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Research In-Press Preview **Inflammation** **Transplantation**

Islet transplantation can restore lost glycemic control in type 1 diabetes subjects, but is restricted in its clinical application by limiting supplies of islets and the need for heavy immune suppression to prevent rejection. *TNFAIP3*, encoding the ubiquitin editing enzyme A20, regulates the activation of immune cells by raising NF-κB signalling thresholds. Here we show that increasing A20 expression in allogeneic islet grafts resulted in permanent survival for approximately 45% of recipients, and > 80% survival when combined with subtherapeutic rapamycin. Allograft survival was dependent upon regulatory T cells, was antigen-specific and grafts showed reduced expression of inflammatory factors. Transplantation of islets with A20 containing a loss-of-function variant (I325N) resulted in increased RIPK1 ubiquitination and NF-κB signalling, graft hyper-inflammation and acute allograft rejection. Overexpression of A20 in human islets potently reduced expression of inflammatory mediators with no impact on glucose stimulated insulin secretion. Therapeutic administration of A20 raises inflammatory signalling thresholds to favour immune tolerance and promotes islet allogeneic survival. Clinically this would allow for reduced immunosuppression and support the use of alternate islet sources.

A20 as an immune tolerance factor can determine islet transplant outcomes.

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Running title: Optimization of the graft microenvironment for immunological tolerance

Abstract

Islet transplantation can restore lost glycemic control in type 1 diabetes subjects, but is restricted in its clinical application by limiting supplies of islets and the need for heavy immune suppression to prevent rejection. *TNFAIP3*, encoding the ubiquitin editing enzyme A20, regulates the activation of immune cells by raising NF-κB signalling thresholds. Here we show that increasing A20 expression in allogeneic islet grafts resulted in permanent survival for ~45 % of recipients, and >80% survival when combined with subtherapeutic rapamycin. Allograft survival was dependent upon regulatory T cells, was antigen-specific and grafts showed reduced expression of inflammatory factors. Transplantation of islets with A20 containing a loss-of-function variant (I325N) resulted in increased RIPK1 ubiquitination and NF-κB signalling, graft hyper-inflammation and acute allograft rejection. Overexpression of A20 in human islets potently reduced expression of inflammatory mediators with no impact on glucose stimulated insulin secretion. Therapeutic administration of A20 raises inflammatory signalling thresholds to favour immune tolerance and promotes islet allogeneic survival. Clinically this would allow for reduced immunosuppression and support the use of alternate islet sources.

Introduction

Type 1 diabetes (T1D) is an autoimmune condition marked by loss of glycemic control caused by immune-mediated destruction of insulin producing beta cells that reside within the pancreatic islets of Langerhans (1). Replacement of lost beta cells by adult islet allogeneic transplantation restores glycemic control, providing fine-tuned release of insulin in response to blood glucose in real-time, something not yet achievable by manual or automatic injection of insulin or its analogues (2-4). Islet transplantation reduces exogenous insulin requirements and reverses hypo-glycemic unawareness, a life-threatening complication of T1D (3, 5, 6). Although highly successful, the need for robust suppression of host immunity to avoid rejection precludes its indication for pediatric T1D patients, restricting the broader application of islet transplantation to adults with life threatening hypoglycemic unawareness (2, 7).

Islet transplantation is further restricted by the scarcity and fragility of islets. Frequently patients require multiple islet infusions extracted from multiple pancreata to achieve clinical outcomes of insulin independence and reversal of hypo-glycemic unawareness (4, 6, 8). Further to this, most islet transplant recipients show relatively poor long-term outcomes compared to solid organ transplant recipients, requiring a return to insulin injections within a few years post islet transplant (2, 4). Evidence suggests the underlying mechanisms leading to reduced islet allograft survival are unique to islet transplantation and include recurrent islet autoimmunity, sensitivity of islets to the intra-portal transplant site and islet-toxicity of immunosuppressive drugs, as well as factors present in solid organ transplantation such as chronic allograft rejection (2). These factors are likely exacerbated by autologous islet inflammation induced via the isolation process and ex vivo culture that may hasten graft failure and increase their immunogenicity after transplant (9-12). Strategies that reduce islet fragility and inflammation could preserve islet graft mass and improve post-transplant function

potentially reducing the reliance on heavy immunosuppression and widening the eligibility criteria for an islet transplant (10).

TNFAIP3, encoding the ubiquitin editing protein A20, is a master regulator of NF-κB signalling. A20 through its ovarian tumor (OTU) and zinc finger 4 domain modifies ubiquitin chains on key intracellular inflammatory signalling mediators, primarily RIP1 and TRAF6 (13, 14) that lie downstream of inflammatory and danger sensing receptors of the TNF receptor family, including TNFR1, IL-1R and TLRs. In hematopoietic cells A20 functions as a negative regulator of immuno-stimulatory factors and thus governs the threshold for immune activation. Reduced expression of A20 in dendritic cells leads to increased expression of costimulatory molecules and an enhanced ability to activate $CD8^+$ and $CD4^+$ T cells during an immune response (15-17). Further, deletion of A20 in B cells, macrophages and granulocytes results in cell intrinsic hyper-activation and spontaneous inflammatory disease in mice (18-21). In human subjects A20 haploinsufficiency is associated with increased serum cytokines, higher frequencies of TH17 cells and autoimmune disease (22, 23). Thus, by regulating NF-κB activation A20 sets the threshold for the generation of a productive immune response. Here we investigated the impact of changed A20 expression levels in islet allografts on immune-stimulatory thresholds and islet allograft survival.

Results

Forced expression of A20 allows permanent islet allograft survival without needing immunosuppression

Transduction of an islet cell line with an adenoviral vector encoding human A20 (rAd.A20) to force A20 expression to high levels suppressed TNF-induced NF-κB and JNK signalling pathways, inhibited activation of a NF-κB and a AP-1 reporter, and suppressed expression of pro-inflammatory factors associated with allograft rejection (Figure 1A-E; Supplemental Figure 1A and B) (9). Forced expression of A20 in primary mouse islets resulted in suppression of TNF-induced pro-inflammatory genes as compared to control islets transduced with rAd.GFP (GFP-expressing) or left non-infected (NI) (Figure 2A, B and Supplemental Figure 1C). To test the impact of A20 on tissue tolerance to transplanted islets, primary NI islets from BALB/c ($H2^d$) donor mice, or islets transduced with rAd.A20 or rAd.GFP were transplanted into diabetic C57Bl/6 ($H2^b$) allogeneic recipients. Adenoviral transduction did not affect islet graft function in vivo as demonstrated by the ability of both rAd.A20, control rAd.GFP and NI grafts to rapidly restore euglycemia in the immediate post transplantation period (Figure 2C). Kaplan-Meier survival analysis showed rapid rejection of control NI and rAd.GFP transduced islet allografts. In contrast, ~ 50% of mice receiving A20 expressing islets failed to reject their grafts and instead exhibited permanent (>200 days) allograft survival (Figure 2D). Graft removal by survival nephrectomy for some recipients at post-operative day (POD) 100 disrupted glucose control, illustrating that A20-transduced surviving islet grafts were both functional and responsible for euglycemia (Figure 2E). Long-term surviving A20-transduced grafts were characterised by normal islet architecture, robust insulin production and distinct pockets of mononuclear cells within the graft microenvironment (Figure 2F). Improved graft morphology was also evident for A20-expressing grafts at POD 10 (Figure 2G). A20 expressing grafts expressed reduced levels of inflammatory mRNAs such as *Cxcl10*, *Icam1*

and *Ccl2* (Figure 2H; Supplemental Table 1). These same mRNAs were also reduced in long-term surviving A20-expressing grafts (Figure 2H). Thus, forced expression of A20 allows permanent and functional survival of an islet allograft without needing immunosuppressive drugs.

Immune features of A20-induced islet allograft survival

We investigated the immunological mechanism for long-term survival of A20-expressing allografts. After 150 days post transplantation, splenic T cells were harvested from mice with A20-expressing BALB/c ($H2^d$) islet grafts and were adoptively transferred to $RAG^{-/-}$ mice previously transplanted with a BALB/c ($H2^d$) islet allograft. Control groups received splenic T cells harvested from C57/BL6 mice (Figure 3A). In this situation $RAG^{-/-}$ mice receiving T cells taken from mice with surviving A20-expressing grafts took longer to reject their islet grafts, and the majority permanently accepted the allograft, compared to $RAG^{-/-}$ mice receiving T cells from C57/BL6 mice (Figure 3B). Thus, A20-induced islet allograft acceptance is T cell dependent. To determine whether graft acceptance was due to T cell anergy, deletion or regulation, we repeated the above experiment but this time transferred T cells depleted of $CD25^+$ cells from mice harbouring long-term surviving A20-expressing or rejecting control non-infected islet grafts. These T cell preparations lacked $CD4^+CD25^+$ T cells with regulatory potential (24, 25). In this experiment all of the recipient mice rejected the second BALB/c allograft regardless of whether they received effector T cells from mice with A20-expressing grafts or control grafts (Figure 3B). This indicated to us that A20-expression engendered T cell dependent tolerance. To test if graft acceptance was specific to the BALB/c ($H2^d$) alloantigen, we established another cohort of long-term surviving A20-expressing islet graft recipient mice to repeat the above experiment. However, in this case the T cells from mice harbouring A20-expressing long-term surviving grafts were adoptively transferred into $RAG^{-/-}$ mice pre-

transplanted with a MHC-disparate graft from a different ($H2^k$) donor strain (Figure 3C). Subsequently, in all cases the $H2^k$ MHC-mismatched grafts were rapidly rejected. We conclude from these experiments that the major mechanism of tolerance induced by A20-intragraft expression is exerted by T cell dependent and antigen specific immune regulation towards the islet allograft. However, we cannot exclude other complementary mechanisms of tolerance, such as effector T cell deletion or T cell anergy.

Forced expression of A20 promotes the accumulation of intragraft $Foxp3^+$ T cells

Both A20- and GFP-expressing islet allografts were infiltrated with $FOXP3^+$ cells at POD 10 after transplantation (Figure 4A) but the number of $FOXP3^+$ cells within the GFP-graft microenvironment subsequently fell during the time period when grafts were being rejected between POD15-25. In contrast, A20-expressing grafts maintained high numbers of $FOXP3^+$ cells (Figure 4, B-D). Also, long-term surviving A20-expressing grafts (>100 days) showed prominent infiltration of $FOXP3^+$ cells congregated within the peri-graft space appearing to surround each individual islet (Figure 4C). The presence of $FOXP3^+$ cells always correlated with improved islet graft architecture, whereas the immunopathology of rejecting grafts revealed reduced number of $FOXP3^+$ cells with fragmented, less defined islet architecture, patchy insulin labelling and increased numbers of graft $CD4^+$ and $CD8^+$ T cells compared to A20-expressing grafts (Figure 4A-C; Supplemental Figure 2A). A20-expressing grafts at POD10, showed a reduced frequency of $CD8^+$ and $CD4^+$ positive cells within the graft site compared to GFP-expressing grafts (Supplemental Figure 2B and C). Increased $FOXP3^+$ cells and a reduced frequency of $CD8^+$ and $CD4^+$ positive cells within the graft site was associated with elevated levels of *Tgfβ* mRNA and a trend to increased levels of *Il10* mRNA within the A20-expressing islet graft microenvironment (Figure 4D and E; Supplemental Figure 2D). *Ccl22*, a chemokine that attracts Tregs (26), was not found to be differentially

expressed between groups. There was also no overall change in the level of dendritic cell activation markers *Cd80* or *Cd86* between A20- or GFP-expressing grafts at POD 10 (Figure 4E).

Within long-term surviving grafts (>100 days) $FOXP3^+$ cells and high levels of *Foxp3* mRNA was readily detected (Figure 4, C and E). In addition, increased $CD4^+CD25^+FOXP3^+$ cells were also detected in the spleen and graft draining (renal) lymph node of mice harbouring long-term surviving grafts (Figure 4F). Therefore, A20 alters the pro-inflammatory milieu, leading to the accumulation of regulatory T cells within the graft microenvironment.

Reducing Tregs reverses A20-mediated graft survival.

To further investigate the role of regulatory T cells in A20-induced tolerance we treated diabetic recipient mice with the αCD25 mAb clone PC61, which depletes $CD25^+FOXP3^+$ regulatory T cells (Supplemental Figure 3) (27), by preventing CD25 binding to interleukin 2 (28, 29). In this experiment, all of the mice receiving A20-transduced grafts and treated with PC61 mAb at the day of transplantation and every 10 days thereafter, rapidly rejected their grafts with similar rejection times to those observed for control GFP-expressing grafts (Figure 5A). In contrast, 40% of recipients of A20 expressing grafts injected with an isotype control antibody exhibited long-term survival (Figure 5A). We conclude that graft intrinsic expression of A20 can promote regulatory T cell dependent tolerance to an MHC mismatched islet allograft.

Rapamycin (also known as sirolimus) inhibits mammalian target of rapamycin (mTOR) and is used clinically as an immunosuppressant to dampen T cell responses in transplantation (4, 6). Preclinical and clinical studies also indicate that rapamycin promotes $FOXP3^+$ regulatory T cells (30-33), therefore we investigated whether graft intrinsic expression of A20 would

157 synergise with the tolerance promoting properties of rapamycin. For the experiment diabetic
158 C57BL/6 recipients received A20-expressing H-2^d BALB/c islet allografts, as well as seven
159 daily injections of a limiting subtherapeutic dose of rapamycin (34) starting on the day of
160 transplantation (Figure 5B). All control GFP-expressing grafts, and those treated with
161 rapamycin alone, were rapidly rejected, whereas mice receiving A20-expressing grafts and
162 treated with a subtherapeutic dose of rapamycin showed superior graft survival compared to
163 grafts transduced with A20 alone (Figure 5B). These data highlight the translational potential
164 of A20 to synergise with clinical approaches that enhance T regulatory cells and promote
165 significant improvements in islet allograft outcomes.

166

167 **A20 promotes tissue tolerance by regulating RIPK1**

168 As increasing intra-graft A20 levels can promote allograft tolerance by increasing the threshold
169 for NF-κB activation we investigated whether A20 reduction would have the reverse effect and
170 promote inflammation with more aggressive allograft rejection. To test this we utilised an *N*-
171 ethyl-*N*-nitrosourea (ENU)-mutagenesis generated mouse line harbouring a germline A20 loss
172 of function mutation (35, 36). This coding mutation lies within the functional OTU ubiquitin
173 editing domain of A20 (13, 36) and substitutes a conserved isoleucine at amino acid position
174 325 for an asparagine (I325N). When transiently expressed in pancreatic beta cell lines the
175 I325N mutation impairs A20's ability to inhibit TNF-induced NF-κB and JNK reporter
176 activation (Figure 6, A and B; Supplemental Figure 4, A and B), and when overexpressed in
177 wild-type mouse islets the I325N A20 variant shows a reduced ability to inhibit TNF-induced
178 inflammatory gene expression as compared to islet expressing wild-type A20 (Figure 6C). A20
179 regulates inflammatory signalling by terminating RIPK1 activation via cleavage of K63
180 ubiquitin chains with its OTU domain and targeting substrates for K48-mediated proteosomal
181 degradation via its zinc finger 4 ubiquitin ligase domain (13). The I325N mutation does not

alter A20 protein stability, nor A20's capacity to interact with key substrates RIPK1 or NEMO in beta cells (Figure 6, D and E; Supplemental Figure 4C and D). Rather, the I325N mutation resulted in increased accumulation of RIPK1, consistent with a reduction in A20's ubiquitin editing function (13). Islets isolated from I325N mice exhibit increased TNF-induced gene expression compared to wild-type islets (Figure. 6F). When transplanted into diabetic allogeneic recipients I325N islets showed accelerated rejection, and a hyper-inflammatory graft microenvironment with heightened expression of *Cxcl10*, *Ifny* and reduced expression of *Tgfb* (Figure 6G-I). Thus, A20 is necessary to control islet homeostasis in response to inflammatory triggers. In the specific context of islet transplantation changing A20 levels can function as an immune modulatory control switch that dictates islet transplant outcomes by regulating islet RIPK1 levels.

Reduced inflammation contributes to long term allograft survival.

We interpret the current data to show that A20 improves islet graft outcomes via changing graft inflammation. Amongst the most differentially expressed inflammatory genes in the two opposing situations of A20 overexpressing grafts, and A20 haploinsufficient grafts, was CXCL10 (Figure. 7A); a chemokine essential for the recruitment of T effector cells to the graft site (37-39). A20 was able to directly inhibit CXCL10 transcription, as transfection of an islet cell line with human A20 was sufficient to inhibit activation of a human CXCL10 reporter stimulated with TNF or IL1β alone, or a cytokine cocktail including TNF, IL-1β and IFNγ (Figure 7, B and C). To test the effect of blocking CXCL10 in our islet allograft model, we administered an anti-CXCL10 mAb (2 mg/kg I.V.) to transplant recipients on the day of transplantation and every 2 days thereafter. Consequently, we found that mice receiving anti CXCL10 mAb showed prolonged graft survival increasing from a MST of 20 days (n = 5) up to maximum of ~50 days (n = 5) days post-transplantation (p = 0.04) (Figure 7. D). These

experiments reveal one protective effect of A20 is to change the graft inflammatory profile, as exemplified by the reduction in CXCL10, with subsequent impact upon transplant outcomes.

Forced expression of A20 in human islets

We transduced human islets with rAd.A20 to test the clinical potential of A20 overexpression. Transduction of human islets with an adenoviral vector encoding GFP at a multiplicity of infection (MOI) of 10:1 was found to infect ~60% of islet cells (Figure 8A). Increasing the MOI up to 30:1 did not significantly improve transduction rates. Immunofluorescent microscopy revealed the majority of infected transduced GFP bright cells to comprise the outer cellular layer of the islet (Figure 8B). Using a MOI of 10:1, we transduced human islets with rAd.A20, which resulted in high levels of A20 mRNA and protein expression (Figure 8, C and D). A20 overexpression did not impact human islet function as shown by the normal glucose stimulated insulin response of rAd.A20 transduced islets (Figure 8E). However, forced expression of A20 blunted the upregulation of inflammatory genes in response to TNF stimulation (Figure 8F; Supplemental Table 2).

Discussion

Here we investigated the impact of changing A20 expression levels in islet allografts as a mechanism to modify immune-stimulatory thresholds and islet allograft survival. A potential role for A20 in transplantation was first indicated in a rodent heart xenotransplantation model where it was found that surviving hearts showed intra-graft A20 expression compared to rejecting hearts (40). Further studies showed that A20 reduces endothelial inflammation during xenotransplantation (41), reduces the severity of graft arteriosclerosis (42) and improves liver graft function (43). In pancreatic islets and beta cells A20 exerts a negative feedback role to

control inflammation. A20 expression is transcriptionally regulated in beta cells by NF-κB activation (44) and once expressed A20 inhibits NF-κB activation in islets (45, 46) by altering RIPK1 stability (36, 47). That A20 forms a part of the natural physiological response of islets to inflammation suggests that manipulating A20 levels in islets would have a good safety profile but also have clinical potential as an approach for the suppression of otherwise deleterious NF-κB-dependent inflammatory genes (9, 10). This is supported by studies showing A20 to be a potent inhibitor of NF-κB-mediated inflammation and cell death in pancreatic islets (46, 48, 49), and that A20 expression reduces syngeneic islet graft apoptosis and improves graft metabolic control (50).

Here we show A20 modulates the local graft micro-environment to generate a state which has features reminiscent of allograft tolerance. The cellular mechanisms by which A20 overexpression increases tissue tolerance includes potent inhibition of NF-κB and JNK/AP1 pathways that reduces inflammation at the graft site. The impact of A20 on NF-κB and JNK-AP1 pathways is dependent upon modification of RIPK1 polyubiquitin editing. As RIPK1 is regulated through ubiquitination (13, 47), this indicates that RIPK1 stability and or function at the TNF receptor represents a potential molecular node for tolerance regulation.

As one specific example of how A20 alters graft inflammation, we found the chemokine CXCL10 to be distinctly differentially regulated between A20 overexpressing and A20 I325N mutant grafts (51). CXCL10 has potent anti-graft effects in transplantation. CXCL10 has been demonstrated to promote immune infiltration and destruction of islets in autoimmune models (37), and is highly expressed in insulitic lesions in patients with recent-onset T1D (52). Further to this CXCL10 promotes immune infiltration in islet isografts (53) and allografts (38, 54), and is linked to poor clinical islet transplant outcomes (9, 55). Suppression of CXCL10 is significant as neutralisation of CXCL10 can prolong graft survival in our model. Together these data show that by controlling RIPK1 polyubiquitination, A20 regulates signal strength through

NF-κB and JNK, which in turn potently supresses graft inflammatory gene expression, such that the islet graft presents a less inflammatory image to the immune system.

Messenger RNA transcripts altered within the graft microenvironment include cytokines that influence T cell polarisation, namely, increased transcripts for TGFβ and IL10, and reduced transcripts for IL6 and TNF (56-59). Bettelli, et al. (56) showed a cytokine-dependent development dichotomy between Tregs and cytotoxic Th17 cells, largely based on a balance between TFGβ and IL6 levels, whereby TGFβ in the presence of IL6 supports Treg differentiation. Together, our data shows A20 can promote tolerance by modulating the inflammatory milieu of the islet graft. The altered graft microenvironment engenders the local maintenance of $Foxp3^+$ Tregs at the graft site providing long term graft acceptance.

The cytokine milieu supporting graft rejection represents a promising therapeutic target, as increasing the levels of BAFF (60), IL-2 (61), or increasing IL-2 while simultaneously blocking IL-15 (62) can result in stable allograft tolerance in rodent transplant models. Different to systemic approaches here we show blunting inflammatory cytokine and chemokine production within the graft microenvironment by increasing intragraft A20 harnesses Foxp3-expressing Tregs and limits inflammatory T cell recruitment.

It is of interest to consider whether once allograft tolerance is established Tregs are required for the continued maintenance phase. In experiments not shown we treated mice with long term surviving (>100 days) and A20 expressing islet allografts with four doses of PC61 mAb every ten days to reduce Tregs, or with a single dose of FTY720 to sequester T cells within lymph nodes (63). In both experiments graft function remained stable and no grafts were lost. These results are consistent with previous studies that manipulate Tregs showing a central role for Tregs in the early acceptance phase of allograft tolerance but not the later phase where established tolerance is relatively robust to Treg depletion (60, 61). These data suggest different mechanisms are involved once stable engraftment has occurred. Indeed, A20 expression has

been previously identified as a factor contributing to long term surviving vascular xenografts without evidence of ongoing immune reactivity in a process referred to as "accommodation" (40).

Different to its known role in controlling immune cell activation thresholds (15-21), our data highlights *TNFAIP3* as a crucial gene for the maintenance of tissue tolerance via its role in dampening islet responses to NF-κB-inducing inflammatory mediators. A20 plays a central role to maintain intestinal tolerance, where a loss of A20 leads to aberrant responses to gut microbiota with intestinal inflammation (21, 64). The requirement of A20 for both tissue homeostasis as well as immune cell activation may be a potent contributing factor driving the GWAS association of A20 with many autoimmune and inflammatory disorders (65). Further to this, A20 haploinsufficient human subjects present with increased Th17 cells and inflammatory disease (22, 23), whereas mice with A20 germline deficiency succumb to uncontrolled inflammation (66).

Here we show that manipulating A20 levels to achieve tissue tolerance can be directed to improve islet transplantation outcomes by changing the inflammatory environment at the graft site, which subsequently alters the instructive signals received by T cells leading to an altered balance of Tregs that favours immunological tolerance. Our findings indicate that the strategy of increasing A20 expression in the graft will likely synergise well with two classes of drugs. 1) T-reg promoting compounds such as rapamycin (30-33), anti-IL2 mAb (61, 62) and or local expression of the regulatory T cell attractant CCL22 (67) and 2) anti-inflammatory drugs that target TNF and IL1β pathways (7, 68).

Methods

Animal models

C57BL/6 and BALB/c mice were purchased from the Animal BioResource Centre (Sydney, Australia). $RAG^{-/-}$ and CBA mice were purchased from the Australian Resources Centre (Perth, Australia). Male, inbred, B6.129S7-$Rag1^{tm1Mom}$/J mice were purchased from Jackson Laboratories (Bar Harbor Main USA). Mice were used at 8-10 weeks of age for all experiments.

Mouse islet isolation and transplantation

Islets were isolated as previously described (49), and counted for islet transplantation or in vitro experiments using a Leica MZ9.5 stereomicroscope. Three hundred islets were transplanted under the kidney capsule of diabetic C57BL/6 littermates (50). This strain combination represents a complete MHC mismatch. Diabetes was induced by intraperitoneal injection of 180 mg/kg streptozotocin (Sigma-Aldrich) dissolved in 0.1 M citrate buffer (pH 4.2) at a concentration of 20 mg/ml. Diabetes was determined as [blood glucose] ≥16 mM on two consecutive days measured by FreeStyle Lite® glucometer and Abbott Diabetes Care test strips following tail tipping. In some experiments transplanted mice were treated with Rapamycin, anti-CD25 (clone: PC61) monocolonal antibody or anti-CXCL0 monoclonal antibody. Rapamycin (LC laboratories) was dissolved in vehicle solution (0.2 % carboxylethyl cellulose, 0.25 % polysorbate-80 in 0.9 % NaCl) and administered by intraperitoneal injection on the day of transplantation and everyday thereafter for 7 days. PC61 was used for in vivo depletion of $CD4^+CD25^+$ T cells, mice were injected with purified rat anti-mouse CD25 IgG1 mAb (PC61; BioExpress) intravenously (200 μg) on the day of transplantation and every 10 days thereafter. The efficacy of CD25 depletion was confirmed by flow cytometry. Anit-CXCL10 was administered at 2 mg/kg by tail vein injection on the day of transplantation and every 2 days

thereafter and was a kind gift from Charles MacKay (Department of Immunology, Monash University, Melbourne, Australia). Islet grafts were retrieved from recipients at indicated time points post-transplantation for analysis of islet morphology, degree of lymphocytic infiltration by histology or gene expression by RT-qPCR. Gene expression in islet grafts was calculated using the average WT ΔCt value. Islets to be used for in vitro studies were cultured overnight in islet overnight culture media (RPMI-1640, 20% FCS, 100 U/ml P/S, 2 mM L-Glutamine) at 37^{o} C + 5% CO2.

Adoptive Transfer

RAG$^{-/-}$ mice were pre-transplanted with an islet graft and left to rest for 14 days. Following 14 days, spleens were obtained from mice harboring long-term surviving grafts. Harvested spleens were prepared by mechanical disruption to single cell suspensions, erythrocytes were removed by osmotic lysis with sterile red blood cell lysis solution (0.156M Ammonium chloride, 0.01M, Sodium hydrogen carbonate, 1mM EDTA) and filtered through 70 µm nylon strainer (Becton Dickinson). CD4^{+} CD8^{+} T cell subsets were isolated via magnetic separation using Pan T cell kit (Miltenyi Biotec). Effector-T-cells (CD25^{-}) were isolated from T-cell populations by positive depletion of CD25^{+} cells using the CD25 MicroBead kit following manufacturer's instructions. Magnetic separations were performed using AUTOMACS (Miltenyi Biotec), to a purity > 95 % as assessed by flow cytometric analysis as described below. Splenocytes T cells (2×10^{7}), T cells (2×10^{6}) or effector T-cells (2×10^{6}) were adoptively transferred via tail vein injection.

Cell lines

Min6 cells, generated by (69) are derived from the pancreatic beta cells of transgenic mice and immortalised by tranduction with T-antigen of simian virus 40 (SV40). MIN6 cells retain the ability to secrete insulin (70, 71). Cells were maintained in Dulbecco's modified Eagles media (DMEM) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 12.5 mM HEPES (Gibco), 0.002% β-mercaptoethanol (Sigma), 1 % (100 U/ml) penicillin/streptomycin (Gibco) and incubated at 37ºC in 5 % CO_2. Passage 31-40 was used for experiments. Higher passage number MIN6 was avoided due to insulin secretion defects reported (71). β-TC_3 cells, derived from insulinoma cells that arise in the pancreatic beta cells of transgenic mice expressing SV40 T antigen under the control of the rat insulin II promoter (RIP) (72, 73). These cells were cultured in Roswell Park Memorial Institute (RPMI) supplemented with 10 % fetal calf serum, 4 mM L-glutamine and 100 U/ml penicillin/streptomycin (Gibco) and incubated at 37ºC in 5 % CO_2. A passage 21-40 used for experiments. Cell line source, American Tissue Culture Collection.

Recombinant adenovirus mediated gene transfer

Islets and insulinoma MIN6 cells were transduced with recombinant adenovirus (rAd.) to over-express GFP (rAd.GFP) or A20 (rAd.A20) as described previously (48, 49). For islet gene transduction, islets were inoculated with virus at the stated multiplicity of infection, and incubated for 1.5 h at 37°C in 0.5 ml serum free RPMI-1640 medium (Gibco). Islets were then ready for further culture or transplantation. MIN6 cells were plated at a density of ~1x106/well in 6-well tissue culture plates (Corning CoStar) and inoculated with virus at the optimal MOI of 100:1 in DMEM (Gibco). After 1.5 h, cells were replenished with DMEM 10% FCS and cultured a further 24 h before use. Adenovirus was propagated by infecting HEK293 cells in six T175 vented flasks (Coring CoStar). Cells were lysed and adenoviruses were extracted using Aenopure kit according to instructions provided (PureSyn Inc.). The purified virus was

titrated and quantified in HEK293 cells using the Adeno-X Rapid Titer Kit (clontech) according to manufacturer's instructions.

Transgene expression

GFP-expression was determined by fluorescent microscopy, images were captured under a Zeiss inverted fluorecence microscope (Carl Zeiss Inc., Jena, Germany). Islets expressing GFP were made to a single cell suspension with 0.1 % trypsin and run through a CytoFLEX (Beckman) or CantoII (BD) flow cytometer to determine GFP expression level. For A20 and IκBα protein expression, primary islets or MIN6 cells were cultured for 24 h after gene transduction and lysed in RIPA buffer, ~10 μg of total protein was resolved on a 10% SDS PAGE gel and then transferred to a nitrocellulose membrane. Membranes were incubated with polyclonal anti-A20 (Abcam, UK) and IκBα (Cell Signalling Technology, USA) respectively, and followed by peroxidase labelled secondary antibodies. Signals were visualised using an ECL detection kit (Amersham Pharmacia Biotech, Australia).

Reporter Assays

Reporter assays were carried out essentially as we have described (44, 74). For NF-κB activity, βTC3 cells were transfected with 0.3 μg of a NF-κB.Luciferase reporter with 0.25 μg CMV.β-galactosidase. pcDNA vectors encoding human A20 or empty vector up to 1ug total DNA. AP-1 activity was determined by the Cignal AP1 Reporter (luc) Kit® (SABiosciences, Australia) according to the manufactures instructions. Transfection was conducted using lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Following transfection cells were stimulated with 200 U/ml of TNF or 200 U/ml of each IL1β, TNF and INFγ (R&D Systems). Luciferase activity was assayed in cell lysates harvested 8 h post stimulation, using a luciferase

399 assay kit (Promega). Results were normalized to β-galactosidase or Renilla activity
400 (Galactostar) to give relative luciferase activity. Expression plasmids and reporters were
401 obtained and maintained as described previously (44, 74). CXCL10 endogenous reporter (GL-
402 IP10) (75) was a kind gift from Richard Ransohoff's lab (Third Rock Ventures, Boston).

403

404 **Immunohistochemistry**

405 Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich), paraffin embedded and
406 parallel sections (5 μm) prepared. Sections were stained with hematoxylin and eosin (H&E;
407 Sigma-Aldrich) or for insulin, FOXP3, CD4 or CD8 followed by counterstain with
408 hematoxylin. To stain for insulin purified rabbit anti-mouse insulin polyclonal antibody was
409 used (4950, Cell Signaling Technology) followed by a HRP-labelled polymer-conjugated goat
410 anti-rabbit IgG (Dako EnVision+ System) with DAB substrate (Sigma-Aldrich) used for
411 visualization. To stain for FOXP3 antigen retrieval was first performed using a pressure cooker
412 (Dako Cytomation), filled with 10 mM citrate, pH 6 (Dako Cytomation) and set to 125^{o}C with
413 30 s at the maximal pressure set to 10 psi. Polyclonal anti-mouse/rat FOXP3 was used for
414 primary antibody staining of FOXP3 antigen (FJK-16S, eBioscience) and followed by
415 secondary biotin anti-rat with spacer to amplify the signal (112-066-071, Jackson
416 ImmunoReseach Laboratories) and visualisation of the signal achieved by using Vectastain
417 Elite ABC kit (Vector Laboratoires, CA). CD4 and CD8 staining of paraffin sections was
418 conducted at St. Vincent Hospital, Darlinghurst, Australia clinical histology core. Images were
419 captured using a Leica DM 4000 (Leica Microsystems).

420 Immunofluorescence was performed on paraffin sections, as previously described (76).
421 Primary antibodies included anti-insulin (IR00261-2, DAKO), anti-glucagon (G2654, Sigma-
422 Aldrich), anti-cytokeratin (CK) 7 anti-A20 (M7018, AbCAM) and anti-A20 (PA-20728,

Thermo Fisher). All appropriate species-specific secondary antibodies were AlexaFluor 488 or 594 conjugates (Thermo Fisher) and diluted 1/200 in 5% normal goat serum. Slides were coverslipped with ProLong Gold antifade reagent with DAPI (4′,6-diamidino2-phenylindole; Thermo Fisher) to counterstain nuclei and preserve fluorescence.

Immunoblot analysis and immunoprecipitation

Primary islets were lysed in islet lysis buffer (50 mM Tris-HCL pH7.5, 1% Triton X, 1043 0.27 M sucrose, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 5 mM 1044 Na4P2O7, 0.1% β-mercaptoethanol; supplemented with EDTA-free protease inhibitor 1045 [Roche]), cell lines were lysed with radioimmunoprecipitation (RIPA) buffer with SDS, following relevant treatment with or without 200 U/ml of recombinant human TNF (R&D Systems). Protein concentration was measured using the Bradford assay (Bio-Rad) and total protein (20-25 μg) resolved on a 7 – 10% SDS PAGE gel and then transferred to a nitrocellulose membrane, Immobilon-P® (Merck Millipore). Immunoprecipitation was conducted by first preclearing lysates with protein A/G-Sepharose (Thermo Fisher Scientific) for 1 h and then incubated with anti-A20 (59A426) antibody (Abcam) or anti-IKKγ (2585) (Cell Signaling Technology) for 2 h at 4 °C. Following incubation with only antibody, 25 μl of protein A/G beads were added and incubated at 4 °C on a roller overnight. Beads were washed 4× with lysis buffer and then eluted with 30 μl of Laemmli reducing gel-loading sample buffer. Samples were vortexed, heated to 100 °C for 5 min, cooled on ice for 10 min, and then loaded onto an 8-10% agarose gel for immunoblotting. Membranes were incubated with anti-A20 (56305/D13H3), anti-IκBα (9242), anti-IKKγ (2585), anti-JNK (9252), anti phospho-JNK (9255) (Cell Signaling Technology); or anti-RIPK1 (610458) (BD bioscience); or anti-beta-actin (AC15) (Sigma-Aldrich), followed by horseradish peroxidase (HRP)-labelled secondary antibody goat-anti mouse IgG Fc (Pierce Antibodies) or donkey-anti-rabbit IgG (GE Life Sciences). HRP

conjugates bound to antigen were detected and visualized by using an ECL detection kit (GE Life Sciences).

Real Time quantitative PCR

Mouse islets or neonatal porcine were isolated and placed into 12-well non-tissue culture-treated plates (150-200 islets/well; Fisher Scientific). Following an overnight culture cells were treated with 200 U/ml recombinant human TNF or of each IL1β, TNF and INFγ (R&D Systems). In some experiments cells were also pre-treated with pharmacological inhibitors, pyrrolidine dithiocarbamate (PDTC) and SP600125 (Sigma-Aldrich). Inhibitors were added at listed concentrations and incubated with cells at 37^{o}C for 1.5 h prior to cytokine stimulation or islet transplantation. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed using Quantitect Reverse Transcription Kit (Qiagen). Primers were designed using Primer3 (77) with sequences obtained from GenBank and synthesized by Sigma Aldrich (Supplemental Table 1 and 2). PCR reactions were performed on the LightCycler® 480 Real Time PCR System (Roche) using the FastStart SYBR Green Master Mix (Roche). Cyclophilin (CPH2) and ACTB were used as housekeeping genes and data analysed using the 2ΔΔCT method. Initial denaturation was =performed at 95° C for 10 sec, followed by a three-step cycle consisting of 95° C for 15 sec =(4.8° C/s, denaturation), 63° C for 30 sec (2.5° C/sec, annealing), and 72° C for 30 sec (4.8° C/s, elongation). A melt-curve was performed after finalization of 45 cycles at 95° C for 2 min, 40° C for 3 min and gradual increase to 95° C with 25 acquisitions/° C.

Flowcytometry:

Flow cytometry staining was performed as described (27). Mouse lymphocytes were incubated with the following fluorochrome-conjugated antibodies: CD4 (RM4-5), CD8 (53-6.7), CD3 (17A2), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), B220 (RA3-6B2), FOXP3 (FJK-165). Antibodies were purchased from BD Bioscience, Biolegend or ebiosciences. Data were acquired with CytoFLEX (Beckman) or CantoII (BD) flow cytometer and analysed using FlowJo software (Tree Star).

Statistics

All data are presented as mean ± s.e.m or ± s.d. 2-way Student's *t*-test or 1- or 2- way ANOVA analysis were performed, depending on experimental design, to determine statistical difference between groups. A P value less than 0.05 was considered significant. For allograft survival, day of rejection was plotted as Kaplan Meier curves and analyzed using the Log-rank test. Tests were conducted on Prism (v8) software (GraphPad Software).

Study approval

All procedures involving animals were carried out according to the guidelines established by the Australian Institutional Animal Ethics Committee guidelines. Animal studies were approved by the Garvan/St Vincent's Animal Ethics Committee. All procedures performed complied with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

Human islets were obtained from Alberta Diabetes Institute IsletCore (University of Alberta)(78). Ethics approval for work with human tissue was obtained from the Health Research Ethics Board, Biomedical Panel, University of Alberta (Study ID Pro00001416).

Author contributions:

Mouse islet isolation, transplantation experiments, adoptive transfer experiments and analysis: N.W.Z, S.N.W and S.T.G Molecular studies for cell lines, islets and analysis: N.W.Z and S.T.G Analysis of I325N mice: N.W.Z and S.T.G. Human islet experimentation and analysis: N.W.Z, K.L.S, G.S.K. and S.T.G. N.W.Z and S.T.G co-wrote the manuscript. All authors read and approved the manuscript. S.T.G. conceived and designed the study.

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Figures and figure legends

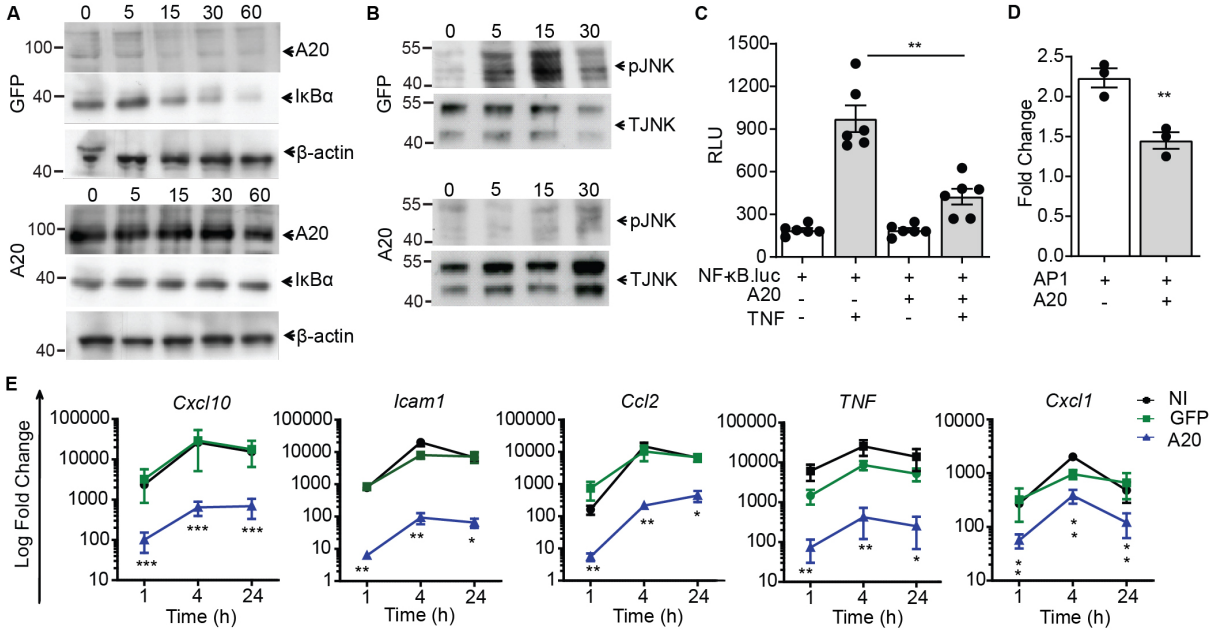

Figure 1. A20 inhibits TNF-induced inflammatory signalling in beta cells. (A, B) Immunoblot of lysates from MIN6 beta cells transduced with recombinant adenovirus encoding GFP or human A20 (MOI 100:1) and stimulated with 200 U/ml TNF for the indicated times and probed with antibodies for A20, IκBα or β-actin (loading control). **(C, D)** βTC3 cells co-transfected with a NF-κB.luciferase reporter (C) or an AP-1.luciferase reporter (D) and a CMV.βgal expression construct ± PCDNA3.1 encoding A20 and stimulated with 200 U/ml TNF for 8 h or left untreated. RLU = Relative light units (Luciferase/β-gal). **(E)** GFP or A20 transduced MIN6 cells, or non-infected (NI) cells, treated with 200U/ml of each TNF, IL-1β and IFNγ for 1, 4 and 24 h and expression of induced genes assessed. Error bars represent mean ± s.e.m. Data represents three independent experiments and statistical significance determined by 1-way ANOVA with Tukey's multiple-comparisons post hoc test (C, E) or 2-tailed Student's *t*-test (D). **P* < 0.05; ***P* < 0.01; *** *P* < 0.001.

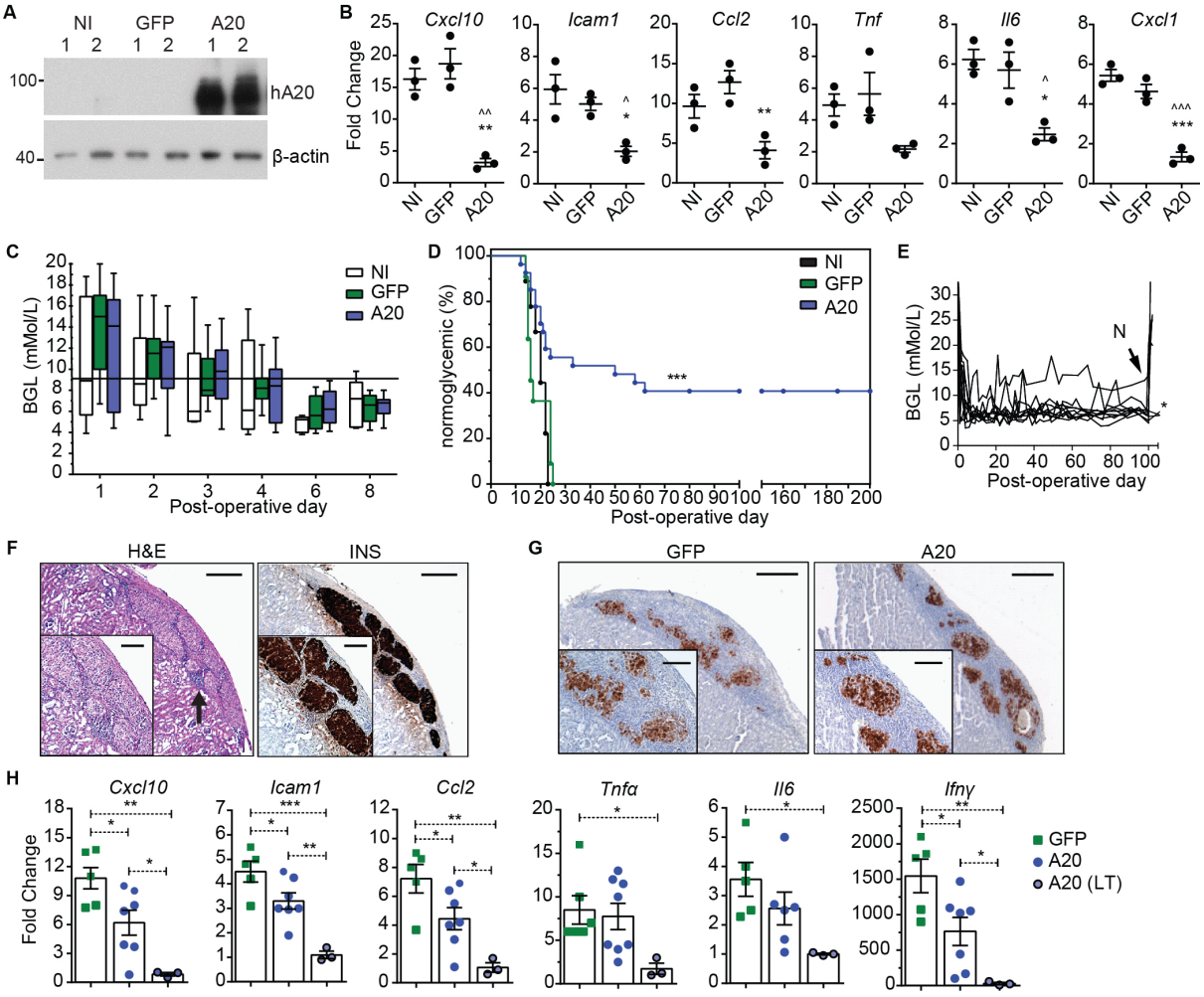

Figure 2. Improved survival characteristics of an A20 expressing islet allograft. Primary islet preparations transduced with adenoviral constructs encoding for GFP, human A20 or left non-infected (NI) were **(A)** lysed in duplicate (1 and 2) and A20 protein levels assessed by immunoblot, or **(B)** treated with 200 U/ml of TNF for 4 h and expression of inflammatory factors measured (* = A20 versus GFP; ^ = A20 versus NI). Data represents 3 independent islet preparations. **(C, D)** 300 NI islets (n = 11) or those expressing GFP (n = 9; p = 0.16) or A20 (n = 27; p = 0.002) were transplanted under the kidney capsule of allogeneic C57BL/6 mice and (C) blood glucose levels (BGL) and (D) percent of mice remaining normoglycemic monitored for the indicated days. Significance determined by Log-rank test. **(E)** Nephrectomies (N) were conducted at post-operative day (POD) 100 for a portion of A20-expressing long-term surviving islet grafts. **(F)** Hematoxylin & Eosin staining (H&E) or insulin labelling (INS) of long-term (>100 days) surviving grafts, representative of 7 long-term surviving grafts. **(G)** Insulin staining of GFP or A20 expressing grafts at POD10. Scale bar = 200 µm (4× magnification) and 100 µm for panel inserts (10× magnification), representative of 4 islet grafts per treatment. **(H)** RNA levels of inflammatory factors from GFP (closed square) or A20 (closed circle) expressing grafts harvested at POD10, as well as A20-transduced long-term surviving grafts harvested at > POD100 (open circle). Each point in a column represents an individual islet graft. Non-transplanted overnight cultured isolated islets were used as base-line. Error bars ± s.e.m and statistical significance determined by 1-way ANOVA with Tukey's multiple-comparisons post hoc test, *$P < 0.05$; **$P < 0.01$.

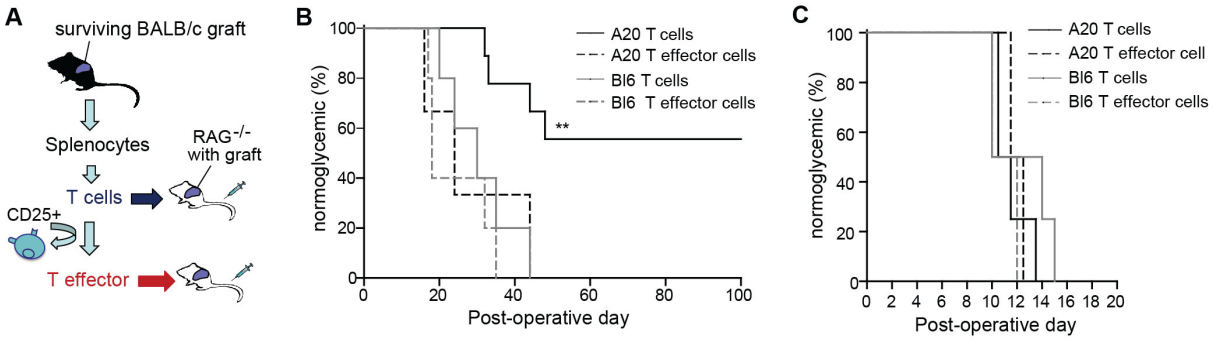

Figure 3. A20-induced islet allograft survival is T cell dependent and is antigen specific. **(A)** Experimental strategy. **(B)** Diabetic RAG$^{-/-}$ mice pre-transplanted with 300 BALB/c islets to restore euglycemia were adoptively transferred with T cells or CD25 depleted T effector cells, from mice harbouring long-term surviving grafts (A20 T cells, n = 9; A20 T effector cells., n = 3) or control C57BL/6 mice (B6 T cells, n = 5; B6 T effector cells n = 5). Blood glucose levels were monitored and percent of mice remaining normoglycemic recorded as a read out of islet graft function. **(C)** Diabetic RAG$^{-/-}$ mice were pre-transplanted with 300 islets from third-party CBA (H2^{k}) donors, and adoptively transferred with T cells or CD25 depleted T effector cells from mice harbouring long-term surviving grafts (A20 T cell, n = 4; A20 T effector cell., n = 2) or control C57BL/6 grafts (Bl6 T cell, n = 4; B6 T effector cell, n = 2). Blood glucose levels were monitored and percent of mice remaining normoglycemic recorded as a read out of islet graft function. Significance determined by Log-rank test (B, C), $^{**}P < 0.01$.

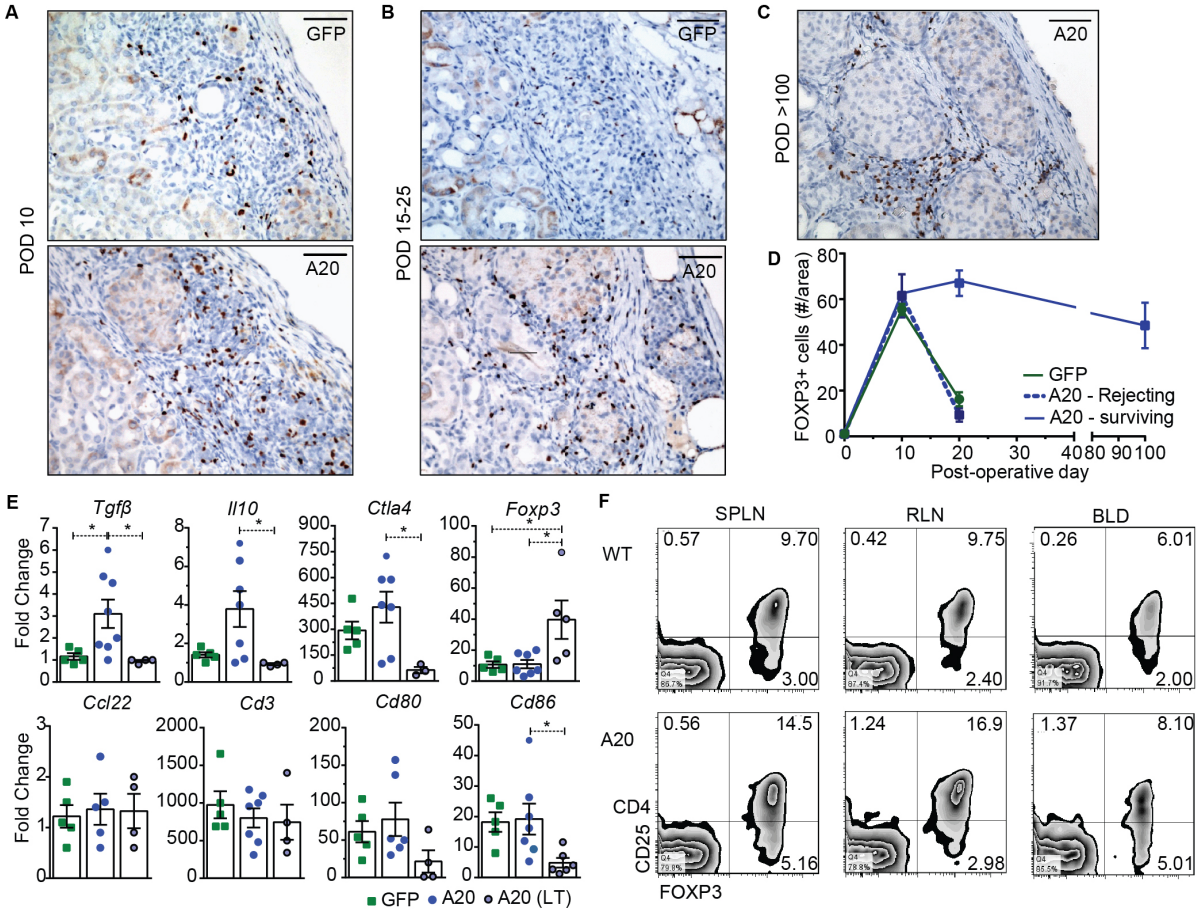

Figure 4. Long-term surviving grafts have graft infiltrating FOXP3$^+$ cells. **(A)** Representative sections of FOXP3 stained GFP or human A20 transduced islet allografts at post-operative day (POD)10 (n = 4 GFP and 4 A20), **(B)** POD 15-25 (GFP grafts taken before rejection; n = 6 GFP and 7 A20) and, **(C)** POD > 100 (n = 6). **(D)** Quantification of FOXP3$^+$ cells. Scale bar = 100 µm. **(E)** GFP or A20 expressing grafts harvested at POD10, as well as, A20-expressing long-term surviving grafts harvested at >POD100 and subjected to RTPCR for known immune regulatory factors. Non-transplanted overnight cultured isolated islets were used as base-line. Each point represents an individual islet graft. **(F)** Flow cytometric analysis of CD4$^+$CD25$^+$Foxp3$^+$ cells from the spleen (SPLN), renal lymph node (RLN), and blood from C57BL/6 mice (WT; n = 5) and C57BL/6 recipients harbouring long-term surviving A20 transduced grafts (A20; n = 5). Error bars ± s.e.m and statistical significance determined by 1-way ANOVA with Tukey's multiple-comparisons post hoc test, *$P < 0.05$.

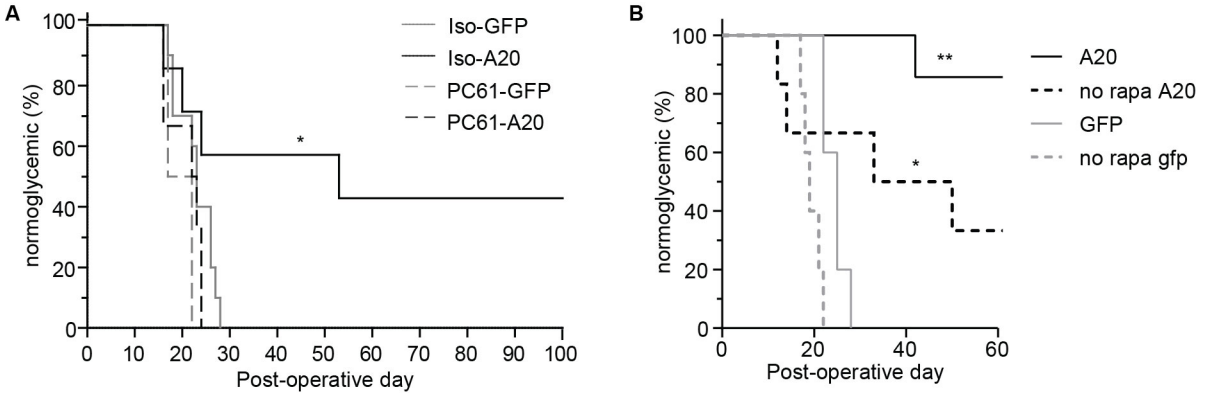

Figure 5. Administration of anti-CD25 ablates, and Rapamycin enhances, the protective potential of intragraft A20 expression. (A) Percent of mice normoglycemic following the transplantation of 300 BALB/c islet expressing GFP or A20 (multiplicity of infection = 10:1) and transplanted under the kidney capsule of allogeneic C57BL/6 recipients. Recipient mice were administered 200 µg αCD25, clone PC61 (PC61-GFP, n = 2; PC61-A20, n = 6) or an isotype-control (Iso-GFP, n = 10; Iso-A20, n = 7) on day 0 and every 10 days thereafter (Supplemental Figure 3). **(B)** Percent of C57BL/6 mice normoglycemic after receiving allogeneic BALB/c islet grafts transduced with GFP or A20 and administered a low dose of Rapamycin (0.1 mg/kg at day of transplantation and every day thereafter for 7 days; A20 n = 7, GFP n = 5), or no Rapamycin (A20 n = 7, GFP n = 5). Data is cumulative over three independent experiments. Significance determined by Log-rank test, *$P < 0.05$; **$P < 0.01$.

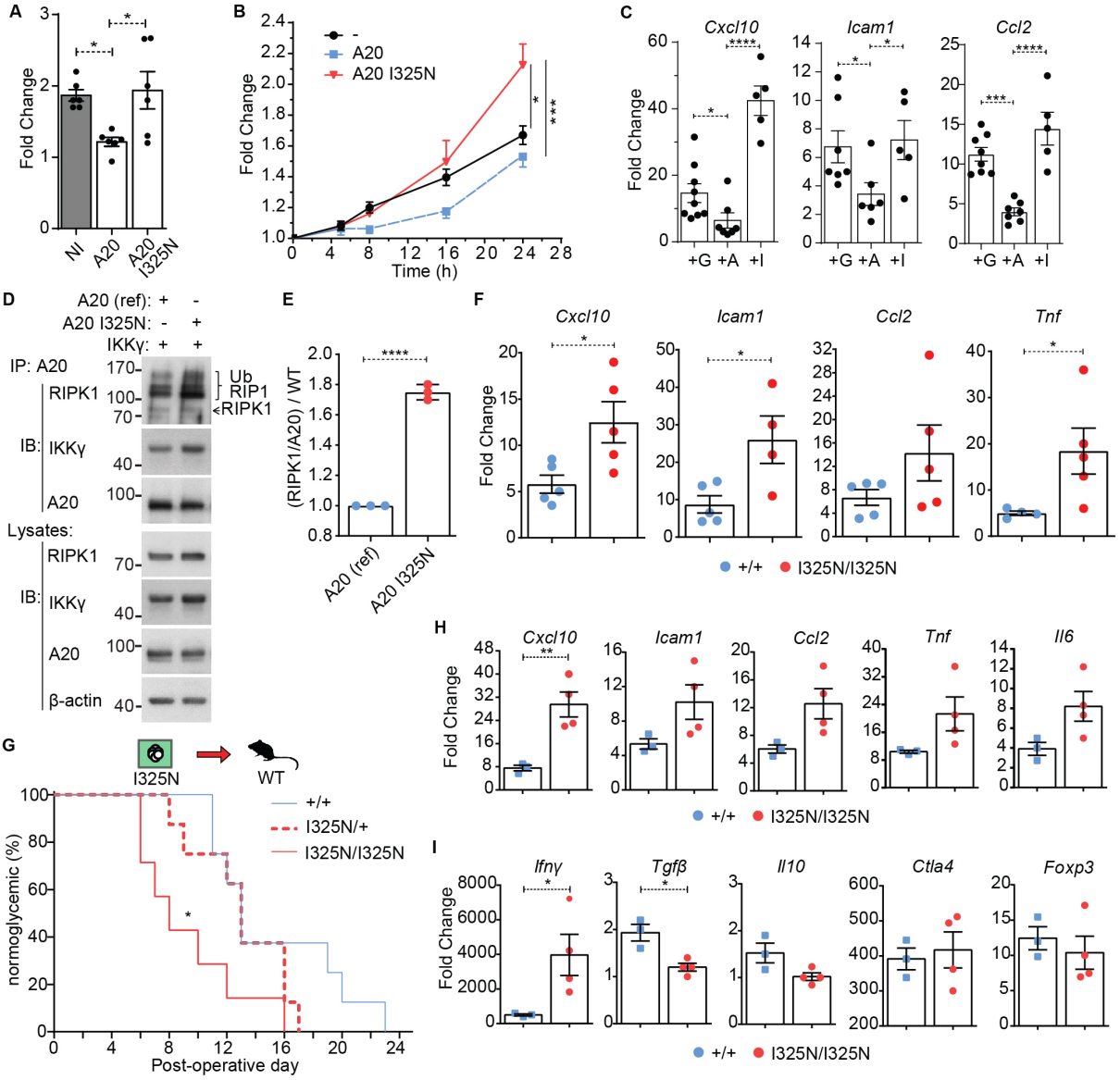

Figure 6. Reduced A20 function leads to rapid islet allograft rejection. (A, B) βTC3 cells co-transfected with an NF-κB.luciferase reporter (A), or an AP-1 luciferase reporter (B) and a CMV.βgal expression construct with or without PCDNA3.1 encoding murine reference A20, or A20 with an I325N coding variant. Cells were stimulated with 200 U/ml TNF for 8 h (A), or for 5, 8, 16 and 24 h (B), or left untreated. Data represents fold change of stimulated versus non-stimulated and three independent experiments. **(C)** Wild-type BALB/c islets were isolated and transduced with rAd.*GFP* (+G), rAd.*TNFAIP3* (+A) or rAd.*TNFAIP3*^{I325N} (+I), incubated overnight and stimulated with 200 U/ml TNF for 0 or 4 h and gene expression of proinflammatory factors assessed. Each point represents a well with 300 islets. Data cumulative over three independent experiments. **(D)** Immunoblot (IB) of A20 immunoprecipitated (IP) lysates and whole-cell lysates from βTC3 cells transfected with reference A20 or A20 I325N and IKKγ and lysed following an overnight incubation. Membranes were probed for RIPK1, IKKγ, A20 or β-actin (loading control). **(E)** Densitometry of co-immunoprecipitated RIPK1 compared A20 pull down, normalised to reference A20 (WT). Data in D and E represents 2 independent experiments and 3 biological replicates. **(F)** Expression of TNF-induced genes in islets from *Tnfaip3*$^{+/+}$ or *Tnfaip3*$^{I325N/I325N}$ mice stimulated with TNF for 4h. Data shown in cumulative with each point in a column representing an independent islet preparation. **(G)** Percent of diabetic recipient CBA ($H2^k$) mice normoglycemic following transplantation of 300 *Tnfaip3*$^{I325N/I325N}$ (n = 8; mean survival time

[MST] = 8), *Tnfaip3*$^{I325N/+}$ (*n* = 8; MST = 13) or *Tnfaip3*$^{+/+}$ (*n* = 8; MST = 13) islet (H2^{b}). **(H, I)** Islet grafts were excised at post-operative day 10 and gene expression of known islet derived inflammatory factors (H) and non-islet derived factors (I) measured. Non-transplanted overnight cultured isolated islets were used as base-line. Statistical significance determined by 1-way ANOVA with Tukey's multiple-comparisons post hoc test (B, C, F), 2-way Student's *t*-test (E, H, I) or Log-rank test (G). Error bars represent s.e.m or s.d. (E), **P*<0.05; ***P*<0.01; ***P<0.001; ****P<0.001.

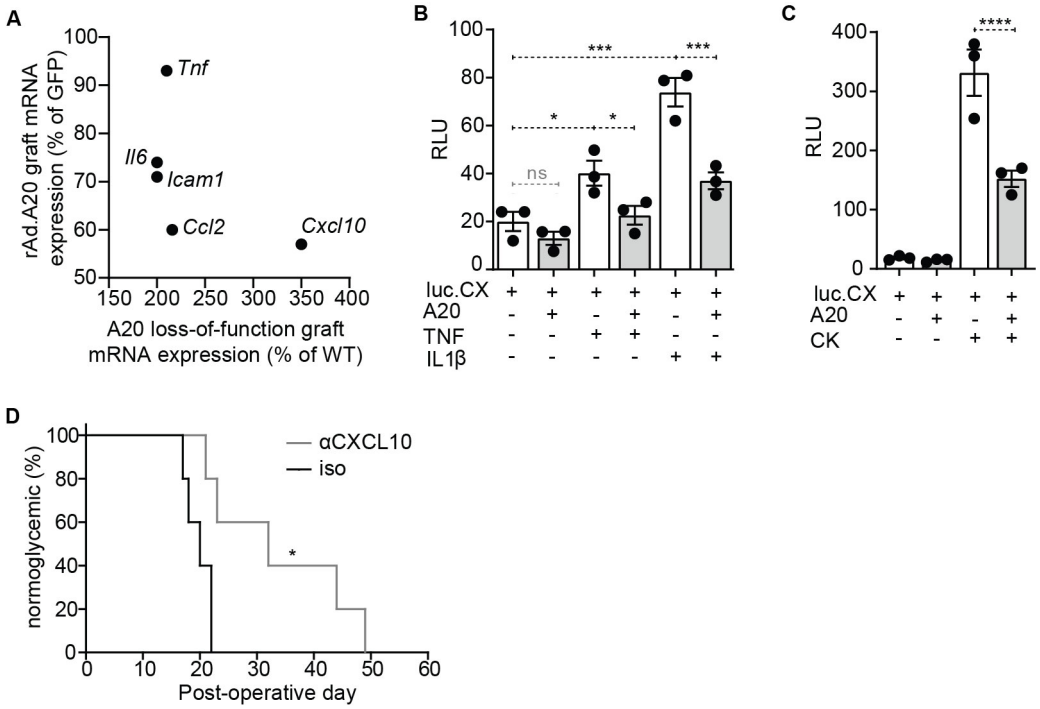

Figure 7. Differential *Cxcl10* expression in A20 expressing and loss-of-function islet grafts. (A) Scatterplot comparing the expression levels (%) of 5 inflammatory genes in allogeneic islet grafts overexpressing A20 (Figure 2H) or harboring loss-of-function A20 (Figure 6H), compared to GFP expressing control grafts harvested at post-operative day 10. **(B, C)** βTC3 cells cotransfected with a *CXCL10*.luciferase reporter encoding the endogenous promoter (75) and a CMV.βgal expression construct ± PCDNA3.1 encoding A20. Transfected cells were stimulated with (B) 200 U/ml TNF or IL-1β, or (C) a cocktail of TNF, IL-1β and IFNγ for 8 h or left untreated**.** Error bars ± s.e.m. Data representative of three independent experiments. Statistical significance determined by 1-way ANOVA with Tukey's multiple-comparisons post hoc test. **(D)** Percent of C57BL/6 mice normoglycemic after receiving allogeneic BALB/c islet grafts and administered 2 mg/kg of an anti-CXCL10 mAb by tail vein injection on the day of transplantation and every 2 days thereafter (n = 5) or an isotype control (iso) (n = 5). Significance determined by Log-rank test, * $P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

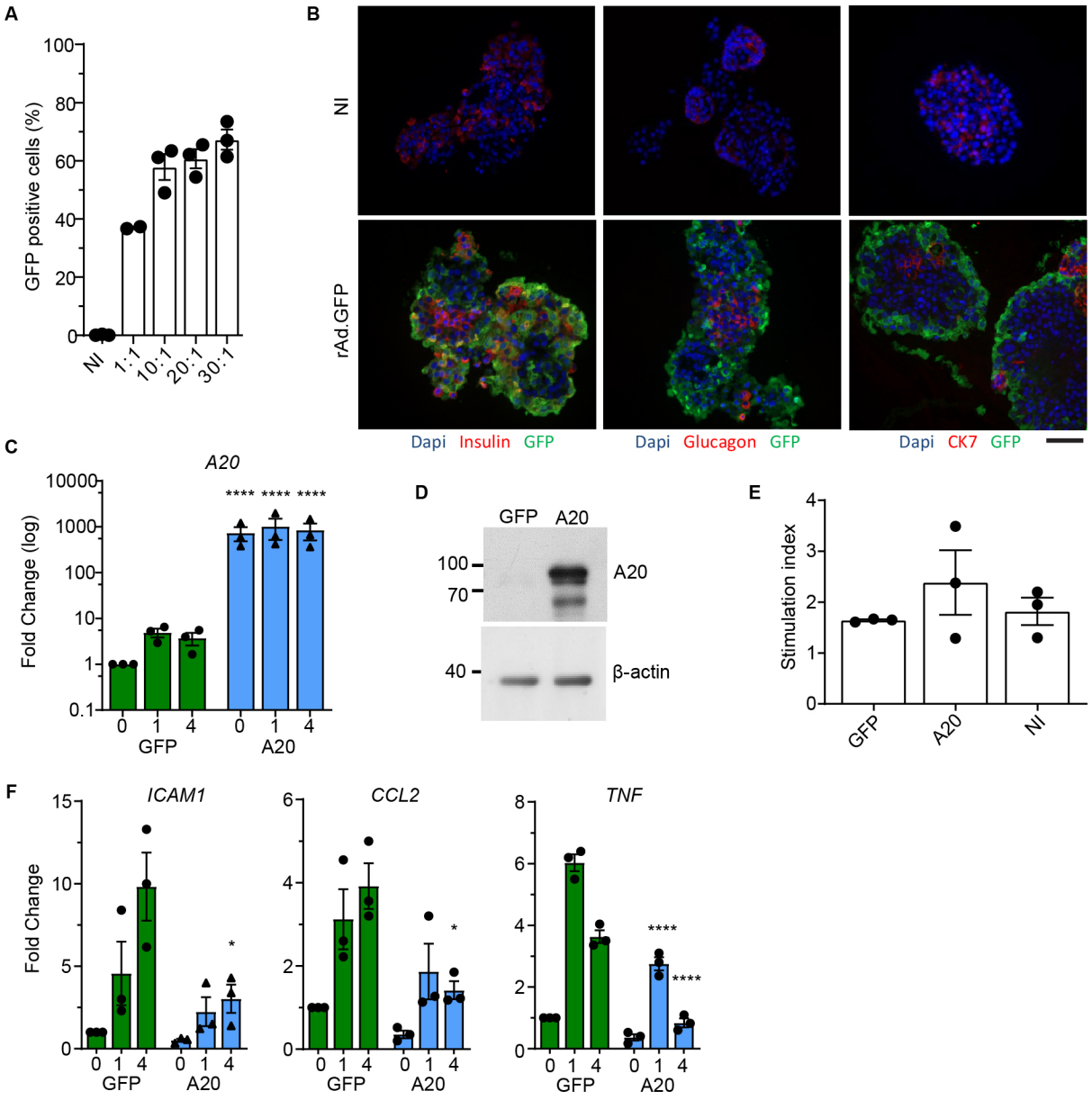

Figure 8. A20 expression inhibits human islet inflammation. (A) Human islets transduced with an adenovirus encoding for GFP (rAd.GFP) at multiplicity of infections indicated, or left non-infected (NI). Twenty-four hours post transduction, islets were digested to single cells and the percent of GFP positive cells determined by flow cytometry. Each dot per column represents an independent human donor. **(B)** Donor human islet preparations were transduced with rAd.GFP at multiplicity of infection of 10:1, or left non-infected (NI). Forty-eight hours post transduction islets were fixed for immunofluorescence analysis. Assessed proteins are indicated below each panel. Scale bar = 50 µm. **(C, D)** Donor human islets transduced with rAd.GFP or A20 at a multiplicity of infection of 10:1. Forty-eight hours post transduction cells were lysed and assessed for (C) A20 RNA expression and (D) protein levels. **(E)** Function of infected islets were assessed in a glucose stimulated secretion assay. Stimulation index = amount of insulin in supernatant in high glucose/ low glucose. **(F)** GFP and A20 transduced donor islets stimulated with TNF for the indicated times and expression of inflammatory genes assessed. Data, A-F, cumulative from three independent human donor islet preparations. Error bars ± s.e.m and statistical significance determined by 2-way ANOVA with Sidak's multiple-comparisons post hoc test, * $P < 0.05$; **** $P < 0.0001$.