IMMUNOLOGY

Combinations of anti-GITR antibody and CD28 superagonist induce permanent allograft acceptance by generating type 1 regulatory T cells

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Type 1 regulatory T (Tr1) cells represent a subset of IL-10–producing CD4+Foxp3− T cells and play key roles in promoting transplant tolerance. However, no effective pharmacological approaches have been able to induce Tr1 cells in vivo. We herein report the combined use of a CD28 superagonist (D665) and anti-glucocorticoid-induced tumor necrosis factor receptor–related protein monoclonal antibody (G3c) to induce Tr1 cells in vivo. Large amounts of IL-10/interferon-γ–co-producing CD4+Foxp3− Tr1 cells were generated by D665-G3c sequential treatment in mice. Mechanistic studies suggested that D665-G3c induced Tr1 cells via transcription factors Prdm1 and Maf. G3c contributed to Tr1 cell generation via the activation of mitogen-activated protein kinase–signal transducer and activator of transcription 3 signaling. Tr1 cells suppressed dendritic cell maturation and T cell responses and mediated permanent allograft acceptance in fully major histocompatibility complex–mismatched mice in an IL-10–dependent manner. In vivo Tr1 cell induction is a promising strategy for achieving transplant tolerance.

INTRODUCTION

Organ transplantation is considered the optimal treatment for a variety of end-stage organ diseases. However, the life-long, systemic immune suppression required after transplantation severely compromises host immune defense and is associated with adverse effects, including organ toxicity, infections, and malignancies. Operational tolerance has long been the ultimate goal in the field of transplantation, which would enable transplant recipients to maintain a stable and acceptable graft function without the need for immunosuppression therapy, thus avoiding undesirable side effects (1).

Allograft immunity is a complex process that results from the interplay of multiple different cell types, including lymphocytes, monocytes, macrophages, and dendritic cells (DCs). Recipient alloreactive T cells recognize non–self-donor alloantigens presented by donor or recipient antigen-presenting cells and initiate the adaptive inflammatory immune response, leading to allograft rejection. Regulatory immune cells regulate or suppress immune responses of other cells and help prevent anti-donor immune responses. Regulatory immune cell–based therapies, via inducing or adaptively transferring regulatory immune cells, are emerging as promising strategies for achieving permanent donor-specific immune tolerance, thus minimizing or even obviating immunosuppressants after organ transplantation.

CD4+ T cells coordinate immune responses by helping to activate and regulate other immune cells and are critical in determining transplantation rejection or tolerance. Type 1 regulatory T (Tr1) cells represent a subset of CD4+Foxp3− T cells and secrete high amounts of interleukin-10 (IL-10), their signature cytokine, with potent immunosuppressive properties. The potent immunosuppressive function of Tr1 cells has been implicated both in vitro and in vivo (2). Tr1 cells have been proven to play key regulatory roles in peripheral immune tolerance and are considered an emerging therapeutic target for improving transplant tolerance (3). However, as with Foxp3+ regulatory T (Treg) cells, in vivo effective pharmacological approaches to induce Tr1 cells are largely lacking.

CD28 superagonist is a monoclonal antibody (mAb) that engages CD28 costimulatory receptors, which laterally bind to the CD28 homodimer, thus allowing its clustering via lattice formation (4, 5). The particular binding topology of CD28 superagonist confers its superagonist properties, resulting in potent T cell activation and expansion independent of concomitant T cell receptor (TCR) engagement (6). CD28 superagonist was shown to expand Treg cells preferentially over effector T (Teff) cells both in vitro and in vivo, a characteristic that was used to prevent autoimmune disease, graft-versus-host disease (GVHD), and allograft rejection (6–8). Glucocorticoid-induced tumor necrosis factor receptor–related protein (GITR), also referred to as TNFRSF18, is a type I transmembrane protein of the tumor necrosis factor receptor (TNFR) superfamily, characterized by three cysteine-rich domain pseudorepeats in its extracellular domain (9). GITR is constitutively expressed at high levels on Treg cells and at low levels on naive and memory T cells (9–11). Its expression is rapidly up-regulated upon TCR activation on both Treg and Teff cells (11, 12). GITR serves as a costimulatory molecule that exerts multiple distinct effects on T cells. GITR engagement results in the proliferation and cytokine production of activated T cells but abrogates the suppressive activity of Treg cells (10, 11, 13, 14). Targeting GITR has been evaluated for its utility in the treatment of cancers, autoimmune diseases, and organ rejection in both animal models and clinical trials (15–19). However, the effects of GITR agonism on T cells, which is cell specific and context dependent, remain controversial (20, 21).

We herein report the combined use of CD28 superagonist D665 and anti-GITR mAb G3c to induce Tr1 cells in vivo. Large amounts of IL-10/interferon-γ (IFN-γ)–co-producing CD4+Foxp3− Tr1 cells were generated by D665-G3c sequential treatment in mice. Tr1 cells suppressed DC maturation and T cell responses in an IL-10–dependent
manner. Further mechanical studies suggested that D665-G3c induced Tr1 cells via the positive regulatory domain zinc finger protein 1 (Prdm1), and musculoaponeurotic fibrosarcoma (Maf) pathways. G3c contributed to Tr1 cell generation via the activation of mitogen-activated protein kinase–signal transducer and activator of transcription 3 (MAPK-STAT3) signaling. The combined use of D665 and G3c induced permanent allograft acceptance in a Tr1-dependent manner. Our study presented an effective pharmacological approach to generate Tr1 cells in vivo by D665-G3c sequential treatment and demonstrated their therapeutic potential in transplantation.

RESULTS

Induction of Tr1 cells in vivo
Foxp3-GFP mice [expressing green fluorescent protein (GFP) under the control of the mouse Foxp3 promoter] and IL-10–Venus mice [expressing yellow fluorescent protein (Venus) under the control of the mouse IL-10 promoter] were treated with D665 (250 µg per mouse) and G3c (250 µg per mouse) on days −3 and 0 sequentially (Fig. 1A). On day 0, before G3c administration, D665 induced a robust expansion of Treg cells over Teff cells (Fig. 1B), consistent with previous reports (22–25). The GITR expression was significantly up-regulated on the surface of both Treg and Teff cells (Fig. 1, C and D).

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When targeting GITR with G3c, large amounts of CD4⁺Foxp3⁺ IL-10⁺ Tr1 cells were generated on days 3 and 7 (Fig. 1, E and F). Although Treg cells were also potently expanded, they were obviously decreased on day 7 in the combination treatment group (Fig. 1, E and G). On day 7, most of the CD4⁺ IL-10⁺ T cells were IL-10/IFN-γ−co-producing CD4⁺Foxp3⁻ Tr1 cells (Fig. 1E and fig. S1A). The gene expression of IL-10 and Ifng exhibited a consistent trend with flow cytometry (FCM) results (fig. S1B). The numbers of splenocytes obtained in each group are shown in fig. S1C.

We further analyzed the phenotype of Treg and Tr1 cells. Treg cells showed a central memory phenotype and strongly expressed the activation markers CD25 and CD69, co-inhibitory markers programmed cell death protein 1 (PD-1), inducible costimulatory molecule (ICOS), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell protein cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG3), and T cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT). The detected GITR expression was slightly lower compared to that in naïve CD4⁺ T cells because of blockade of FCM antibody binding to GITR by G3c (Fig. 2A and fig. S2A). Tr1 cells comprised both central and effector memory phenotypes and strongly expressed co-inhibitory markers PD-1, ICOS, CTLA-4, and LAG3 but weakly expressed activation markers CD25 and CD69 (Fig. 2B and fig. S2B). The coexpression of CD49b and LAG3, identified as biomarkers for a population of murine and human memory Tr1 cells, was not observed (26).

**Function of induced Tr1 cells in vitro**

Tr1 cells are characterized by the production of high amounts of IL-10, which has a potent immunosuppressive function. IL-10 exerts its major suppressive effects on the DC maturation and accessory functions (27, 28). To determine the suppressive function of Tr1 cells on DCs, we isolated CD4⁺IL-10⁺ T cell, CD4⁺IL-10⁻ T cells, and naïve CD4⁺ T cells and cocultured them with bone marrow–derived DCs (BMDCs) stimulated with lipopolysaccharide (LPS) on day 5. CD11b⁺CD11c⁺ population cells were identified as BMDCs to assess maturation state on day 7 (fig. S3). Tr1 cells prevented LPS-mediated BMDC maturation, as measured by the decreased median fluorescent intensity of surface CD40, CD80, CD86, and major histocompatibility complex class II (MHC-II) molecules (Fig. 3, A and B). Furthermore, the addition of anti–IL-10–neutralizing antibody diminished the Tr1 cell–mediated prevention of BMDC maturation.

IL-10 also exerts inhibitory effects by suppressing T cell proliferation (29, 30). Cytotoxic CD8⁺ T cells are the principal driving force of allograft destruction and regulated by helper CD4⁺ T cells. We therefore next investigated the suppressive effects of Tr1 cells on CD8⁺ T cells using a one-way mixed lymphocyte reaction (MLR) system, where purified B6/J mice CD8⁺ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as responders and cocultured with BALB/c BMDC stimulators. FCM-sorted CD4⁺IL-10⁺ T cells, CD4⁺IL-10⁻ T cells, and naïve CD4⁺ T cells were added to the MLR systems as regulators and cocultured for 3 days. As shown in Fig. 3 (C and D), CD8⁺ T cell proliferation was significantly suppressed in the presence of CD4⁺IL-10⁺ Tr1 cells, whereas the addition of an anti–IL-10 antibody restored the CD8⁺ T cell proliferation. These data indicated that Tr1 cells mediated the suppressive function mainly dependent on IL-10 signaling.

**Mechanism underlying Tr1 cell generation**

To investigate the mechanism underlying the generation of Tr1 cells by D665-G3c sequential treatment, we isolated CD4⁺IL-10⁺...
Tr1 cells and CD4^+IL-10^- T cells from D665-G3c-treated IL-10–Venus mice on day 7 and conducted a transcriptome RNA sequencing (RNA-seq) analysis (Fig. 4A). The gene expression of IL-10 and Ifng in both cell populations was confirmed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 4B). Following the RNA-seq analysis, a magnitude component (principal component #1) separated samples by cell type, explaining 83% of the total variation (fig. S4). Compared to CD4^+IL-10^- T cells, 1963
Fig. 4. Results of an RNA-seq analysis of CD4^{+}IL-10^{+} and CD4^{+}IL-10^{−} T cells. (A) Total CD4^{+} T cells were purified by magnetic-activated cell sorting (MACS) from the splenocytes of D665-G3c–treated IL-10–Venus mice on day 7 and then subjected to CD4^{+}IL-10^{+} and CD4^{+}IL-10^{−} T cell sorting based on the Venus expression. The isolated CD4^{+}IL-10^{+} and CD4^{+}IL-10^{−} T cell samples were prepared in three duplicates for the subsequent transcriptome RNA-seq analysis. (B) The relative mRNA expression of IL-10 and Ifng in CD4^{+}IL-10^{+} and CD4^{+}IL-10^{−} T cells, detected by qRT-PCR, normalized with 18S for each sample (n = 4 for each group). Paired Student’s t test; *P < 0.05 and **P < 0.01. (C) The volcano plot shows the DEGs, with a threshold of absolute log2FC of >0.5 and adjusted P value of <0.05, between CD4^{+}IL-10^{+} T cells versus CD4^{+}IL-10^{−} T cells. ns, not significant. (D) The heatmap shows 1963 DEGs, among which 525 were up-regulated and 1438 were down-regulated, between CD4^{+}IL-10^{+} cells versus CD4^{+}IL-10^{−} cells. (E) GO cluster plot displaying a circular dendrogram of the clustering of the DEGs with color-coded log2FC (inner ring) and the assigned functional terms (outer ring). (F) Heatmap of differentially expressed TFs known to regulate Tr1 differentiation. (G) The relative mRNA expression of differentially expressed TFs identified in (F), validated by qRT-PCR, and normalized with 18S for each sample (n = 4 for each group). Paired Student’s t test; *P < 0.05 and **P < 0.01. Values are shown as the mean ± SEM.
differentially expressed genes (DEGs) were obtained in CD4^+IL-10^+ Tr1 cells, with a threshold of absolute log2 fold change (log2FC) > 0.5 and adjusted P value of <0.05, including 525 genes that were up-regulated and 1438 that were down-regulated (Fig. 4, C and D). Consistent with the cell-sorting strategy, IL-10 was the most up-regulated gene compared to the CD4^+IL-10^- T cells (Fig. 4C).

We then performed a gene ontology (GO) enrichment analysis to gain insight into the molecular functions of the DEGs that were enriched in CD4^+IL-10^-Tr1 cells (Fig. 4E). The most enriched GO terms were those involving signaling receptor activity, molecular transducer activity, transmembrane signaling activity, cell adhesion, molecular binding, immune receptor activity, cytokine binding, G protein–coupled receptor activity, growth factor binding, cytokine receptor activity, and cytokine activity. To identify transcription factors (TFs) that might be responsible for IL-10 production, we extracted TFs known to regulate Tr1 differentiation from DEGs, in factors (TFs) that might be responsible for IL-10 production, we extracted TFs known to regulate Tr1 differentiation from DEGs, including interferon regulatory factor 4 (Irf4), early growth response 2 (Egr2), aryl hydrocarbon receptors (Ahr), T-box transcription factors (TFs) that might be responsible for IL-10 production, we extracted TFs known to regulate Tr1 differentiation from DEGs, including interferon regulatory factor 4 (Irf4), early growth response 2 (Egr2), aryl hydrocarbon receptors (Ahr), T-box transcription factor 21 (Tbx21), Eomesoderm (Eomes), Prdm1, and Maf. Of these, Prdm1, Maf, Eomes, and Tbx21 were up-regulated with IL-10, whereas Irf4, Egr2, and Ahr were down-regulated in CD4^+IL-10^- Tr1 cells (Fig. 4F). The gene expression of the TFs was further validated by qRT-PCR. Prdm1, Maf, Eomes, and Tbx21 were verified to be significantly up-regulated in CD4^+IL-10^- Tr1 cells (Fig. 4G).

To further explore the role of D665 and G3c in Tr1 cell generation, we examined the expression of TFs in different treatment groups on days 3 and 7 (Fig. 5A). Single-D665 treatment induced Prdm1 and Maf up-regulation, and the combination treatment further increased their expression. We also observed an elevated Prdm1 expression in the single-G3c treatment group compared to the naïve group on day 7. The Eomes expression was only up-regulated in the combination treatment group. Next, we detected the Blimp1 (encoded by the Prdm1 gene) and c-Maf (encoded by the Maf gene) protein expression by Western blotting (Fig. 5, B and C). The expression of both Blimp1 and c-Maf protein was significantly up-regulated in the combination treatment group. It has been shown that STAT3 plays a critical role in Tr1 cell generation and is a potent inducer of both Blimp1 and c-Maf expression during Tr1 cell differentiation (31–33). In our study, high STAT3 phosphorylation was observed in the single-G3c treatment group as well as the combination treatment group (Fig. 5, B and C), possibly hinting at the essential role of GITR signaling in Tr1 cell generation. Extracellular signal–related kinase (ERK) MAPK, as downstream signaling of GITR activation, have been shown to induce and maintain IL-10 production in Tr1 cells (34). Our results revealed that the phosphorylation of ERK was significantly increased in the single-G3c treatment group as well as the combination treatment group (Fig. 5, B and C). In addition, although IL-27 was highlighted as an important driver of Tr1 cell generation in previous studies (35, 36), we only observed a slightly increased expression of IL-27p28 in the combination treatment group on day 7 (Fig. 5D). The level of Epstein-Barr virus–induced gene 3 (Ebi3), a subunit of IL-27 heterodimer, was comparable among groups.

**Generation of Tr1 cells for permanent allograft acceptance**

Previous studies have shown that Tr1 cells play a critical role in promoting and maintaining tolerance (3). We therefore next determined whether or not Tr1 cells generated by the combination of D665 and G3c treatment could induce permanent allograft acceptance.

By using a fully MHC-mismatched (donor: BALB/c, H-2k^d; recipient: B6/J, H-2k^b) mouse model of heterotopic heart transplantation (Fig. 6A), we found that the use of D665 alone prolonged the survival of heart allograft whereas the single use of G3c showed a minimal effect on the heart allograft survival. Unexpectedly, combinations of D665 and G3c treatments induced permanent allograft acceptance (Fig. 6B). A histological analysis of the heart allograft on postoperative day 7 (POD7) indicated pronounced inflammatory infiltration and severe myocyte damage in the no-treatment control transplantation group, effects that were markedly ameliorated in the D665-G3c–treated group (Fig. 6C and fig. S5A). D665 potently expanded Treg cells, and G3c further enforced the expansion of Treg cells on POD3. However, on POD7, Treg cells were largely diminished in the combined treatment group (Fig. 6D and fig. S5B). Large amounts of IL-10/IFN-γ co-producing CD4^+Foxp3^-Tr1 cells were observed both in the cardiac graft-infiltrating lymphocytes (GLls) and splenocytes in the D665-G3c–treated group on POD7 (Fig. 6E and fig. S5C).

Further FCM analyses revealed that the CD4^-/CD8^- T cells ratio were significantly increased in both the graft and spleen in the combination treatment group on POD7 (Fig. 6F and fig. S5D). The numbers of splenocytes and GLls obtained in each group are shown in fig. S5E. Consistently, myocardial infiltration of both of cytotoxic CD8^- T cell and proliferating cytotoxic CD8^- T cells, considered the major executor of transplantation rejection, were notably decreased in the combination treatment group on POD7 detected by immunohistochemical staining (Fig. 6G).

Because Foxp3^- Treg cells were also transiently expanded in our model, we next examined the contribution of Treg and Tr1 cells to the induction of heart allograft acceptance. As shown in Fig. 6H, depletion of Treg cells on POD–1, POD3, and POD7, respectively, using anti-CD25 treatment did not disrupt heart tolerance, whereas the neutralization of IL-10 by anti-IL-10 or IL-10 knockout resulted in heart rejection. These results indicated that the combination of D665 and G3c treatment induced permanent allograft acceptance in a Tr1 cell–dependent rather than Treg cell–dependent manner.

**DISCUSSION**

Tr1 cells are potent IL-10–producing cells capable of suppressing immune responses to self, foreign, and allogeneic antigens. We found in the present study that combinations of CD28 superagonist D665 and anti–GITR antibody G3c could generate large amounts of IL-10/IFN-γ co-producing CD4^+Foxp3^-Tr1 cells in vivo. Mechanistic studies suggested that D665 and G3c treatment induced Tr1 cells via TFs Prdm1 and Maf. G3c contribute to Tr1 cell generation via the activation of MAPK-STAT3 signaling, Tr1 cells suppressed DC maturation and T cell proliferation in an IL-10–dependent manner. Furthermore, in a mouse heart transplantation model, combinations of D665 and G3c treatments induced permanent allograft acceptance in fully MHC-mismatched mice in a Tr1 cell–dependent manner rather than a Treg cell–dependent manner.

CD28 superagonist bivalently binds to the laterally exposed C′D loop of the extracellular Ig-like domains of the CD28 homodimer and forms a stable lattice on the T cell membrane, which provides strong activating signals and leads to potent polyclonal T cell expansion (4, 5, 37). The CD28 superagonist D665 has been shown to preferentially expand Treg cells over Teff cells in various rodent models for Treg–based interference with autoimmune and inflammatory
We also observed robust T<sub>reg</sub> cell expansion consistent with previously published data (Fig. 1). However, use of D665 alone resulted in a prolonged cardiac allograft survival rather than permanent allograft acceptance in the BALB/c to B6/J strain combination with strong rejection responses (Fig. 6). Following D665 treatment, the GITR expression was strongly up-regulated on both T<sub>reg</sub> and Teff cells. GITR signaling is complicated, and its functions are cell specific and context dependent (21). The conventional anti-GITR antibody DTA-1 has been reported to activate Teff lymphocytes while depleting GITR-expressing T<sub>reg</sub> cells, thereby promoting anti-tumor immune responses. G3c, another agonist anti-GITR antibody, showed a stronger costimulatory activity than DTA-1 for both Teff and T<sub>reg</sub> cells but failed to remove T<sub>reg</sub> cells in vivo and cure tumor-bearing mice (38). The application of G3c targeting GITR following disease (22–25). We also observed robust T<sub>reg</sub> cell expansion consistent with previously published data (Fig. 1). However, use of D665 alone resulted in a prolonged cardiac allograft survival rather than permanent allograft acceptance in the BALB/c to B6/J strain combination with strong rejection responses (Fig. 6). Following D665 treatment, the GITR expression was strongly up-regulated on both T<sub>reg</sub> and Teff cells. GITR signaling is complicated, and its functions are cell specific and context dependent (21). The conventional anti-GITR antibody DTA-1 has been reported to activate Teff lymphocytes while depleting GITR-expressing T<sub>reg</sub> cells, thereby promoting anti-tumor immune responses. G3c, another agonist anti-GITR antibody, showed a stronger costimulatory activity than DTA-1 for both Teff and T<sub>reg</sub> cells but failed to remove T<sub>reg</sub> cells in vivo and cure tumor-bearing mice (38). The application of G3c targeting GITR following
**Fig. 6.** D665-G3c treatment induced permanent allograft acceptance. (A) Treatment protocol of D665 and G3c in mouse heart transplantation. D665 (250 μg per mouse) and G3c (250 μg per mouse) were intraperitoneally injected on days −3 and 0 sequentially. Heart transplantation was performed on day 0. (B) The survival of cardiac allografts in each group (n = 9 to 12 for each group). The graft survival of each group was evaluated using Kaplan-Meier curves and log-rank tests. ****P < 0.0001. (C) Representative hematoxylin and eosin staining of cardiac grafts in each treatment group on POD7 (n = 6 for each group). Scale bars, 100 μm. (D) A representative FCM analysis of CD4+CD25+Foxp3+ Treg cells in the splenocytes of each treatment group on POD3 and POD7. (E) A representative FCM analysis of IL-10/IFN-γ–co-producing CD4+ Tr1 cells in the splenocytes (SPs) and GILs of each treatment group on POD7 (n = 4 for each group). (F) A representative FCM analysis of CD4+ T and CD8+ T cells in the splenocytes and GILs of each treatment group on POD7 (n = 4 for each group). (G) Representative CD8 (blue), 5-bromo-2′-deoxyuridine (BrdU) (red), and type IV collagen (yellow) triple immunohistochemistry staining of heart grafts in each group on POD7 (n = 4 for each group). Scale bars, 100 μm. (H) Survival of cardiac allografts in each group (n = 6 for each group). The graft survival of each group was evaluated using Kaplan-Meier curves and log-rank tests. ****P < 0.001.
D665 generated large amounts of Tr1 cells. G3c played an important role in the induction of Tr1 cells based on D665 treatment.

The strong synthesis of IL-10 is a hallmark of Tr1 cells. Over the past few years, many studies have explored the mechanism underlying the IL-10 expression in Tr1 cells. While the molecular mechanisms underlying the development of Tr1 cells are still unclear, a plethora of TFs have been identified as being involved in IL-10 production (2). Notably, several TFs have been identified that contribute to the development of Tr1 cells activated by IL-27 signaling (35). Prdm1 and Maf were identified as two central regulators that cooperatively drive the expression of IL-10 expression and Tr1 signature genes induced by IL-27 (36). IL-27 induced potent Egr2 expression via STAT3 signaling, which is required for IL-27-induced Prdm1-mediated IL-10 production in CD4⁺ T cells (32). Furthermore, Ahr and Maf, which are located downstream of IL-27 signaling, collaboratively promoted the development of Tr1 cells (39). In addition, Eomes and Prdm1 cooperated to generate Tr1 cells and mediated the expression of granzyyme B, which is another feature of Tr1 cells (40). The induction of Eomes in Tr1 cells requires Tbx21 and IL-27 signaling. Recently, Eomes has been reported as a lineage-defining TF of the unique granzyyme K⁺ Tr1 cells (41). In the present study, Prdm1, Maf, Tbx21, and Eomes were up-regulated in Tr1 cells (Fig. 4), accompanied by strong phosphorylation of STAT3 (Fig. 5), whereas Egr2 and Ahr were down-regulated in Tr1 cells (Fig. 4). Of these, Egr2 was reported to be essential for IL-27–induced Blimp-1–dependent IL-10 induction. A slightly elevated IL-27 expression was only observed in the combination treatment group on day 7, whereas a considerable amount of Tr1 cells had been generated on day 3. We suspect that the cell-intrinsic function of GITR signaling may be responsible for the development of Tr1 cells. GITR induced activation of ERK via TNFR-associated factor 5, which is an adaptor protein and signal transducer of TNFRs (42). The ERK/MAPK signaling pathways were able to induce IL-10 production through STAT3 activation (43). We therefore proposed that GITR signaling might contribute to Tr1 cell generation via MAPK-STAT3 signaling (Fig. 7). The high expression of Tbx21, the lineage-defining TF of T helper type 1 (Th1) cells, and IFN-γ, the signature of T1 cytokine, suggested that T1 cells might be the origin of Tr1 cells. Switching of IFN-γ–secreting T1 cells into IL-10/IFN-γ–co-producing CD4⁺Foxp3⁺ Tr1 cells via the activation of MAPK-STAT3 signaling, TF Blimp1, c-Maf, Eomes, and Tbx21 are responsible for IL-10/IFN-γ production in D665-G3c–induced Tr1 cells. TRAF5, TNFR-associated factor 5.

The strong synthesis of IL-10 is a hallmark of Tr1 cells. Over the past few years, many studies have explored the mechanism underlying the IL-10 expression in Tr1 cells. While the molecular mechanisms underlying the development of Tr1 cells are still unclear, a plethora of TFs have been identified as being involved in IL-10 production. Notably, several TFs have been identified that contribute to the development of Tr1 cells activated by IL-27 signaling. We therefore proposed that GITR signaling pathways were able to induce IL-10 production through MAPK-STAT3 signaling pathways. The ERK/MAPK signaling pathways were able to induce IL-10 production through STAT3 activation. We therefore proposed that GITR signaling might contribute to Tr1 cell generation via MAPK-STAT3 signaling. The CD28 superagonist D665 induced up-regulation of GITR along with activation and expansion of Treg cells. The application of G3c targeting GITR contributes the conversion of T1 cells into IL-10/IFN-γ–co-producing CD4⁺Foxp3⁺ Tr1 cells via the activation of MAPK-STAT3 signaling. TF Blimp1, c-Maf, Eomes, and Tbx21 are responsible for IL-10/IFN-γ production in D665-G3c–induced Tr1 cells. TRAF5, TNFR-associated factor 5.

The therapeutic effects of Tr1 cells have been investigated in many preclinical models of immune-mediated diseases, including autoimmune diseases, allergic diseases, GVHD, and transplantation. Tr1 cells, via de novo induction or adoptive transfer, promote pancreatic islet graft tolerance in mouse transplantation models and human antigen-specific Tr1 cells in vivo. We demonstrated that D665-G3c–induced Tr1 cells mediated permanent cardiac allograft acceptance. D665-G3c induced both Treg and Tr1 cells, but the Treg cells were only transiently elevated, and the heart transplantation tolerance was dependent on Tr1 rather than Treg cells. Further translational studies concerning donor specificity and the safety of Tr1 cells generated by the D665-G3c induction strategy are needed.

In the present study, we found that the combination of D665 and G3c treatment generated large amounts of Tr1 cells, resulting in permanent cardiac allograft acceptance. We proposed that D665-G3c induced Tr1 cells via TFs Prdm1 and Maf. G3c contributed to Tr1 cell generation via the activation of MAPK-STAT3 signaling. Tr1 cells suppressed both innate and adaptive immunity, thus causing permanent cardiac allograft acceptance in an IL-10–dependent manner. Further studies should focus on the associated molecular mechanisms and clinical translation of Tr1 cells. We believe that the in vivo pharmacological induction of Tr1 cells opens up possibilities for transplantation tolerance and can be successfully translated to the clinical setting.
Lebanon, NH, catalog no. BE0049) on pre- and posttransplantation
intraperitoneally injected with 250 μg of anti–IL-10 (Bio X Cell, Kyoto, Japan), respectively. For IL-10 neutralization, mice were
supernatant of hybridomas, gifts from T. Hunig (University of
Würzburg, Würzburg, Germany) and J. Shimizu (Kyoto University,
Osaka, Japan) in 24-well tissue culture plates. On day 2, floating
granulocyte clusters were drained away, and half of the medium was
refreshed. On day 5, for maturation, cells were collected and reseeded
(1 × 10^5 per well) in a 96-well flat-bottom plate with 100 μl per well
of fresh medium in the presence of LPS (10 ng/ml; Sigma-Aldrich)
and stimulated for 2 days. Different-sorted CD4^+ T cells (1 × 10^5 per
well) were also added to the medium to test their effect on BMDC
maturation. On day 7, different groups of BMDCs were harvested
for subsequent analyses.

Mixed lymphocyte reaction
In the one-way MLR, MACS-isolated CD8^+ T cells from B6/J mouse
splenocytes were labeled with CellTrace CFSE (Thermo Fisher
Scientific) as responders. BALB/c-derived BMDCs were irradiated
with 20-Gy x-ray and used as stimulators. Naïve CD4^+ T cells,
CD4^+IL-10^+ cells, and CD4^+IL-10^- cells were seeded to serve as
regulators. Stimulator BMDCs (1 × 10^6 per well), responder CD8^+ T
cells (1 × 10^5 per well), and regulators CD4^+ T cells (1 × 10^6 per
well) were cocultured in a 96-well U-bottom plate with 100 μl per
well of complete RPMI 1640 medium at 37°C for 3 days. For neu-
ralization of secreted cytokines, anti–IL-10 (20 ng/ml; Bio X Cell,
catalog no. BE0049) mAb was added at the start of the culture. At
the end of the assay, cells were collected for measurement of CFSE
dilution by FCM.

Isolation of mouse cardiac GILs
Cardiac grafts were harvested on POD7 and cut into 1- to 2-mm pieces
on ice. The tissue was then mechanically disrupted and digested
at 37°C for 20 min in 10 ml of digestion solution, which included
collagenase IV (0.5 mg/ml; Sigma-Aldrich) and deoxyribonuclease
I (50 U/ml; Thermo Fisher Scientific) in phosphate-buffered saline
(PBS). Subsequently, 10 ml of iced RPMI 1640 with 5% FCS was
added to stop digestion. The digested material was filtered through
a nylon mesh (100 μm) to remove aggregates and centrifuged at
200g for 10 min to pellet the cells. The pellet was then suspended in
5 ml of PBS, loaded onto 5 ml of Lympholyte-M (Cedarlane, Ontario,
Canada), and centrifuged at 1500g for 25 min at room temperature.
GILs were collected from the Lympholyte-M interface and washed
twice in PBS before staining.

Flow cytometry
Cells were stained with LIVE/DEAD staining (Thermo Fisher
Scientific) for labeling dead cells and blocked with anti–CD16/CD32
(BioLegend, San Diego, CA; catalog no. 101302) Fc Block antibody
to prevent nonspecific antibody binding. For cell surface staining, the
cells were incubated with different combinations of fluorochrome-
conjugated antibodies against mouse CD45 (BioLegend, catalog no.
103136), CD3 (BioLegend, catalog no. 100328), CD4 (BioLegend,
catalog no. 100526), CD8α (BioLegend, catalog no. 100730), CD11b
(BioLegend, catalog no. 101216), CD11c (BioLegend, catalog no.
117310), CD40 (BioLegend, catalog no. 124610), CD80 (BioLegend,
catalog no. 104722), CD86 (BioLegend, catalog no. 105030), MHC-II
(BioLegend, catalog no. 107606), CD44 (BioLegend, catalog no.
103012), CD62L (BioLegend, catalog no. 104408), GITR (BioLegend,
catalog no. 126310), CD39 (BioLegend, catalog no. 143804), CD25
(BioLegend, catalog no. 102012), ICOS (BioLegend, catalog no.
313508), TIGIT (BioLegend, catalog no. 142104), CD49b (BioLegend,
catalog no. 103515), PD-1 (BioLegend, catalog no. 109104), TIM-3
(BioLegend, catalog no. 119718), Neuropilin-1 (BioLegend, catalog

Cell preparation and cell sorting
Total CD4^+ and CD8^- T cells were purified from the mouse spleno-
cytes using magnetic-activated cell sorting (MACS) mouse CD4^+
(Milenyi Biotec, Bergisch Gladbach, Germany, catalog no. 130-104-
454) and CD8^- (Milenyi Biotec, catalog no. 130-095-236) T cell
isolation kits according to the manufacturer’s instructions. For
CD4^+IL-10^- cells and CD4^+IL-10^- cell preparation, CD4^+ T cells
were isolated from D665- and G3c-treated IL-10^-Venus mice on
day 7 using MACS and then subjected to cell sorting for CD4^+Venus^+
and CD4^-Venus^- populations using a FACSARia (BD Biosciences,
Franklin Lakes, NJ). Because most CD4^-Venus^- T cells (>95%) were
IL-10/IFN-γ^-co-producing CD4^-Foxp3^- Tr1 cells (Fig. 1E and fig.
S1A), the sorted CD4^-Venus^- T cells were considered Tr1 cells.

BMDC culture
Bone marrow cells were obtained from the femur and tibia of male
BALB/c mice, and then, erythrocytes were removed by lysis. To
generate BMDCs, bone marrow cells (1 × 10^8 per well) were cultured
in RPMI 1640 medium supplemented with 10% fetal calf serum
(FCS) (Thermo Fisher Scientific, Waltham, MA), granulocyte macro-
phage colony-stimulating factor (10 ng/ml; PeproTech, Cranbury,
NJ), IL-4 (10 ng/ml; PeproTech), and 50 μM β-mercaptoethanol (Wako,
These RNA samples were then subjected to RNA-seq aligner HISAT2 (v2.1.0). Read counts for each gene were calculated with Feature Counts. DEGs were identified by DESeq2 (v1.32.0) with the following criteria: absolute log 2 FC of ≥0.5 and their quality with FastQC (v0.11.8), trimmed with TrimGalore (v0.5.0), and aligned to the GRCm38 reference genome using the sequence platform. The raw sequencing reads were checked for throughput sequencing by BGI Japan (Kobe, Japan) on the DNBseq sequencing platform. The relative gene expression was calculated by the ΔΔCt calculation method. The sequences of 18S and target gene primers used in this research are shown in Table 1.

**RNA-seq analyses**

CD4^+IL-10^+ and CD4^+IL-10^− T cell samples were prepared in three duplicates. Total RNA extraction and DNA removal were performed as described above. These RNA samples were then subjected to preprocessing, DNA nanoball-based library construction, and high-throughput sequencing by BGI Japan (Kobe, Japan) on the DNaseq sequencing platform. The raw sequencing reads were checked for their quality with FastQC (v0.11.8), trimmed with TrimGalore (v0.5.0), and aligned to the GRCm38 reference genome using the RNA-seq aligner HISAT2 (v2.1.0). Read counts for each gene were calculated with Feature Counts. DEGs were identified by DESeq2 (v1.32.0) with the following criteria: absolute log2 FC of ≥0.5 and adjusted P value of ≤0.05. Variance stabilization–transformed expression data were Z-transferred and used to generate a heatmap by heatmap (v1.0.12). A GO enrichment analysis was performed using ClusterProfiler (v4.0.5). The top 10 enriched clusters were visualized with GOplot (v1.0.2).

**RNA purification and qRT-PCR**

Total RNA from heart grafts was extracted using reagent Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan). Total RNA from the cell suspension was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. After treating samples with a DNA-free kit (Ambion, Life Technologies, Carlsbad, CA), total RNA was reverse-transcribed to generate complementary DNA using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). qRT–PCR was performed in an Applied Biosystem PRISM 7700 instrument (Applied Biosystems, Foster City, CA) using SYBR Green system. The threshold cycle (Ct) values of the target genes were normalized to the Ct value of 18S ribosomal RNA. The relative gene expression was calculated by the ΔΔCt calculation method. The sequences of 18S and target gene primers used in this research are shown in Table 1.

**Western blotting**

Total cell protein was extracted with radioimmunoprecipitation assay lysis buffer (Wako) containing 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail 1, and 1% phosphatase inhibitor cocktail 2 (Sigma-Aldrich), measured concentrations with the BCA Protein Assay (Thermo Fisher Scientific). Twenty micrograms of protein was resolved on 10% SDS–polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). After blocking, the membranes were incubated with primary antibodies containing phosphorylated STAT3 (p-STAT3) (Cell Signaling Technology, Danvers, MA, catalog no. 9131), STAT3 (Cell Signaling Technology, catalog no. 4904), p-ERK (Cell Signaling Technology, catalog no. 4370), ERK (Cell Signaling Technology, catalog no. 4695), Blimp1 (Cell Signaling Technology, catalog no. 9115), c-Maf (Bethyl Laboratories, Montgomery, TX; catalog no. A300-613A-M), and β-actin (Cell Signaling Technology, catalog no. 4970) overnight at 4°C, followed by horseradish peroxidase–linked anti-rabbit IgG secondary antibody (Cell Signaling Technology, catalog no. 7074) probing for 1 hour at room temperature. The chemiluminescence signal intensity of protein bands were detected with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) and the ImageQuant LAS4000 System (GE Healthcare). The protein expression was quantitated with the ImageJ software program (National Institutes of Health, Bethesda, MD).

**Histological analyses**

Heart grafts were fixed in 10% neutral buffered formalin (Wako), dehydrated, and then embedded in paraffin. Paraffin sections (4 μm

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**Table 1. Primer sequences for qRT-PCR.**

| Gene | Forward primer (5′ to 3′) | Reverse primer (3′ to 5′) |
|------|-------------------------|-------------------------|
| Prdm1 | CCTCAGATCCCTGGTAGGAGTCTCTA | ACGTGAGCCATCCAGAGTGG |
| Maf | CGAACAGAAGCAGCCGAGCTG | CGAGTCCTGAGGCGACTAGC |
| Eomes | GCCGATTTCTTTTCTTGTGAG | GGTCCGCCGAAGACCACCTTC |
| Ahr | AGCCGCTGAGGAGAAACAGTAA | AGCGGTCTCCTGCTGTTT |
| Egr2 | GCAAGGCGCTAGGAACAAATC | CCCCTCCTGCTCTGCTG |
| Tbx21 | ACGAGAGCCGGCGCAAGTGT | GGGTTGCAATATAAAGCGCT |
| Irf4 | TGCAGATGGTGTTATGCAG | CCTCAGAGTGATGGCCTGCT |
| Il-27p28 | CCTGACATGGCCAGGAGGTACAGGACC | TCACTCGAGTTAGAATAACCCAGGCTG |
| Ebi3 | CTTCAGCGGCGGCGGCTG | GTGACATTAGCAGGGCAA |
| Il-10 | GTGCTTCATGCCTGACCTG | CGGAGCTCAGAGGACATG |
| Ilfng | AAGCGTCTTGGACCAACCATCAG | ACCCTTGGTTTGAGACCTCAA |
| 18S | ACATCGACACCTCACCAGAGG | TCCATCCTTCACATCTTC |
thick) were stained with hematoxylin and eosin stain. Heart allograft rejection was graded by two investigators blinded to the group and labeling allocation during the experiment, according to the International Society for Heart and Lung Transplantation criteria (60).

**Immunohistochemistry**

Heart grafts were frozen in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and then cut into 4-μm thick cryosections. Cryosections were rehydrated, fixed with formaldehyde calcium solution, and then blocked using block ace for 10 min. For CD8α T cell staining, the sections were immunostained with CD8α (BioLegend, catalog no. 100777) as the primary antibody and then incubated with alkaline phosphatase (ALP)-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA; catalog no. 712-055-153) as the secondary antibody. The positive antigens were visualized with the Vector Blue Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. For type IV collagen staining, the sections were immunostained with rabbit–anti-mouse type IV collagen polyclonal antibody (Cosmo Bio, Tokyo, Japan, catalog no. LSI-LB-1403) and then incubated with POD-conjugated goat–anti-rabbit Ig (Jackson ImmunoResearch, catalog no. 111-036-144) as the secondary antibody. The positive antigens were visualized with diaminobenzidine (DAB) (Dojindo, Kumamoto, Japan) substrate reaction. For BrdU staining, the sections were digested with a pepsin (Sigma–Aldrich) solution, immunostained with anti-BrdU (Bio-Rad Laboratories, catalog no. OB70030CX) as the primary antibody, and then incubated with ALP-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, catalog no. 712-055-153) as the secondary antibody. The positive antigens were visualized with New Fuchsin (Dako, Santa Clara, CA) in a substrate reaction. Last, the sections were fixed in formaldehyde calcium solution and mounted with Aquatex (Merck, Whitehouse Station, NJ).

All of the sections were imaged with a DP70 camera (Olympus, Osaka, Japan). The images were processed and analyzed using the Imagej software program. Positive cells were determined by counting nine random 400× high-power fields on each slide.

**Statistical analyses**

All data were analyzed using the GraphPad Prism software program (v7.0, GraphPad Software, San Diego, CA). The results were presented as the mean ± SEM. Student’s t test (normal distribution data) was used for comparisons between the two groups. A one-way analysis of variance (ANOVA), followed by a post hoc test was used for comparisons between multiple groups. A log-rank (Mantel-Cox) test was used for the survival data. In all experiments, differences were considered statistically significant at *p* < 0.05, **p** < 0.01, ***p*** < 0.001, and ****p*** < 0.0001.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abo4413

View request a protocol for this paper from Bio-protocol.

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