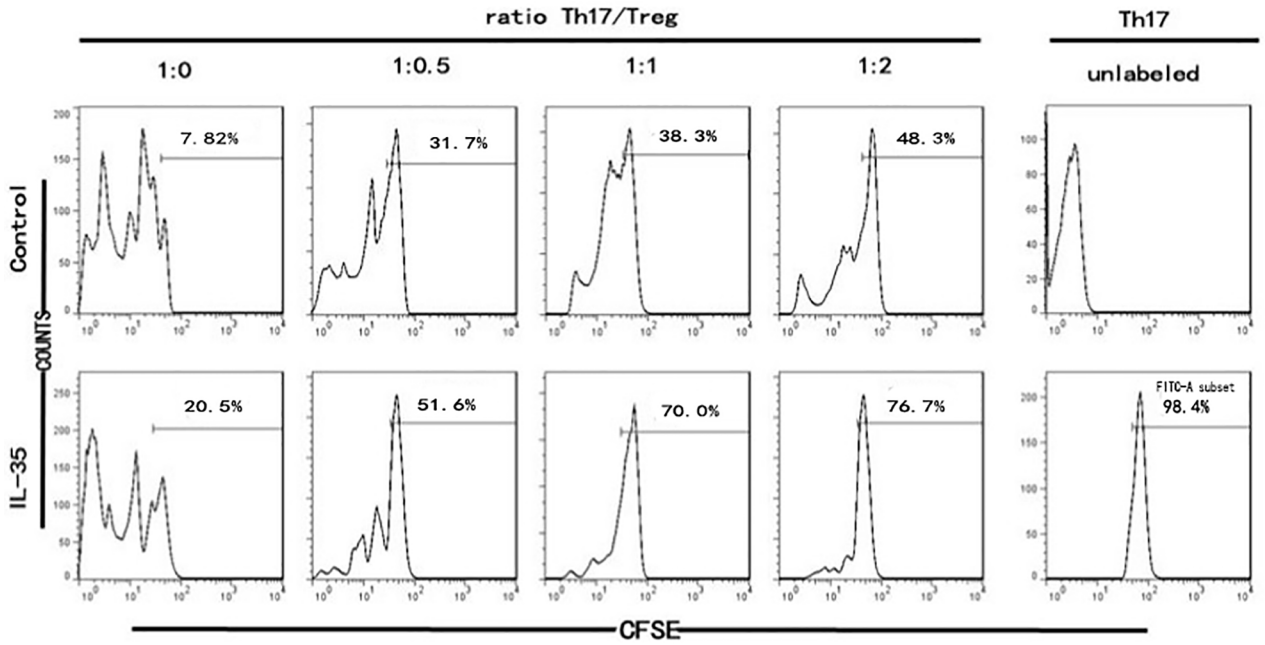

Fig 6. Treg inhibited the proliferation of Th17 and IL-35 enhanced the suppressive function of Treg. Representative histograms showing different Th17/Treg ratio (1:0, 1:0.5, 1:1, 1:2) in the co-culture system. With an increase of Treg ratio, on day 5, the proliferation of the CFSE-labeled Th17 cells was suppressed markedly (suppression ratio rising from 7.82% in 1:0 group to 48.2% in 1:2 group); besides, stronger suppression was observed when IL-35 was added, compared to that in the control group (suppression ratio rising from 20.5% in 1:0 group to 76.7% in 1:2 group).

https://doi.org/10.1371/journal.pone.0189617.g006

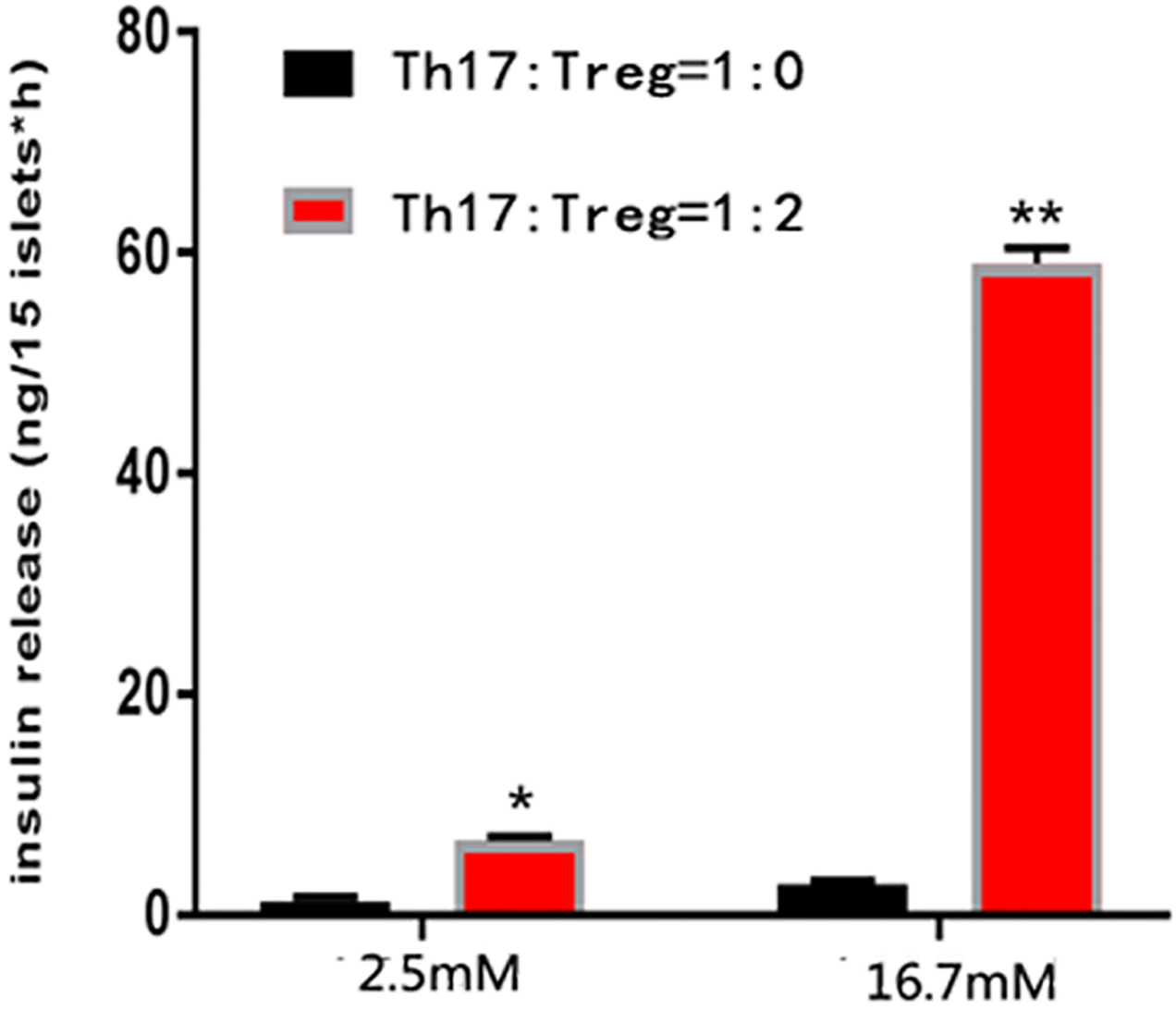

Fig 7. Up-regulating the prevalence of Treg ameliorated the function of islets. The function of co-cultured islet cells in Th17:Treg = 1:0 group and Th17:Treg = 1:2 group in low glucose level (2.5mM) or high glucose level (16.7mM). The results showed that regardless of the glucose level, islet cells in the Th17/Treg = 1:2 group had better insulin secretory function than that in the Th17/Treg = 1:0 group. Results are expressed as means ± SEM, from two experiments (n = 3 times/group/experiment). Unpaired t-tests were performed for comparisons between control- and IL-35- groups on corresponding days. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

https://doi.org/10.1371/journal.pone.0189617.g007

[61], T1D [41, 43] and acute myeloid leukemia blasts [62]. However, the role of IL-35 in solid organ transplantation is poorly understood. Studies have demonstrated the role of IL-35 in conversion of human and murine CD4+CD25- T cells into IL-35-induced Treg (iTr35) cells [63–65]. Ma et al. showed that rhIL-35 could induce the expression of EBI3 and P35 in CD4+CD25- T cells (including Th17 cells) and relative Tregs were capable of inducing a further increase in IL-35 levels [63]. IL-35 treatment activated the phosphorylation of STAT1 and STAT3 in CD4+ T cells, which then allowed the differentiation of naïve CD4+ T cells into Tregs. Additionally, exogenous IL-35 also favored the conversion of Tregs into iTr35 cells. Interestingly, the induced iTr35 cells still possessed the ability to release IL-35 and induce a positive feedback to promote CD4+CD25- T cell differentiation into Tregs and IL-35 production [37–39, 42, 45, 66]. All these processes are involved in islet transplant rejection (Fig 7). Furthermore, as demonstrated by

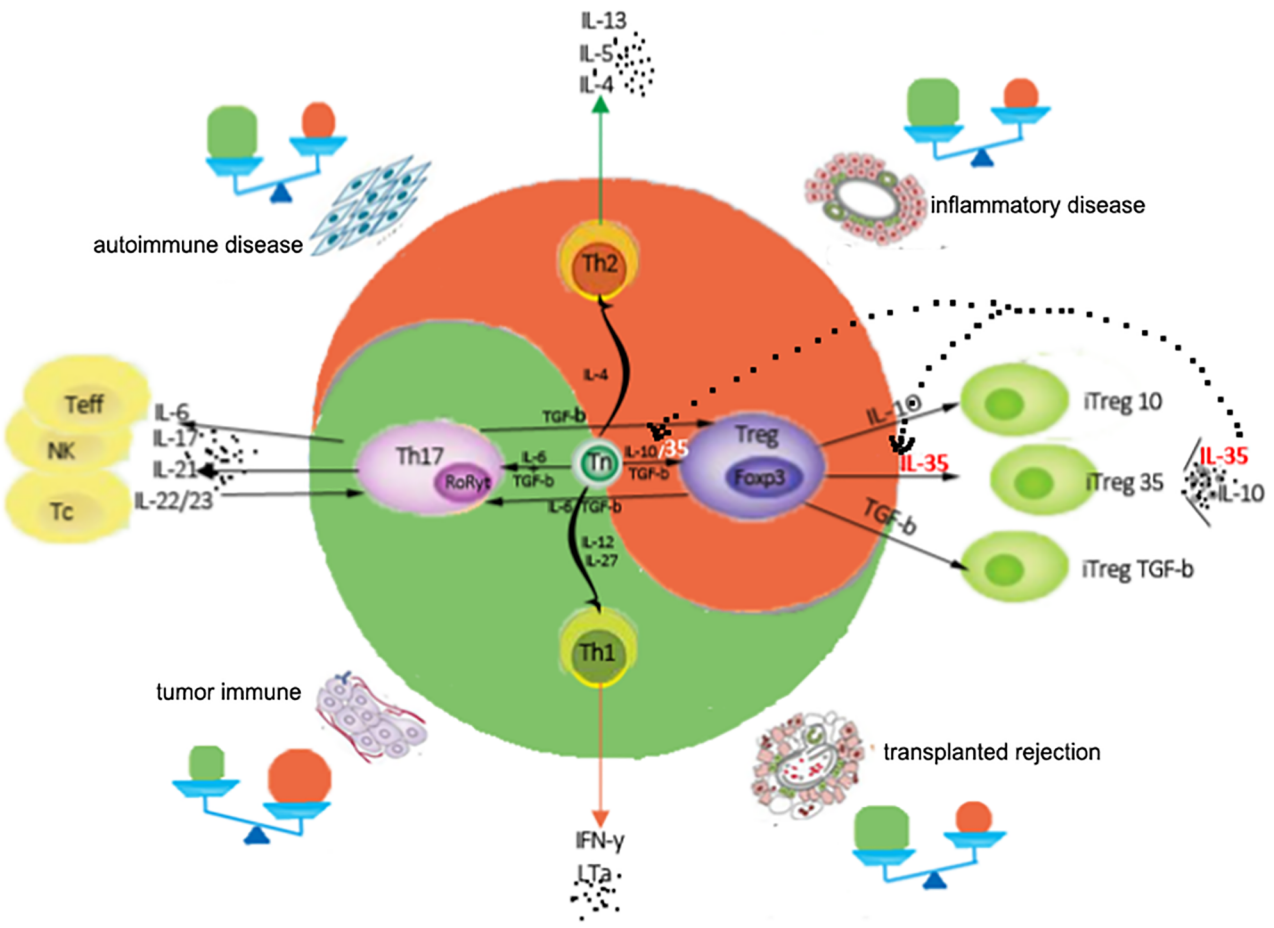

Fig 8. Model of IL-35 regulating Th17/Treg differentiation.

https://doi.org/10.1371/journal.pone.0189617.g008

Kailash et al. [41], IL-35 administration could counteract established T1D. Although the exact mechanism is still unknown, several mechanisms might be involved. According to Kailash et al., Tregs might play a role, thus preventing the autoimmune destruction of β cells of patients. Moreover, it is suggested that IL-35 might promote the differentiation of other pancreatic cells (e.g. α cells) into β-cells through the GABA pathway [67, 68] or other signaling pathways *in vivo*. Taken together, IL-35 might not only improve islet transplanted rejection, but also can help reverse the destruction of β cells of T1D patients themselves.

Conclusions

IL-35 mitigates the function of murine transplanted islet cells via regulation of the Th17/Treg ratio. This might serve as a potential and promising therapeutic strategy for islet transplant rejection and T1D, thus raising the need for conducting more in-vivo studies in this context.

Supporting information

S1 Table. NC3Rs ARRIVE guidelines checklist (fillable).
(PDF)

Acknowledgments

We would like to thank all members in our lab for their help with the performing of assay and the drafting of the manuscript.

Author Contributions

Conceptualization: Yin Zongyi, Li Baifeng.

Data curation: Yin Zongyi.

Formal analysis: Yin Zongyi.

Funding acquisition: Li Baifeng.

Investigation: Yin Zongyi, Zou Funian, Wang Xin.

Methodology: Yin Zongyi.

Project administration: Li Baifeng.

Resources: Yin Zongyi, Cheng Ying, Zhang Jialin, Liu Yongfeng, Li Baifeng.

Software: Yin Zongyi, Li Hao.

Supervision: Cheng Ying, Zhang Jialin, Liu Yongfeng, Li Baifeng.

Validation: Yin Zongyi, Li Baifeng.

Visualization: Yin Zongyi.

Writing – original draft: Yin Zongyi.

Writing – review & editing: Yin Zongyi, Li Baifeng.

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