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ARTICLE

Targeted deletion of *Traf2* allows immunosuppression-free islet allograft survival in mice

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Abstract
Aims/hypothesis Administration of anti-CD40 ligand (CD40L) antibodies has been reported to allow long-term islet allograft survival in non-human primates without the need for exogenous immunosuppression. However, the use of anti-CD40L antibodies was associated with thromboembolic complications. Targeting downstream intracellular components shared between CD40 and other TNF family co-stimulatory molecules could bypass these complications. TNF receptor associated factor 2 (TRAF2) integrates multiple TNF receptor family signalling pathways that are critical for T cell activation and may be a central node of alloimmune responses.
Methods T cell-specific *Traf2*-deficient mice (*Traf2*TKO) were generated to define the role of TRAF2 in $CD4^+$ T cell effector responses that mediate islet allograft rejection in vivo. In vitro allograft responses were tested using mixed lymphocyte reactions and analysis of IFN-γ and granzyme B effector molecule expression. T cell function was assessed using anti-CD3/CD28-mediated proliferation and T cell polarisation studies.
Results *Traf2*TKO mice exhibited permanent survival of full MHC-mismatched pancreatic islet allografts without exogenous immunosuppression. *Traf2*TKO $CD4^+$ T cells exhibited reduced proliferation, activation and acquisition of effector function following T cell receptor stimulation; however, both *Traf2*TKO $CD4^+$ and $CD8^+$ T cells exhibited impaired alloantigen-mediated proliferation and acquisition of effector function. In polarisation studies, *Traf2*TKO $CD4^+$ T cells preferentially converted to a T helper (Th)2 phenotype, but exhibited impaired Th17 differentiation. Without TRAF2, thymocytes exhibited dysregulated TNF-mediated induction of c-Jun N-terminal kinase (JNK) and canonical NFκB pathways. Critically, targeting TRAF2 in T cells did not impair the acute phase of CD8-dependent viral immunity. These data highlight a specific requirement for a TRAF2–NFκB and TRAF2–JNK signalling cascade in T cell activation and effector function in rejecting islet allografts.
Conclusion/interpretation Targeting TRAF2 may be useful as a therapeutic approach for immunosuppression-free islet allograft survival that avoids the thromboembolic complications associated with the use of anti-CD40L antibodies.

Keywords Allograft · Effector function · Immunosuppression · Islet · T cell · TRAF2

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Abbreviations

CFSE	Carboxyfluorescein diacetate succinimidyl ester
H&E	Haematoxylin and eosin
JNK	c-Jun N-terminal kinase
L	Ligand
MLR	Mixed lymphocyte reaction
MST	Median survival time
siRNA	Small interfering RNA
Th	T helper
TNFR	TNF receptor
TRAF2	TNF receptor associated factor 2
Treg	T regulatory cell

Introduction

Allogeneic islet transplantation is emerging as a promising treatment for type 1 diabetes following the successful outcomes reported by the Collaborative Islet Transplant Registry [1]. A major obstacle to islet transplantation is immune rejection of the transplanted tissue by the recipient's immune system, which can only be overcome with the use of toxic immunosuppression. Greater understanding of the immune response towards transplanted tissues could lead to new therapeutic approaches. TNF receptor (TNFR) family molecules participate in T cell co-stimulation and provide new therapeutic avenues for transplantation. Blockade of CD27/CD70 and OX40/OX40 ligand (L) interactions, together with CD28 blockade, have been reported to prolong islet allograft survival [2, 3]. Anti-CD40L antibody administration has been reported to allow long-term islet allograft survival in non-human primates without the need for exogenous immunosuppression [4]. However, the use of a humanised anti-CD40L monoclonal antibody in human clinical trials was halted because of an increased incidence of thromboembolic events [5, 6].

Targeting intracellular T cell co-stimulation components could bypass the complications that occur when targeting extracellular co-stimulation components. A key intracellular component of TNFR family signalling is TNFR associated factor 2 (TRAF2) [7]. TRAF2 mediates downstream signalling by binding to the cytoplasmic domains of key T cell surface receptors, including TNFR, CD27, CD30, CD40, GITR, OX40 and 4-1BB [8]. TNFR family members play a critical role in allowing optimal T cell responses through co-stimulation. Without this T cell responses are impaired [8], thus providing a mechanistic basis for the efficacy of anti-CD40L antibodies in preclinical islet transplant studies [4]. T cells from dominant negative *Traf2* mutant mice have been reported to exhibit reduced proliferation and IFN-γ production in response to alloantigens in vitro, but also show impaired T cell receptor-mediated proliferation, highlighting the therapeutic potential of targeting TRAF2 in preventing T cell allograft responses [9, 10]. While promising, these studies did not test whether TRAF2 loss impairs islet allograft rejection in vivo [9]. In this study, we examined whether targeting TRAF2 in T cells would impact islet allograft rejection.

Methods

Mice *Traf2*$^{lox/lox}$ mice were crossed with *Lck*-cre (C57BL/6) mice (Jackson Laboratory, Bar Harbor, ME, USA) [11] to generate T cell-specific deletion of *Traf2* (*Traf2*TKO). BALB/c, C57BL/6 and *Rag1*$^{-/-}$ mice were from Australian BioResources (Moss Vale, NSW, Australia). Male and female mice were used for the studies. Mice were housed at 22°C in a 12 h light/dark cycle, and were fed ad libitum and received humane care in compliance with guidelines from the Australian National Health and Medical Research Council. The Garvan/St Vincent's Hospital Animal Ethics Committee approved all protocols for animal experiments.

Islet transplantation and histopathology Islets from female BALB/c mice were transplanted to streptozotocin (Sigma-Chemical, St Louis, MO, USA)-induced diabetic floxed, *Traf2*TKO or *Rag1*$^{-/-}$ male recipients as previously described [12]. T regulatory cell (Treg) depletion studies were performed as previously described [13] using 100 μg PC61. Graft sections were stained with haematoxylin and eosin (H&E) or labelled for insulin (1:100; Cell Signaling, Beverly, MA, USA) using the DAKOCytomation EnVision+ System-HRP kit (DAKOCytomation, Glostrup, Denmark), and images were captured using a Leica DM 4000 microscope and DCF450 digital microscope camera using Leica Application Suite software (Leica Microsystems, North Ryde, NSW, Australia).

Western blot Western blots were performed using standard approaches [14]. Antibodies were used at 1:1000 dilution unless otherwise specified: anti-TRAF2 (C-20; Santa Cruz Biotechnology, Dallas, TX, USA); anti-phospho (p)-p38 MAPK (T180/Y182; Cell Signaling Technologies); p38 MAPK (Cell Signaling Technologies); anti-p-c-Jun N-terminal kinase (JNK) (T183/Y185) (Cell Signaling Technologies); SAPK/JNK (Cell Signaling Technologies); anti-IκBα (9242; Cell Signaling Technologies); anti-β-actin (1:10,000; Sigma-Aldrich, Castle Hill, NSW, Australia); anti-rabbit IgG-horseradish peroxidase (1:5000; GE Healthcare, Rydalmere, NSW, Australia); and anti-mouse IgG-horseradish peroxidase (1:5000; Thermo Scientific, Scoresby, VIC, Australia).

Flow cytometry Lymphocyte subpopulations were analysed using a FACSCanto system (BD Biosciences, San Jose, CA,

USA) using antibodies and methods as previously described [12]. Data analysis was performed using FlowJo analysis software (TreeStar, Ashland, OR, USA). Light-scatter gating to include live lymphocytes and exclude doublet cells, dead cells and debris was performed unless otherwise specified.

Lymphocyte BrdU incorporation Floxed control and *Traf2*TKO mice (age 6–8 weeks) were given 0.2 ml i.p. BrdU (Sigma-Aldrich; 10 mg/ml PBS) in five injections at 12 h intervals. Lymphocytes were harvested 12 h after the final injection and BrdU incorporation was assessed using the BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions.

T cell proliferation assays Purified T cells (Pan T Isolation Kit II; Miltenyi Biotec, Macquarie Park, NSW, Australia) were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (0.5 μmol/l; Sigma-Aldrich), and 2×10^5 cells/well were stimulated with immobilised anti-CD3 (0.1, 0.3, 1 and 3 μg/ml; BD Biosciences) with or without anti-CD28 (5 μg/ml; BD Biosciences) for 72 h. For mixed lymphocyte reactions (MLRs), T cells were co-cultured with mitomycin C (Sigma-Aldrich; 25 μg/ml)-treated BALB/c splenocytes in a 1:1 responder to stimulator ratio. T cell proliferation was assessed by flow cytometry based on CFSE dilution.

Treg suppression assay FACS-purified floxed control $CD4^+CD25^-$ (Teffector) cells were CFSE-labelled and co-cultured with FACS-purified floxed or *Traf2*TKO $CD4^+CD25^+$ (Treg) cells at various ratios. Proliferation was calculated as a percentage of suppression vs proliferation of T cells alone ($100 \times [1–\%CFSE^{low}CD4^+CD25^-$ T cells in co-culture/$\%CFSE^{low}CD4^+CD25^-$ T cells alone]).

T cell polarisation assay FACS-sorted floxed or *Traf2*TKO $CD4^+$-naive T cells ($CD3^+CD4^+CD25^-CD62L^+CD44^-$) were seeded at 1×10^5 cells/well in a flat-bottom, 96-well plate pre-coated with 1 μg/ml anti-CD3 (BD Biosciences) and co-cultured with 3 μg/ml anti-CD28 or 4×10^5 irradiated (3000 cGy) autologous antigen-presenting cells at 37°C with 5% CO_2 for 68 h under the following conditions: N (media), T helper (Th)0 (5 μg/ml anti-IFN-γ, 5 μg/ml anti-IL-4), Th1 (5 μg/ml anti-IL-4, 3.5 ng/ml IL-12), Th2 (5 μg/ml anti-IFN-γ, 3.5 ng/ml IL-4), Th17 (5 μg/ml anti-IFN-γ, 5 μg/ml anti-IL-4, 1 ng/ml TGF-β, 10 ng/ml IL-6) and Treg (5 μg/ml anti-IFN-γ, 5 μg/ml anti-IL-4, 1 ng/ml TGF-β). Cells were treated with phorbol 12-myristate-13-acetate (PMA)/ionomycin and GolgiStop (BD Biosciences) for 4 h prior to intracellular cytokine labelling.

Inhibition of signalling Purified floxed control T cells were incubated with pyrrolidine dithiocarbamate (NFκB inhibitor; Sigma-Aldrich), SP600125 (JNK inhibitor; Sigma-Aldrich) or SB203580 (p38 inhibitor; Cell Signaling) for 1 h at 37°C and 5% CO_2, and then stimulated with 3 μg/ml immobilised anti-CD3 for 72 h.

Influenza infection model Floxed and *Traf2*TKO mice were infected with 1×10^4 plaque-forming units of the HKx31 (H3N2) strain of the influenza A virus intranasally, and lymphocytes were harvested for staining with phycoerythrin (PE)-conjugated D^bNP_{366} or D^bPA_{224} tetramers, peptide stimulation and intracellular cytokine staining as previously described [15].

Statistical analysis Kaplan–Meier survival curve analysis using a logrank (Mantel–Cox) test and Dunnett's multiple comparison or unpaired *t* tests (Mann–Whitney) were performed using GraphPad Prism v6.0b for Macintosh (GraphPad Software, La Jolla, CA, USA). Results were considered statistically significant when $p < 0.05$.

Results

T cell-specific deletion of *Traf2* promotes long-term allograft survival BALB/c (H-2^d) islets were transplanted under the kidney capsule of streptozotocin-induced diabetic *Traf2*TKO or littermate floxed control (both H-2^b) mice. All floxed control recipient mice rejected their islet allograft, with a median survival time (MST) of 20.5 days ($n = 4$). In contrast, approximately 77% of *Traf2*TKO recipient mice accepted an islet allograft long term (e.g. >100 days; $p = 0.0012$; $n = 13$) (Fig. 1a). Graft function was demonstrated by nephrectomy at postoperative day 100 (Fig. 1b). Mononuclear cells were abundant in rejecting grafts from floxed control mice and early (postoperative day <20) islet allografts in *Traf2*TKO mice (Fig. 1c), whereas long-term surviving islet allografts were characterised by reduced mononuclear cell infiltrate, normal islet morphology and robust insulin production in situ (Fig. 1d). The prolongation of allograft survival in *Traf2*TKO mice also extended to models of vascularised allografts. Compared with controls, *Traf2*TKO mice exhibited prolonged skin allograft survival (MST 19 vs 14 days; $p = 0.0201$) and heterotopic heart allograft survival (MST 9.5 vs 7 days; $p = 0.017$; Table 1). Thus, targeted deletion of *Traf2* on T cells allowed long-term islet allograft survival across a full MHC barrier without the need for exogenous immunosuppression.

In another set of experiments, streptozotocin-induced diabetic *Rag1*$^{-/-}$ mice transplanted with BALB/c islet allografts received purified T cells from either floxed or *Traf2*TKO mice 14 days post-transplant. All recipients receiving floxed control T cells rejected the islet allograft (MST 19 days; $n = 4$); however, approximately 83% of mice receiving *Traf2*TKO T cells exhibited intact and functional islets by histological analysis (MST >100 days; $n = 6$;

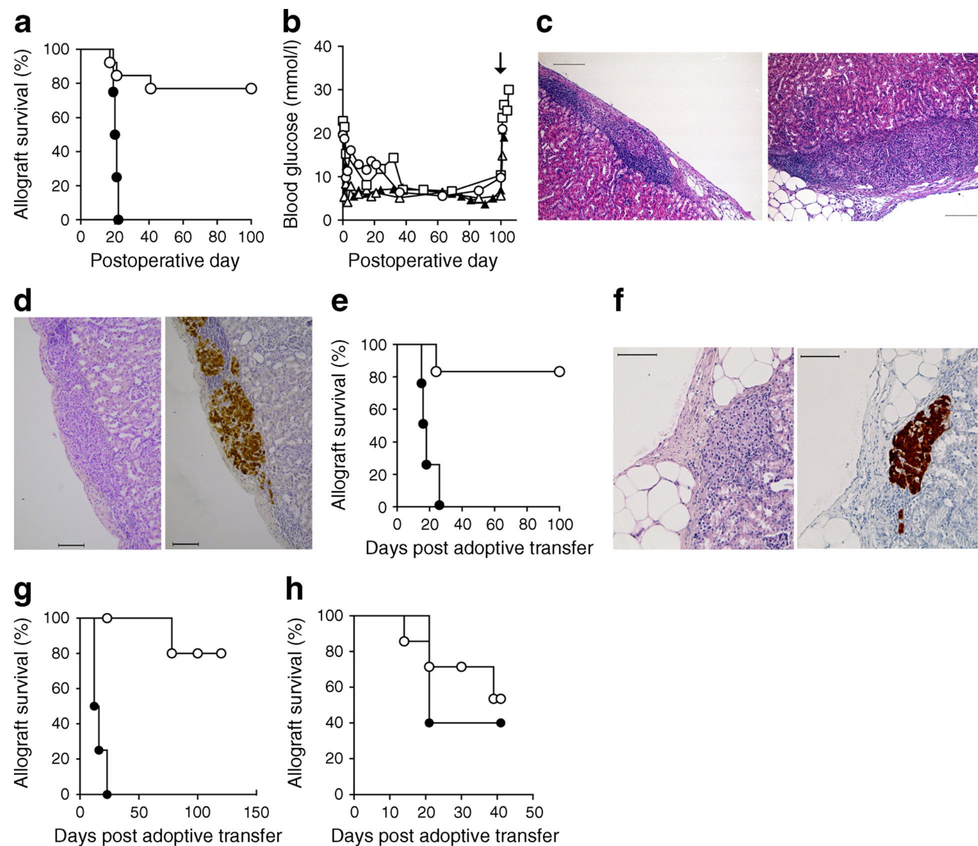

Fig. 1 Long-term islet allograft survival in *Traf2*TKO mice. (**a**) Islet allograft survival in floxed control (black circles, $n = 4$) and *Traf2*TKO (white circles, $n = 13$) mice; $p = 0.0012$. (**b**) Blood glucose readings from the *Traf2*TKO cohort ($n = 4$; each symbol reflects an individual mouse) followed for 100 days post transplantation and after survival nephrectomy on postoperative day 100 (arrow). (**c**) H&E staining of islet allografts in floxed control (left) and *Traf2*TKO (right) mice ($n = 3$) at postoperative day <20; scale bars, 100 μm. (**d**) H&E (left) and insulin labelling (brown; right) of >100 day surviving islet allografts in *Traf2*TKO mice; scale bars, 100 μm. (**e**) Islet allograft survival in transplanted *Rag1*$^{-/-}$ mice receiving 2×10^6 floxed control (black circles; $n = 4$) or *Traf2*TKO (white circles; $n = 6$) T cells; $p = 0.0046$. (**f**) H&E (left) and insulin labelling (brown; right) of >100 day surviving islet allografts in *Rag1*$^{-/-}$ mice receiving *Traf2*TKO T cells; scale bars, 100 μm. (**g**) Islet allograft survival in transplanted *Rag1*$^{-/-}$ mice receiving 2×10^6 floxed control (black circles; $n = 4$) or *Traf2*TKO (white circles; $n = 6$) $CD4^+$ T cells; $p = 0.0011$. (**h**) Islet allograft survival in transplanted *Rag1*$^{-/-}$ mice receiving 2×10^6 floxed control (black circles; $n = 5$) or *Traf2*TKO (white circles; $n = 7$) $CD8^+$ T cells; $p = 0.6536$

$p = 0.0046$; Fig. 1e, f), indicating that T cell intrinsic TRAF2 is required for islet allograft rejection.

To dissect the role of individual T cell subsets in islet allograft rejection, purified $CD4^+$ or $CD8^+$ T cells were adoptively transferred to *Rag1*$^{-/-}$ transplant recipients. All mice receiving floxed control $CD4^+$ T cells rejected their islet allograft (MST 14 days; $n = 4$), whereas 80% of mice receiving *Traf2*TKO $CD4^+$ T cells maintained their islet allograft for >100 days ($n = 6$; $p = 0.0011$; Fig. 1g). In contrast, adoptive transfer of floxed control or *Traf2*TKO $CD8^+$ T cells resulted in approximately 50% islet allograft rejection ($n = 5$–7; $p = 0.6884$; Fig. 1h). These data suggest that $CD4^+$ T cells have a specific requirement for TRAF2 to mediate the in vivo islet allograft response.

Table 1 Prolonged survival of vascularised allografts in *Traf2*TKO mice.

Group	Experiment	Allograft rejection days	MST (days)	*n*	*p* value[a]
1a	Floxed + BALB/c skin allograft	10, 12, 12, 12, 14, 14, 14, 17, 17, 17, 17	14	11	0.0201
1b	*Traf2*TKO + BALB/c skin allograft	12, 12, 14, 19, 19, 19, 21, 23	19	8	
2a	Floxed + BALB/c HHTx	6, 7, 7, 7, 7, 9, 10	7	7	0.0170
2b	*Traf2*TKO + BALB/c HHTx	7, 7, 8, 8, 8, 11, 11, 12, 12, 14	9.5	10	

[a] Logrank Mantel–Cox test comparing group a vs b

HHTx, heterotopic heart transplant

Without TRAF2, CD4$^+$ T cells fail to proliferate after T cell receptor stimulation Floxed and *Traf2*TKO total T cells exhibited comparable proliferation following anti-CD3 alone (Fig. 2a); however, *Traf2*TKO total T cells showed approximately 20% reduced proliferation at lower anti-CD3 concentrations (0.1 μg/ml) with anti-CD28 (Fig. 2b). In addition, anti-CD3 stimulated *Traf2*TKO CD4$^+$ T cells proliferated less than floxed controls (Fig. 2c). CD28 co-stimulation boosted *Traf2*TKO CD4$^+$ T cell proliferation, but it still failed to reach floxed control levels (Fig. 2d). When anti-CD3/CD28-mediated proliferation was compared with anti-CD3 stimulation alone, *Traf2*TKO CD4$^+$ T cells exhibited a similar proliferation fold-change compared with floxed controls (data not shown), suggesting that TRAF2 loss maintained CD28-mediated responses. In contrast to CD4$^+$ T cell proliferation, we have previously shown that *Traf2*TKO CD8$^+$ T cells showed normal proliferative responses to anti-CD3 and anti-CD3/CD28 [11]. Furthermore, the reduced anti-CD3/CD28-mediated proliferation of *Traf2*TKO CD4$^+$ T cells was not due to decreased CD4$^+$ T cell numbers as *Traf2*TKO and floxed control mice harboured comparable numbers of peripheral CD4$^+$ T cells, as shown in previous studies [11, 16]. T cell activation marker analysis over a 96 h period following anti-CD3 stimulation indicated a reduced ability of *Traf2*TKO CD4$^+$ T cells to upregulate CD25, CD44 and CD69 as effectively as floxed controls; however, CD62L downregulation was normal (Fig. 2e–h). In contrast to CD4$^+$ T cells, our previous studies demonstrate that activation-induced changes in *Traf2*TKO CD8$^+$ T cells were equivalent to those of floxed controls [11]. Therefore CD4$^+$ T cells, but not CD8$^+$ T cells, require TRAF2 for optimal activation and proliferation after T cell receptor engagement in vitro.

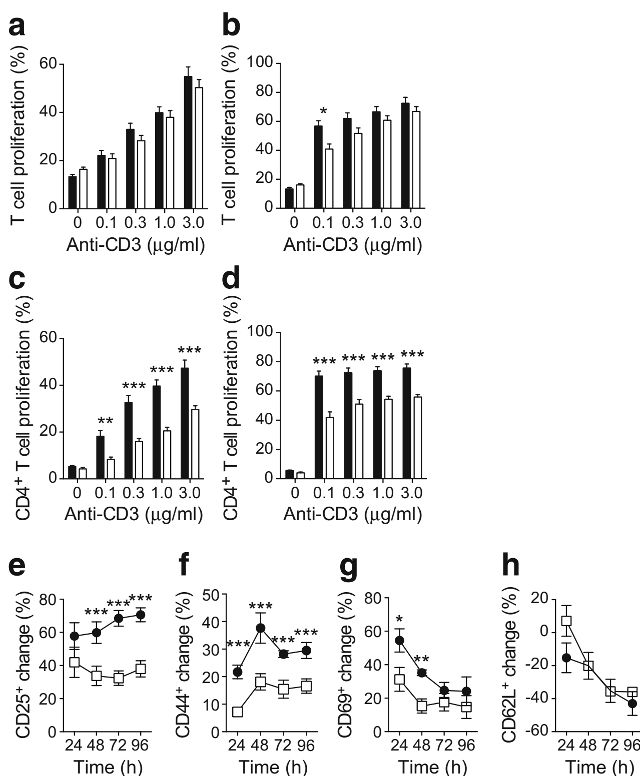

Fig. 2 Reduced anti-CD3-mediated proliferation and activation of *Traf2*TKO CD4$^+$ T cells. (**a**, **b**) T cell proliferation in floxed control (black bars) and *Traf2*TKO (white bars) mice after (**a**) anti-CD3 stimulation and (**b**) anti-CD3/CD28 stimulation. Bars represent means ± SEM. (**c**, **d**) CD4$^+$ T cell proliferation in floxed control (black bars) and *Traf2*TKO (white bars) mice after (**c**) anti-CD3 stimulation and (**d**) anti-CD3/CD28 stimulation. Bars represent means ± SEM. (**e**–**h**) Expression of (**e**) CD25, (**f**) CD44, (**g**) CD69 and (**h**) CD62L in anti-CD3 proliferated floxed control (black circles) and *Traf2*TKO (white squares) CD4$^+$ T cells analysed at 24 h intervals by flow cytometry as the percentage change from baseline (unstimulated) expression. Cumulative data from $n = 3$–5 mice over three to five independent experiments. Error bars show SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

TRAF2 is necessary for in vitro T cell effector responses to alloantigen We next measured proliferation and IFN-γ and granzyme B expression following co-culture with allogeneic BALB/c splenocytes to determine T cell effector function. *Traf2*TKO CD4$^+$ T cells exhibited approximately 40% reduced proliferation to alloantigen compared with floxed CD4$^+$ T cells (Fig. 3a, b). Furthermore, *Traf2*TKO CD4$^+$ T cells showed impaired IFN-γ (Fig. 3c, d) and granzyme B (Fig. 3e, f) expression relative to floxed control CD4$^+$ T cells. The impaired effector molecule expression was particularly evident in a small proportion of *Traf2*TKO CD4$^+$ T cells that underwent extensive proliferation but lacked expression of either IFN-γ or granzyme B, an effect that was not observed in the corresponding floxed control MLR. For CD8$^+$ T cells, *Traf2*TKO CD8$^+$ T cell proliferation was reduced by approximately 50% ($p < 0.0001$) compared with floxed controls (Fig. 3g, h), with impaired IFN-γ (Fig. 3i, j) and granzyme B expression (Fig. 3k, l) also observed following in vitro alloantigen exposure. Thus, for in vitro alloresponses, TRAF2 is required for full acquisition of CD4$^+$ and CD8$^+$ T cell effector function.

Loss of TRAF2 does not alter T cell sensitivity to apoptosis To assess whether impaired alloresponses in *Traf2*TKO mice could be due to reduced peripheral T cell turnover, floxed control and *Traf2*TKO mice were treated with the thymidine analogue BrdU. Analysis of BrdU incorporation revealed no discernible differences in BrdU$^+$CD4$^+$ and BrdU$^+$CD8$^+$ T cells between floxed control and *Traf2*TKO mice (see electronic supplementary material [ESM] Fig. 1a, b). Furthermore, no differences were observed in Bcl-2 expression between floxed control and *Traf2*TKO CD4$^+$ mice (ESM Fig. 1c), or in CD8$^+$ T cells [11] or lymphocyte viability (ESM Fig. 2). These data suggest that the impaired T cell alloresponse seen in *Traf2*TKO mice is not due to reduced peripheral T cell turnover or T cell survival.

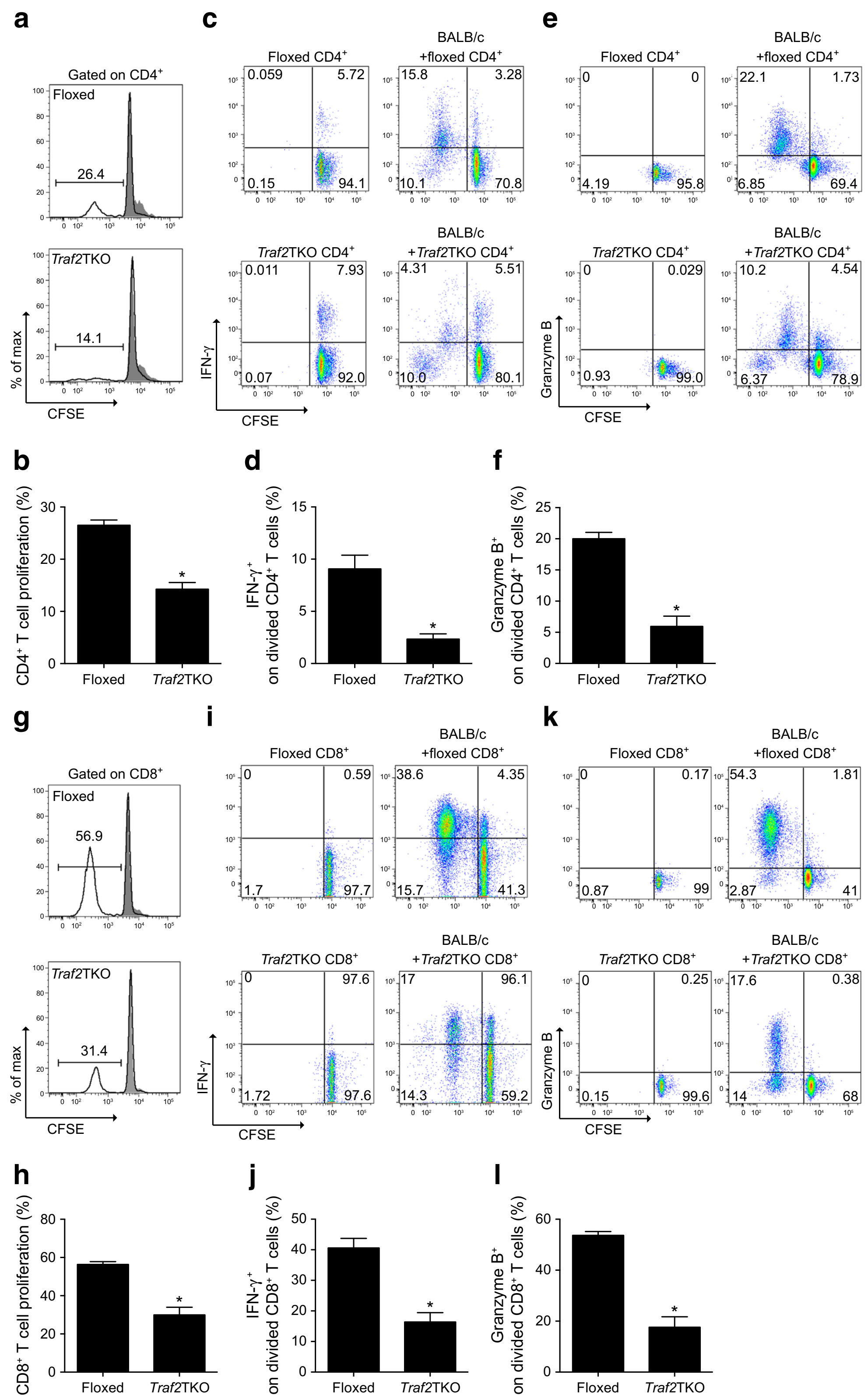

Fig. 3 Impaired *Traf2*TKO CD4$^+$ T effector responses to alloantigen in an MLR. Proliferation of CFSE-labelled floxed control or *Traf2*TKO T cells (both H-2^b) co-cultured with mitomycin-C-treated BALB/c splenocytes (H-2^d) for 3 days. (**a**) Representative CD4$^+$ T cell proliferation FACS plots. Shaded histogram represents unstimulated T cells. Cumulative data are shown in (**b**). (**c**) Representative CD4$^+$ T cell IFN-γ^+ expression FACS plots. (**d**) Cumulative data from (**c**) of CFSE$^-$IFN-γ^+ CD4$^+$ T cells. (**e**) Representative CD4$^+$ T cell granzyme B expression FACS plots. (**f**) Cumulative data from (**e**) of CFSE$^-$granzyme B$^+$ CD4$^+$ T cells. (**g**) Representative CD8$^+$ T cell proliferation FACS plots. Shaded histogram represents unstimulated T cells. Cumulative data are shown in (**h**). (**i**) Representative CD8$^+$ T cell IFN-γ^+ expression FACS plots. (**j**) Cumulative data from (**i**) of CFSE$^-$IFN-γ^+ CD8$^+$ T cells. (**k**) Representative CD8$^+$ T cell granzyme B expression FACS plots. (**l**) Cumulative data from (**k**) of CFSE$^-$granzyme B$^+$ CD8$^+$ T cells. Bars represent means ± SEM; $n = 3$ mice over two independent experiments; $^*p < 0.0001$

There is a specific requirement for the TRAF2–JNK and TRAF2–NFκB signalling cascades for T cell activation Following receptor ligation of TNFR family members, TRAF2 mediates signalling through the canonical NFκB, p38/MAPK and JNK pathways [17]. Compared with floxed controls, *Traf2*TKO thymocytes displayed reduced TNF-α-induced p38 phosphorylation, whereas the kinetics of canonical NFκB (IκBα degradation) and JNK

phosphorylation were significantly delayed (Fig. 4a). Thus, TRAF2 governs the timing of intracellular signalling pathways following TNF-α stimulation.

Pharmacological inhibition of these signalling pathways demonstrated that both JNK and NFκB activation, but not p38, were critical for CD4$^+$ T cell blasting (Fig. 4b), as well as for high expression levels of CD25 and CD44 (Fig. 4c, d). For CD8$^+$ T cells, blasting and high CD44 expression was dependent upon NFκB and JNK signalling but not p38 signalling, whereas CD25 expression was most dependent upon NFκB signalling (Fig. 4e–g). These data suggest a specific requirement for the TRAF2–JNK and TRAF2–NFκB signalling cascades in T cell activation and islet allograft rejection.

TRAF2 deficiency promotes Th2 and suppresses Th17 T cell differentiation Because CD4$^+$ T cells were found to rely upon TRAF2-dependent signals, the ability of *Traf2*TKO CD4$^+$ T cells to differentiate into effector subsets was examined. Purified naive CD4$^+$ T cells from floxed control and *Traf2*TKO mice were cultured under different Th-polarising conditions, and T cell fate (N, Th0, Th1, Th2, Th17 and Treg) was determined by flow cytometry (Fig. 5). Under Th1 conditions, *Traf2*TKO and floxed control naive CD4$^+$ T cells showed comparable frequencies of IFN-γ^+ Th1-type cells (Fig. 5a, c). Under Th2 conditions, *Traf2*TKO naive CD4$^+$ T cells showed an approximately sevenfold increase in IL-13$^+$ Th2-type cells compared with controls (Fig. 5a, d). Under Th17 polarising conditions, *Traf2*TKO naive CD4$^+$ T cells failed to differentiate into IL-17$^+$ Th17-type cells to floxed control levels (Fig. 5b, e); however, under the same Th17 conditions, *Traf2*TKO cells preferentially expressed the Treg marker FOXP3 (Fig. 5b, f). The increased propensity towards FOXP3$^+$ differentiation was also observed under Treg conditions, although this was not statistically significant (Fig. 5g). The differentiation profile of *Traf2*TKO cells favouring Th2-type and Treg cells and Th17-type inhibition was also observed following polarisation using anti-CD3 with autologous antigen-presenting cells (data not shown). These data indicate that under specific Th-polarising conditions, loss of TRAF2 strongly skews CD4$^+$ T cells towards Th2 differentiation, but strongly inhibits Th17 cell differentiation.

Islet allograft survival in *Traf2*TKO mice is Treg independent *Traf2*TKO mice showed increased frequencies of naturally occurring Tregs (CD4$^+$CD25$^+$FOXP3$^+$) compared with floxed control mice (Fig. 6a, b) but normal

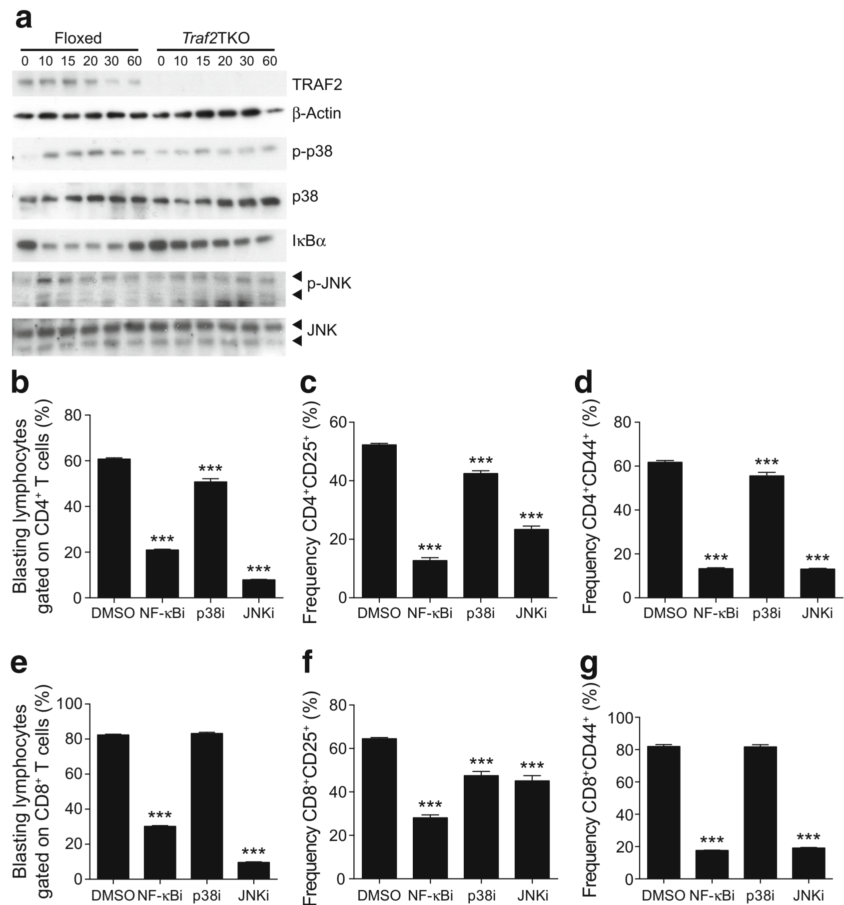

Fig. 4 Specific requirement for TRAF2–JNK and TRAF2–NFκB signalling cascades for T cell activation. (**a**) Western blot analysis of TRAF2, p-p38, p38, IκBα, pJNK, JNK and β-actin in floxed control and *Traf2*TKO thymocytes following 200 U TNF-α stimulation. Data are representative of three independent experiments. (**b**–**g**) Purified floxed control T cells pretreated with pyrrolidine dithiocarbamate (NFκB inhibitor [i]), SB203580 (p38i), SP600125 (JNKi) or vehicle control (DMSO) and anti-CD3-mediated lymphocyte blasting and activation assessed by flow cytometry after 72 h. (**b**) CD4$^+$ T cell blasting; (**c**) CD4$^+$CD25$^+$ T cell percentage; (**d**) CD4$^+$CD44$^+$ T cell percentage; (**e**) CD8$^+$ T cell blasting; (**f**) CD8$^+$CD25$^+$ T cell percentage; (**g**) CD8$^+$CD44$^+$ T cell percentage; $n = 3$ mice. Bars represent means ± SEM. ***$p < 0.001$

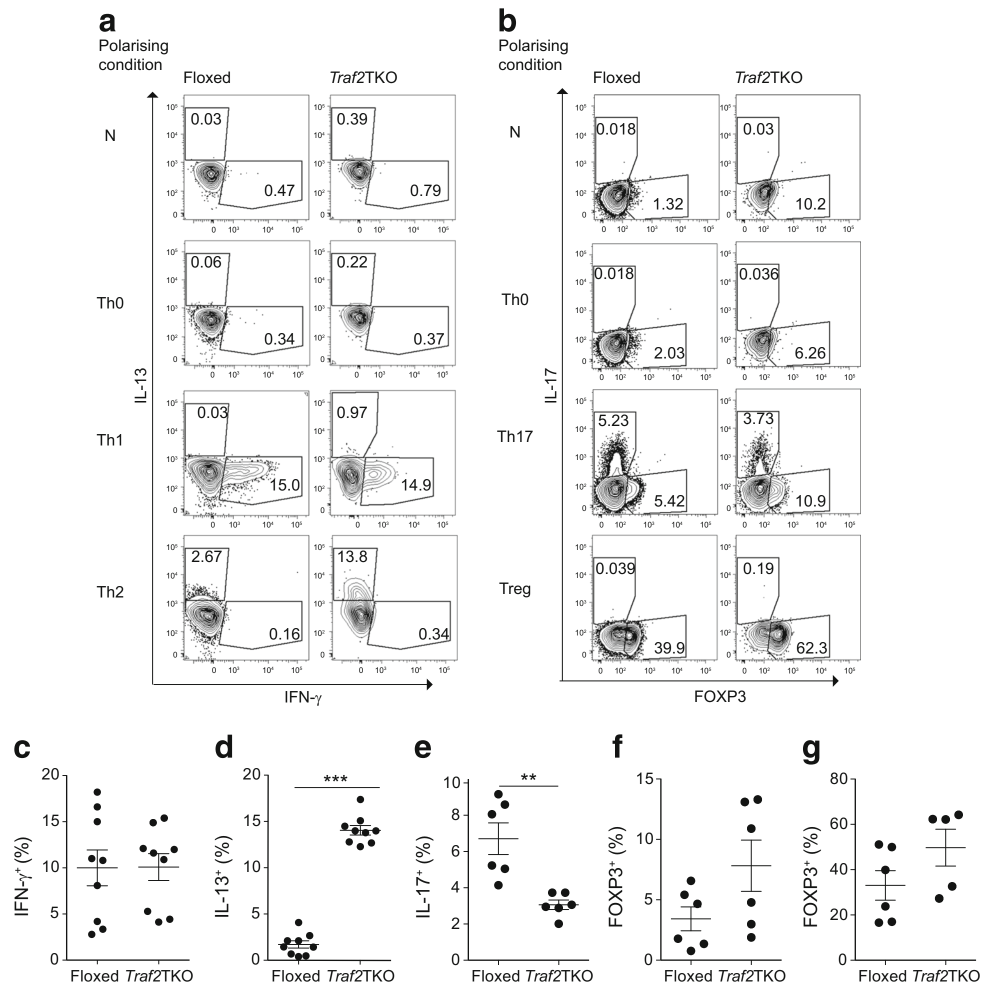

Fig. 5 Loss of TRAF2 strongly enhances Th2 and inhibits Th17 T cell differentiation. FACS-sorted naive CD4$^+$ T cells (CD4$^+$CD25$^-$CD62L$^+$CD44$^-$) were cultured with anti-CD3/CD28 for 72 h under N, Th0, Th1, Th2, Th17 and Treg polarising conditions. (**a**, **b**) Representative FACS plots of (**a**) Th1 and Th2 and (**b**) Th17 and Treg polarisation. (**c**, **d**) Cumulative data of (**c**) IFN-γ$^+$ and (**d**) IL-13$^+$CD4$^+$ T cells in Th1 and Th2 conditions. (**e**–**g**) Cumulative data of (**e**) IL-17$^+$ and (**f**) FOXP3$^+$CD4$^+$ T cells in Th17 conditions and (**g**) FOXP3$^+$CD4$^+$ T cells Treg conditions. Data are from $n = 3$–4 mice pooled over two independent experiments shown as means ± SEM. **$p < 0.01$, ***$p < 0.001$

Treg absolute numbers (Fig. 6c) and normal levels of well-characterised Treg-associated activation markers, with the exception of reduced CD44 expression (Fig. 6d) suggestive of Treg suppressive activity [18]. However, *Traf2*TKO Tregs exhibited normal in vitro T cell suppression (Fig. 6e). Furthermore, in vivo Treg depletion with the anti-CD25 monoclonal antibody PC61 (Fig. 6f) did not impact islet allograft survival in *Traf2*TKO mice (Fig. 6g). These data indicate that islet allograft acceptance in *Traf2*TKO mice is Treg independent and suggest impaired T cell effector function. Indeed, in an MLR, purified *Traf2*TKO effector (CD4$^+$CD25$^-$) T cells showed markedly reduced proliferation (Fig. 6h) and activation (CD44 expression) (Fig. 6i, j) compared with their floxed control counterparts, supporting this conclusion.

Maintenance of protective immunity in *Traf2*TKO mice Our study shows that functional TRAF2 is necessary for T cell-mediated islet allograft rejection, and highlights targeting TRAF2 as a possible therapeutic approach. To address the critical question of whether TRAF2 deletion impairs protective immune responses, we used a well-studied model of viral immunity. Following infection with HKx31 influenza A (H3N2), both floxed and *Traf2*TKO mice demonstrated similar antigen-specific CD8$^+$ T cell responsiveness during the acute phase (day 10) of infection, with no differences observed in splenic and bronchoalveolar lavage D^{b}NP366- and N^{b}PA244-specific CD8$^+$ T cell numbers (ESM Fig. 3a, b) or viral-specific CD8$^+$IFN-γ$^+$ cell numbers (ESM Fig. 3c, d). Therefore, CD8$^+$ T cell-dependent acute influenza-specific responses remained intact following T cell-specific deletion of *Traf2*. Thus, targeting TRAF2 allowed islet allograft survival but did not impair the acute antiviral CD8$^+$ T cell response.

Discussion

The impaired islet allograft rejection in *Traf2*TKO mice was not due to absolute immune anergy, since lymphocyte migration during the early islet allograft response was intact and regulatory T cell numbers and function were maintained. Loss of TRAF2 impaired in vitro CD4$^+$ and CD8$^+$ T cell alloreactivity; however, the requirement for TRAF2 for

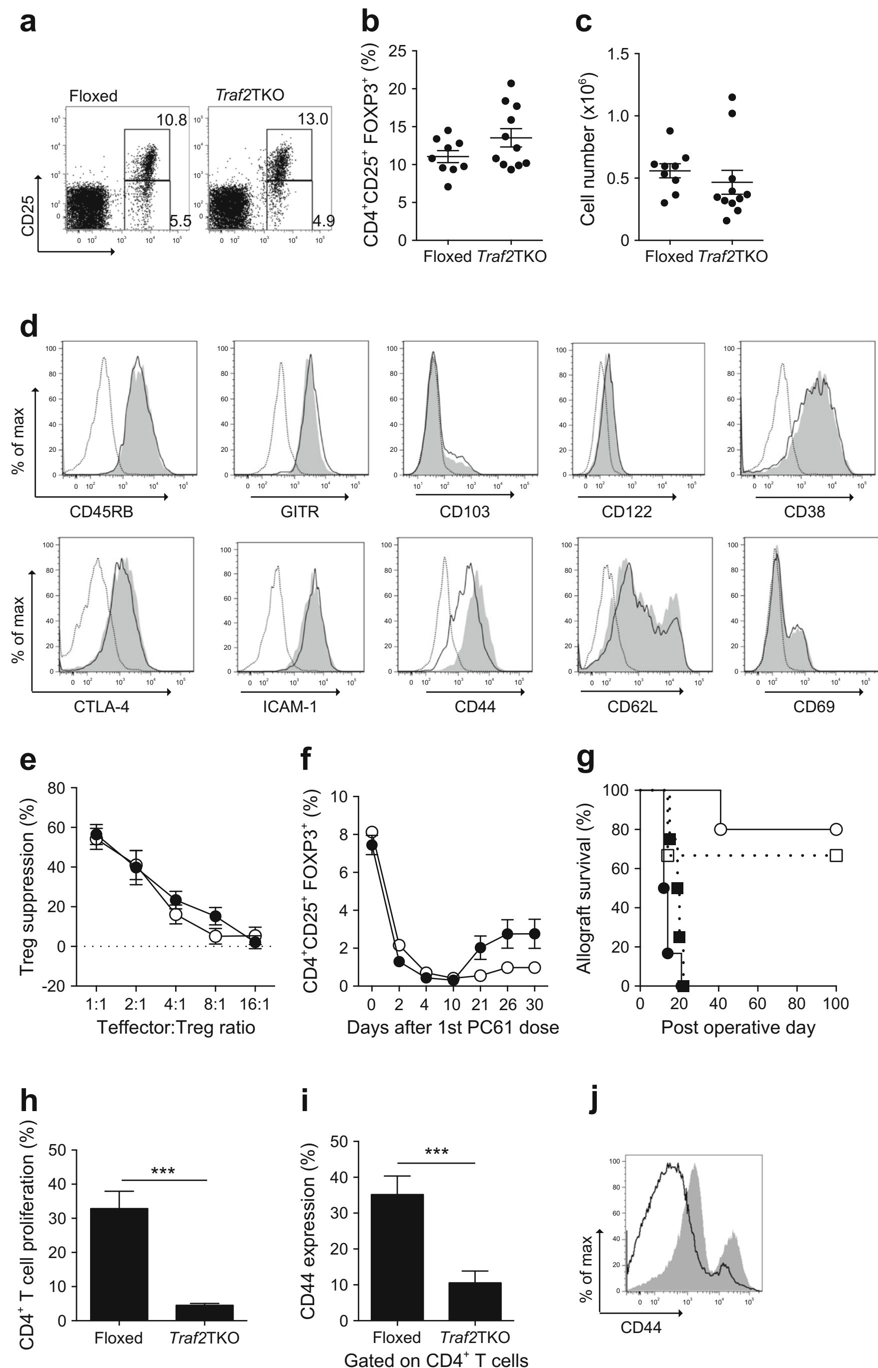

Fig. 6 Prolonged allograft survival in *Traf2*TKO mice is Treg independent. (**a**–**e**) Flow cytometric analysis of floxed and *Traf2*TKO CD4$^+$CD25$^+$FOXP3$^+$ Treg (**a**, **b**) frequency, (**c**) cell number and (**d**) cell surface activation markers. Black solid line, *Traf2*TKO; grey area, floxed; black dotted line, isotype control; $n=9$–11 mice over three independent experiments. (**e**) Treg-mediated suppression of floxed (black circles) or *Traf2*TKO (white circles) CD4$^+$CD25$^+$ T cells co-cultured with CFSE-labelled floxed CD4$^+$CD25$^-$ T cells. Error bars show SEM. Statistical analysis using unpaired *t* test; pooled cells from $n=14$–16 mice over three independent experiments. (**f**) Peripheral blood CD4$^+$CD25$^+$FOXP3$^+$ T cell percentage of floxed (black circles) and *Traf2*TKO (white circles) mice after 100 μg PC61. Error bars show SEM; $n=5$ mice. (**g**) BALB/c islet allograft survival in floxed or C57BL/6 wild-type and *Traf2*TKO mice treated with PC61 or isotype control 3 days before receiving allografts. Statistical analysis using logrank (Mantel–Cox) test; black squares, C57BL/6 untreated ($n=4$); white squares, *Traf2*TKO untreated ($n=3$); black circles, floxed PC61 treated ($n=6$); white circles, *Traf2*TKO PC61 treated ($n=5$). (**h**, **i**) CFSE-labelled floxed or *Traf2*TKO CD4$^+$CD25$^-$ T cells co-cultured with mitomycin-C-treated BALB/c splenocytes, with (**h**) proliferation and (**i**, **j**) CD44 expression analysed by flow cytometry after 3 days. Line, *Traf2*TKO; grey area, floxed; $n=5$ mice over three independent experiments; $^{***}p<0.001$

in vivo alloresponses was specific for CD4$^+$ T cells. In addition, while loss of TRAF2 limited CD4$^+$ anti-CD3-mediated proliferation and T effector function, *Traf2*TKO CD8$^+$ T cells maintained normal T cell activation and proliferation to CD3/CD28 signals, and were able to mount an effective acute-phase response to viral antigen, a naive CD8$^+$ T cell-dependent process [19]. In contrast to *Traf2*TKO CD8$^+$ T cells, *Traf2*TKO CD4$^+$ T cells displayed a reduced activation status (i.e. reduced CD25 and CD44 expression but normal CD62L expression) and decreased CD3/CD28-mediated proliferation. The activation defects of *Traf2*TKO CD4$^+$ T cells may reflect the involvement of TRAF2 with TNFR family co-stimulation molecules (i.e. OX40 and 4-1BB) that enhance their expression on T cells after initial activation [20, 21].

The dysregulated downstream signalling pathways in the absence of TRAF2 may consequently result in the altered T cell activation status observed in this study. *Traf2* deletion preferentially skewed T cells towards a Th2-like profile—a possible consequence of aberrant JNK signalling since JNK2-knockout T cells are highly Th2-like [22]. Th2

cytokines such as IL-13 and IL-4 are coordinately regulated [23] and are associated with prolonged allograft survival. Impaired cardiac allograft rejection has previously been observed, with long-term surviving cardiac allografts exhibiting high expression of IL-4 [24, 25] and following IL-13 administration in rodents [26], whereas impaired donor-derived IL-13 levels have been correlated with an increased severity of graft-vs-host disease [27]. Furthermore, similar to *CD30*$^{-/-}$ CD4$^+$ T cells [28], *Traf2*TKO CD4$^+$ T cells were unable to differentiate into IL-17$^+$ Th17 cells. Blockade of IL-17 has been shown to prolong cardiac allograft survival [29, 30]. These studies suggest that Th17 inhibition and an increased Th2 phenotype propensity of *Traf2*TKO CD4$^+$ T cells could contribute to impaired islet allograft rejection.

Our studies highlight TRAF2 as a potential drug target to improve islet transplant outcomes. While complete ablation of TRAF2 is embryonic-lethal, due to the critical and non-redundant role of TRAF2 in embryogenesis [31], postpartum TRAF2 blockade may not carry these risks. Furthermore, whether transient inhibition of TRAF2 prior to transplantation would promote delayed allograft rejection is also unknown. Future studies could potentially harness the use of small interfering (si)RNA technologies to block TRAF2 specifically on T cells, similar to the T cell-specific siRNA treatment that is used to reduce HIV infection [32]. The natural biology of TRAF2 may also offer up potential therapeutic opportunities. New drugs targeting ubiquitin-editing enzymes such as lenalidomide, which targets the E3 ubiquitin ligase cereblon, are in clinical trials [33]. Furthermore, simultaneous targeting of canonical NFκB and JNK signalling may replicate TRAF2 blockade and offer an alternative therapeutic avenue [34, 35]. The information gathered here about the processes in which TRAF2 is critical for T cell-mediated islet allograft destruction can be used to identify targets for immune modulation that could be applied in the clinical setting of islet transplantation.

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Data availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Author contributions JEV and SNW conducted and analysed islet and skin transplant and histological studies. MS and SIA conducted and analysed heart transplant studies. JEV conducted and analysed in vitro T cells assays, cell signalling studies and T cell polarisation studies. NWZ and EKM established and analysed inhibitor and signalling studies. KAW and NLL designed, conducted, analysed and interpreted viral immunity experiments. RB generated critical genetic tools for the study. JEV and STG interpreted all findings and co-wrote the manuscript. All authors were involved in revising the article critically for important intellectual content and gave final approval of the version to be published. STG designed and led the study and, as guarantor, takes responsibility for the contents of the article.

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