Brief communication

MOLECULAR METABOLISM

Macrophages are essential for CTGF-mediated adult β-cell proliferation after injury

Kimberly G. Riley[2], **Raymond C. Pasek**[3], **Matthew F. Maulis**[3], **Jennifer C. Dunn**[3], **W. Reid Bolus**[1,4], **Peggy L. Kendall**[3], **Alyssa H. Hasty**[1,4], **Maureen Gannon**[1,2,3,4,*]

ABSTRACT

Objective: Promotion of endogenous β-cell mass expansion could facilitate regeneration in patients with diabetes. We discovered that the secreted protein CTGF (aka CCN2) promotes adult β-cell replication and mass regeneration after injury via increasing β-cell immaturity and shortening the replicative refractory period. However, the mechanism of CTGF-mediated β-cell proliferation is unknown. Here we focused on whether CTGF alters cells of the immune system to enhance β-cell replication.

Methods: Using mouse models for 50% β-cell ablation and conditional, β-cell-specific CTGF induction, we assessed changes in immune cell populations by performing immunolabeling and gene expression analyses. We tested the requirement for macrophages in CTGF-mediated β-cell proliferation via clodronate-based macrophage depletion.

Results: CTGF induction after 50% β-cell ablation increased both macrophages and T-cells in islets. An upregulation in the expression of several macrophage and T-cell chemoattractant genes was also observed in islets. Gene expression analyses suggest an increase in M1 and a decrease in M2 macrophage markers. Depletion of macrophages (without changes in T cell number) blocked CTGF-mediated β-cell proliferation and prevented the increase in β-cell immaturity.

Conclusions: Our data show that macrophages are critical for CTGF-mediated adult β-cell proliferation in the setting of partial β-cell ablation. This is the first study to link a specific β-cell proliferative factor with immune-mediated β-cell proliferation in a β-cell injury model.

Keywords CTGF; β-cell; Proliferation; Regeneration; Macrophages; T-cells

1. INTRODUCTION

Connective Tissue Growth Factor (CTGF/CCN2), a member of the CCN family of secreted extracellular matrix (ECM)-associated proteins, is a β-cell proliferative factor [1–3]. Although CTGF induction in adult β-cells does not increase proliferation [4], CTGF treatment after 50% β-cell ablation promotes β-cell proliferation and regeneration by modifying β-cell intrinsic characteristics, including maturity state and replication refractory period [2]. We hypothesized that CTGF induction might also elicit β-cell mass regeneration by altering extrinsic factors of the islet micro-environment, in particular immune cell populations. In other models of tissue damage, CTGF promotes wound repair via immune cell modulation [5]. For example, in the kidney, CTGF induction promoted infiltration of both macrophages and T cells [6], suggesting that CTGF serves as an immune cell chemoattractant. Further, in an ethanol model of pancreatic injury, CTGF over-expression increased recruitment of neutrophils and T-cells to the pancreas [7]. CTGF may thus promote the recruitment of particular immune cell populations and/or alter their characteristics, resulting in increased β-cell proliferation and regeneration.

The role of the immune system in β-cell regeneration is well-appreciated [8–10]. Specifically, macrophages are critical for proper β-cell mass regeneration after injury by partial duct ligation [10] and VEGF-mediated islet endothelial cell expansion [9]. Additionally, several groups have proposed that T cells promote β-cell mass regeneration in the setting of diabetes mellitus [8,11]. Following 50% β-cell ablation, we observed an increase in macrophages, which was further heightened by CTGF induction after β-cell destruction. In addition, we observed an increase in T cells in the parenchyma only in pancreata from animals with CTGF induction after 50% β-cell ablation. Whole islet gene expression analysis revealed that CTGF induction after β-cell ablation increased the expression of several markers of M1 macrophages and T cells as well as chemoattractant genes. Thus, it appears that CTGF induction after 50% β-cell ablation promotes an increase in both T cells and macrophages.

We assessed the requirement for macrophages in CTGF-mediated β-cell regeneration via macrophage depletion. We observed that a reduction in macrophages (with no changes in T cell number) in this model of β-cell destruction inhibits the proliferative effects elicited by CTGF induction. Also, we propose that macrophage depletion, promotes a more mature β-cell phenotype, via an unknown mechanism,

[1]Department of Veterans Affairs, Tennessee Valley Health Authority, Nashville, TN, USA [2]Department of Cell and Developmental Biology at Vanderbilt University, Nashville, TN, USA [3]Department of Medicine at Vanderbilt University, Nashville, TN, USA [4]Departments of Molecular Physiology and Biophysics at Vanderbilt University, Nashville, TN, USA

*Corresponding author. Department of Medicine, Division of Diabetes, Endocrinology, and Metabolism, 2213 Garland Avenue 7465 MRBIV, Vanderbilt University, Nashville, Tennessee, TN 37232-0475, USA. Tel.: +1 615 936 2676; fax: +1 615 936 1667. E-mail: maureen.gannon@vanderbilt.edu (M. Gannon).

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contributing to the inability of β-cells to proliferate in response to CTGF. Together these data suggest a critical role for macrophages during CTGF-mediated β-cell proliferation and regeneration.

2. MATERIALS AND METHODS

2.1. Animals

Generation of RIP-rtTA [12], TetO-CTGF [1], and RIP-DTR [13] transgenic mice has been described previously. Primers are available upon request. Female mice were administered 2 mg/ml of doxycycline (DOX) in 2% Splenda (to avoid taste aversion) in drinking water after diphtheria toxin (DT) administration. Mice were treated with DOX for 2 days. DT (126 ng; Sigma) was given I.P. three times at 8 weeks of age. PBS- or clodronate-liposomes (250 μL; clodronateliposomes.com) were given I.P. once a day for 8 days. Liposomes (artificially prepared lipid vesicles) contained either PBS or clodrinate, a non-toxic bisphosphonate. After injection, liposomes are ingested and digested by macrophages. Clodronate is released and accumulates intracellularly, where at high intracellular concentrations, it induces apoptosis [14]. These studies were all approved by the Vanderbilt University Institutional Animal Care and Use Committee.

2.2. Immunolabeling

Pancreata were dissected and fixed for 1 h in 4% paraformaldehyde at 4°C, and placed in a 30% sucrose solution overnight at 4°C. Pancreata were embedded in O.C.T. (Tissue-Tek) and serial sectioned on a cryostat at 7 μM. Indirect protein localization was obtained by incubations with primary antibodies overnight at 4°C: Guinea Pig α-Insulin (DAKO; 1:500), Rabbit α-Ki67 (AbCam; 1:500), Rabbit α-MafA (Bethyl Laboratories; 1:400), Rat α-CD45 (BD Pharmingen; 1:100), Rat α-F4/80 (Invitrogen; 1:100), Rat α-B220 (BD Pharmingen; 1:100), Rat α-CD3 (BD Pharmingen; 1:00). Nuclei were visualized with DAPI (Molecular Probes). Immunohistochemistry for Neutrophil Marker (Sigma) was completed by Vanderbilt University's Translational Pathology Shared Resource. Imaging was with a ScanScope FL scanner (Aperio Technologies, Inc.) and quantified using Metamorph 6.1 (Molecular Devices). Unless otherwise noted, 4 sections more than 250 μM apart were selected and immunolabeled, with ≥4,000 cells per animal quantified.

2.3. β-cell proliferation

Five slides (at least 250 μm apart) per animal were immunolabeled for insulin and Ki67. A minimum of 4,000 cells were counted using Metamorph 6.1 software (Molecular Devices). The percentage of proliferating cells was determined by dividing the number of Ki67/insulin double-positive cells by the total number of insulin+ cells.

2.4. Analysis of β-cell maturity

Sections were immunolabeled for insulin, and MafA or MafB. Percentage of mature and immature β-cells was determined by dividing number of MafA/insulin double-positive cells or MafB/insulin double positive cells by total number of insulin+ cells, respectively.

2.5. Gene expression analysis

Islets were isolated from 10 week old females and immediately prepared for RNA isolation by dissolving in Trizol reagent. RNA was isolated using the RNeasy Mini kits (Qiagen). Greater than 250 ng cDNA was prepared from islet RNA using the SuperScript III First Stand Synthesis System (Invitrogen). TLDAs were conducted on a 7900HT Fast Real-Time PCR system. Data was analyzed with SDS RQ Study software (Applied Biosystems, Life Technologies). cDNA for qRT-PCR was generated using the iScript cDNA synthesis kit (BioRad). Relative gene expression was assayed by the FAM-conjugated TaqMan Gene Expression Assay (Life Technologies) on a BioRad CFX Real Time PCR Instrument (BioRad). Gene expression for qRT-PCR analysis is relative to Emr1, after first normalizing to GAPDH. Statistical comparisons were analyzed by the Pfaffl method. Primer sequences available upon request.

2.6. Quantification of immune cell populations

Sections were immunolabeled for insulin and immune cell markers; CD45 (pan-immune), B220 (B cells), CD3 (T cells), Neutrophil Marker (neutrophils), and F4/80 (macrophages) as described earlier. One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). Five random insulin+ areas (4000^2 pixels) were extracted per slide. Through Metamorph 6.1 software (Molecular Devices) immune cells were binned as either islet associated or within the exocrine compartment. Macrophage proliferation was quantified by dual labeling for F4/80 and Ki67. Sections were imaged via a ScanScope FL slide scanner. Five random pancreatic areas (4000^2 pixels) were extracted per slide and the percentage of Ki67 positive F4/80 positive cells out of the total number of F4/80 positive cells was quantified using Metamorph 6.1

2.7. Statistics

Results are expressed as mean ± SEM. Statistical significance was calculated by Student's T test, One-way, or Two-way ANOVA analysis where applicable. $p \leq 0.05$ was considered significant.

3. RESULTS

3.1. CTGF induction increases islet-associated macrophages and T cells at the peak of β-cell proliferation

We previously showed that CTGF promotes β-cell mass regeneration, without altering α-cell proliferation or number [2]. This was achieved by using a diphtheria toxin (DT)-mediated mouse model (RIP-DTR) of 50% β-cell ablation paired with our previously described β-cell specific doxycycline (Dox)-inducible CTGF bi-transgenic model (RIP-rtTA; TetO-CTGF) [1,4,13]. This model of β-cell ablation does not result in alterations in glucose homeostasis [2]. At 8 weeks of age, DT was administered to RIP-DTR; RIP-rtTA controls ("Ablation") and RIP-DTR; RIP-rtTA; TetO-CTGF experimental animals ("Ablation + CTGF") and CTGF induced for 2 days (Figure 1A). This time was chosen as it is the peak of β-cell proliferation in Ablation + CTGF pancreata [2]. Non-DT injected animals included controls ("Control") and those in which CTGF was induced without ablation ("CTGF"). β-cell regeneration occurs only in the Ablation + CTGF animals, neither CTGF induction under normal conditions nor 50% β-cell ablation alone elicits β-cell proliferation or mass expansion [2].

To assess whether CTGF induction increased the population of immune cells in the pancreas, Hematoxylin and Eosin (H&E) staining was conducted (Figure 1B–E). In both the Ablation and Ablation + CTGF cohorts, analysis of H&E staining indicated the presence of inflammation and increased immune cells, in the absence of fibrosis (Figure 1D,E [2]). In order to confirm the increase in immune cells in the pancreas parenchyma, immunohistochemistry with the pan-leukocyte marker, CD45, was conducted at the 2 day time point (Figure 2A–D). CTGF induction under normal conditions in adult islets elicited no increase in leukocyte number (Figure 2A). However, 50% β-cell ablation alone did promote an increase in the number of leukocytes in the pancreas (Figure 2A) although these cells were not targeted preferentially to the islets (Figure 2B). The increase in immune cell

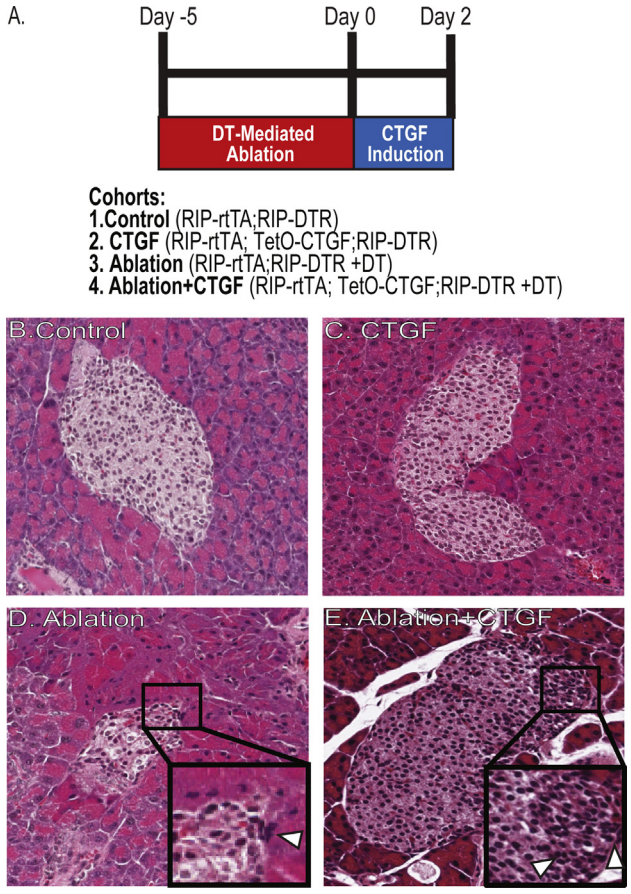

Figure 1: Experimental design for β-cell ablation and CTGF overexpression. **(A)** Experimental outline and cohorts. **(B–E)** H&E staining for immune cell detection adjacent to islets. Representative images of Control (B), CTGF (C), Ablation (D), and Ablation + CTGF (E) islets after 2 days of CTGF induction. Insets highlight small, dark and closely clustered nuclei that are indicative of immune cells (white arrows).

number in response to ablation was further heightened upon CTGF induction after injury (Figure 2A,D), and there was a greater proportion of CD45+ cells localized to islets (Figure 2B,D).

To determine which specific immune populations were increasing following ablation and CTGF, immunohistochemistry for immune cell populations was conducted. The largest proportion of immune cells in any of the cohorts was macrophages, as detected by immunolabeling against F4/80 (Figure 2E–H). 50% β-cell ablation did elicit an increase in pancreatic macrophages (Figure 2E); CTGF induction further enhanced this increase (Figure 2E,H). Additionally, a greater proportion of macrophages were islet associated in the Ablation + CTGF cohort as compared to all other groups (Figure 2F,H). Interestingly, only CTGF induction after β-cell ablation elicited a modest increase in T cells (Figure 21,K), as assessed by CD3 immunolabeling (Figure 2J–K). The increased T cells were not specifically targeted to islets (not shown). Very few B cells, as detected by B220 immunolabeling (Supplemental Figure 1A,C–F), were observed within the pancreatic parenchyma of any cohort, and rarely were they observed close to islets (Supplemental Figure 1B,C-F) Additionally, as CTGF has been shown to recruit neutrophils in other models of pancreatic injury, we assessed this immune population via immunohistochemistry (Supplemental Figure 1I–L). However, in our model of β-cell ablation, no significant increase of neutrophils was observed (Supplemental Figure 1G–L). Finally, we observed no presence of eosinophils, as assessed by H&E staining, in any cohort (not shown).

3.2. Gene expression analyses reveal changes associated with increased macrophages and T cells

To gain further insight into which immune cell populations and associated signaling pathways are altered by CTGF with or without β-cell ablation, gene expression analysis was conducted on islets isolated from animals following 2 days of CTGF induction in vivo. We specifically assessed changes in expression of genes associated with the innate and adaptive immune cell response, including cytokine expression changes (Figure 3; Supplemental Figure 2A). In addition, gene expression alterations in ECM components, vascular markers, and the stress response were determined (Supplemental Figure 2B,C). There were several changes in key innate and adaptive immune response genes following ablation and/or CTGF induction (Figure 3A,B). CTGF induction under normal conditions or after β-cell ablation elicited an increase in *Mcp1* (Macrophage Chemoattractant Protein 1), *RANTES* (Regulated on Activation, Normal T cell Expressed and Secreted/CCL5), and *Ccr2* (C–C chemokine receptor type 2). MCP1 and its receptor, Ccr2, serve as chemoattractants for macrophages [15,16], in agreement with the immunolabeling results showing increased macrophages in islets. In addition, RANTES promotes macrophage activation along with T cell recruitment [17], further corroborating the observed increase in T cells in our Ablation + CTGF cohort. β-cell ablation alone and in conjunction with CTGF induction increased expression of *CD45*, a pan-leukocyte marker, and *CD68*, a macrophage marker, once again aligning with immunolabeling findings. These results were highly suggestive of a critical role for macrophages in CTGF-mediated β-cell proliferation. The unique setting of CTGF induction after β-cell ablation resulted in the specific upregulation of *C3* (Complement Component 3), *TNFα* (Tissue Necrosis Factor α), and *Selp* (Selectin P). These genes are all associated with inflammation [18,19], while *Selp* also serves as a leukocyte chemoattractant [20].

Alterations in expression of genes associated with the adaptive immune response focused primarily on T cells (Figure 3B). CTGF induction under normal conditions did not promote the expression of any genes associated with the adaptive immune response (Figure 3B). However, β-cell ablation alone or with CTGF induction increased the expression of *CD3e*, a marker of T cells, and *Stat4*, a promoter of Th_1 development (Figure 3B; Ablation; [21]). Expression of several genes was increased only in the Ablation + CTGF cohort (Figure 3B; Ablation + CTGF). These included several additional markers of T cells, including *CD4* (T helper cells), *CD28* (costimulator necessary for T cell activation), and *CD8a* (Cytotoxic T cells). Additionally, CTGF induction after β-cell ablation elicited the increased expression of macrophage-expressed genes that promote T cell activation (*CD86*) and trafficking (*Ccl19*) [22,23]. Finally, CTGF induction alone promoted the expression of *Ctla4* (Cytotoxic T Lymphocyte Associated protein 4), which downregulates T cell activation [24] (Figure 3A). As predicted by immunolabeling, we did not observe changes in expression of genes associated with B cells (Figure 3B, CD19, CD40, CD38). We also assessed changes in the expression of several cytokines (Supplemental Figure 2A). However, the only observed alteration was with *IL-12b* (Interluekin-12b), which was induced by CTGF expression after β-cell ablation and under normal settings (Supplemental Figure 2A). *IL-12b* is expressed by macrophages and aids T helper cell development [25]. Overall, these findings align well with our observed increase in T cells in the Ablation + CTGF cohort (Figure 2I), suggesting that CTGF induction promotes β-cell regeneration through macrophages and/or T cells.

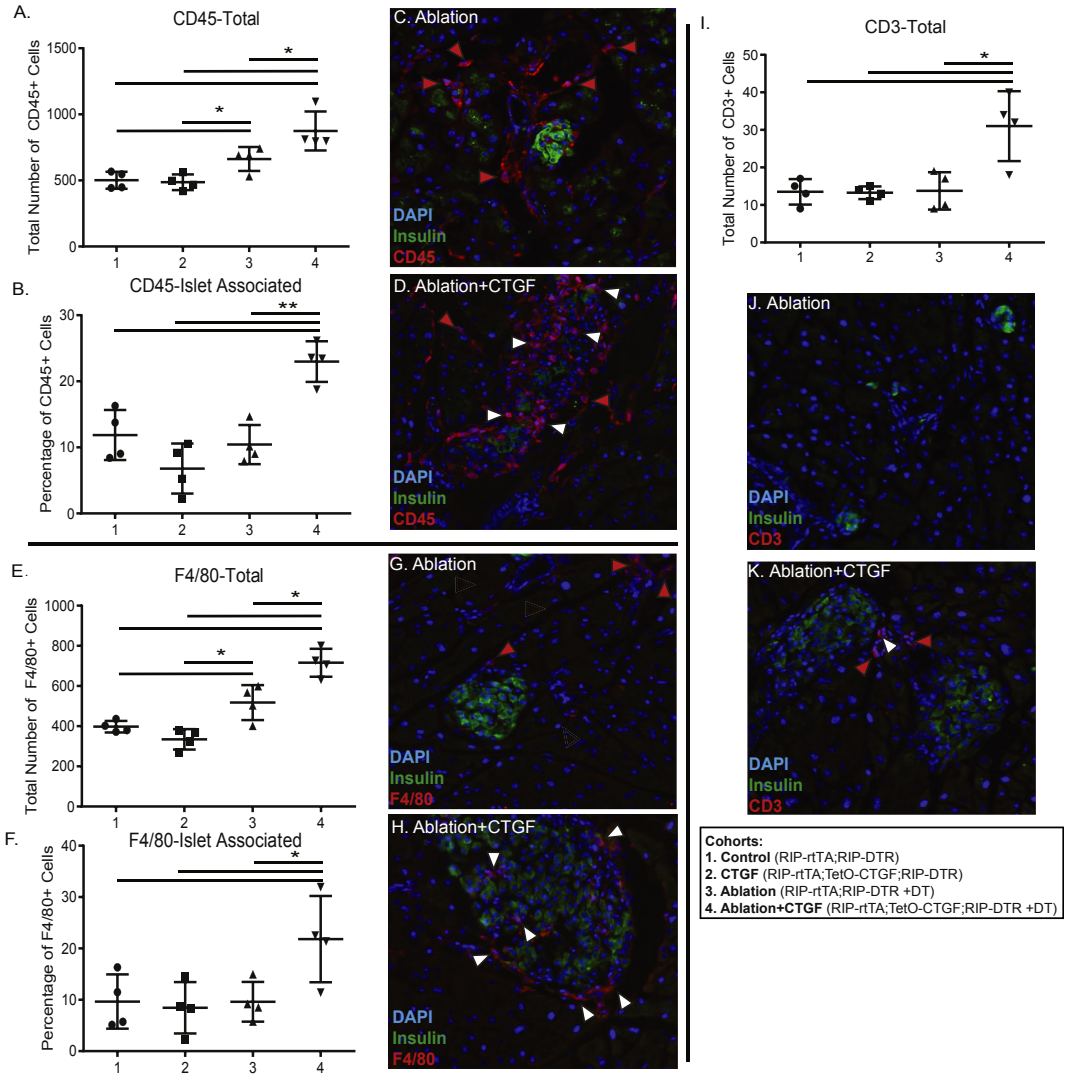

Figure 2: Alterations to immune cell populations by β-cell ablation and CTGF induction. **(A)** Total number of pancreatic CD45 + cells and **(B)** Proportion of islet-localized CD45-positive cells. **(C–D)** Representative images of Ablation (C) and Ablation + CTGF (D) islets after 2 days CTGF. Insulin (green), CD45 (red). **(E)** Total number of F4/80-positive cells and **(F)** Proportion of islet-localized macrophages. **(G–H)** Representative images of Ablation (G) and Ablation + CTGF (H) islets after 2 days CTGF. Insulin (green), F4/80 (red). **(I)** Total number of CD3-positive cells. **(J–K)** Representative images of Ablation (J) and Ablation + CTGF (K) islets after 2 days CTGF. Insulin (green), CD3 (red). Immune cells within the exocrine or endocrine compartments are demarked by red and white arrows, respectively. n = 4. *p < 0.05, **p < 0.01.

Finally, we assessed alterations to genes associated with the ECM and vasculature, which play key roles in immune cell trafficking (Supplemental Figure 2B). In our model *Vcam1* (Vascular Cell Adhesion Molecule 1) was the sole gene significantly upregulated, and only with CTGF induction after β-cell ablation (Supplemental Figure 2B). Vcam1 is critical for adhesion of leukocytes to endothelial cells and subsequent signal transduction, leading to extravasation [26]. Increased Vcam1 expression, suggested to us that the increase in macrophages was due to increased extravasation from the pancreatic vasculature. As an alternative, we examined whether CTGF increased macrophage proliferation, but failed to detect any proliferating macrophages (Supplemental Figure 3). Thus, increased macrophage recruitment, rather than proliferation of resident pancreatic macrophages in response to CTGF, appears to cause the increase in islet-associated macrophages in our model.

We also assessed whether our model of CTGF mediated β-cell regeneration involved induction or alterations to the cellular stress response (Supplemental Figure 2C). However, no alterations were observed. Thus, it appears that in CTGF-mediated β-cell mass expansion after β-cell ablation, CTGF induction promotes an increase in and activation of primarily macrophages and T cells.

3.3. Macrophages are required for CTGF-mediated β-cell proliferation

In order to assess whether infiltrating macrophages are involved in CTGF-mediated β-cell proliferation, we conducted macrophage depletion using liposomes containing clodronate. Clodronate liposomes were administered, one day prior to, during, and for 2 days following DT injections in 8 week old RIP-DTR; RIP-rtTA controls ("Ablation + Clodronate") and RIP-DTR; RIP-rtTA; TetO-CTGF experimental animals ("Ablation + CTGF + Clodronate") and CTGF induced by Dox induction for 2 days after DT injection. Additional controls included RIP-DTR; RIP-rtTA animals injected with PBS-containing liposomes ("Ablation + PBS") and DTR; RIP-rtTA; TetO-CTGF animals

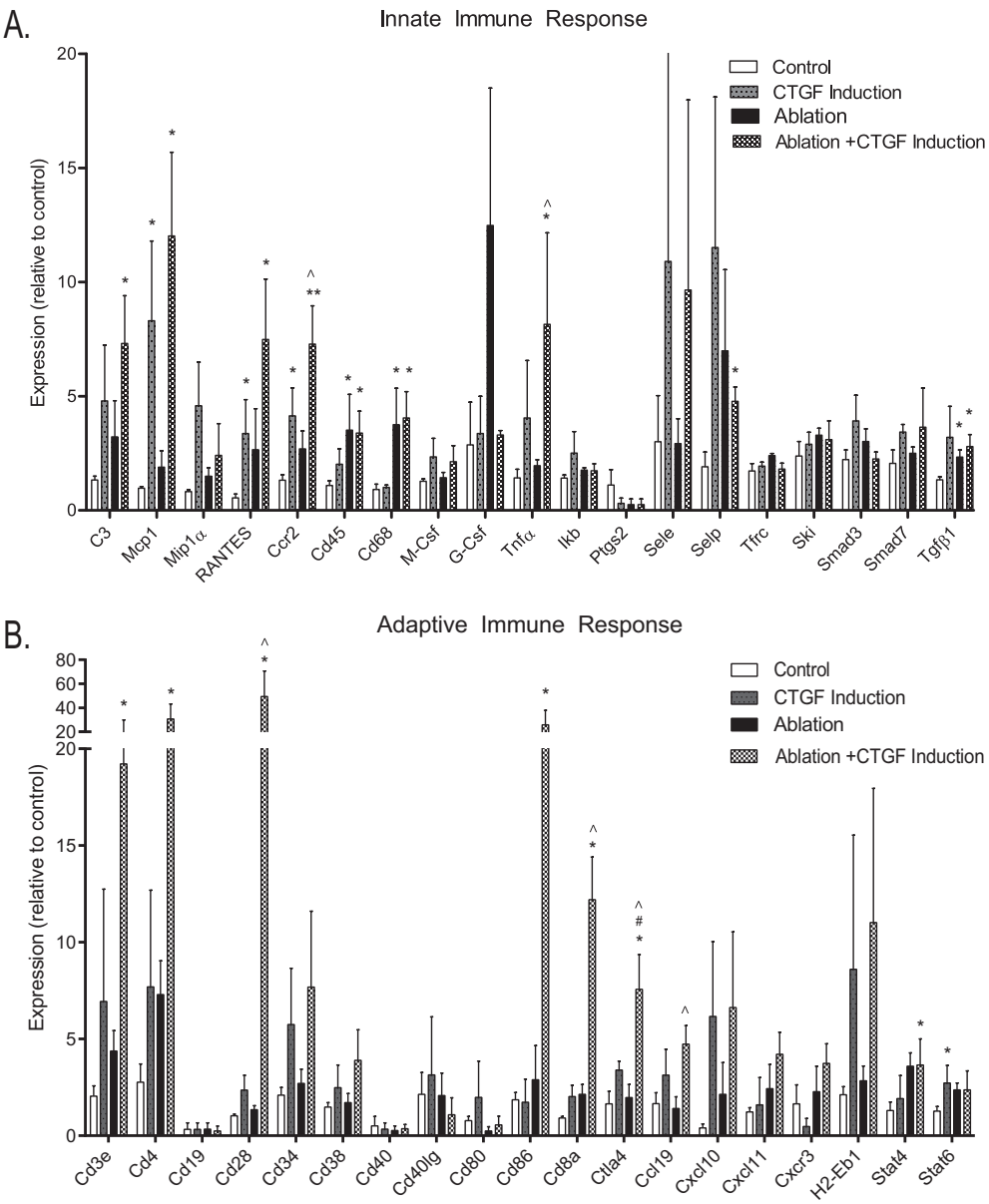

Figure 3: CTGF induces expression of genes involved in the innate **(A)** and adaptive **(B)** immune response. Gene expression analysis on whole islets using Taqman Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation ± CTGF induction for 2 days. All samples were run in duplicate. n = 4. * compared to Control, # compared to CTGF induction, ^ compared to Ablation. *, #, ^ $p < 0.05$, **$p < 0.01$.

injected with PBS-containing liposomes in which CTGF was induced ("Ablation + CTGF + PBS") (Figure 4A).

F4/80 immunolabeling confirmed clodronate induction-specific depletion of macrophages (Figure 4B). Additionally, since macrophages can serve as T cell chemoattractors [27], we assessed whether removal of macrophages also decreased the observed increase in T cells in our "Ablation + CTGF" cohort (Figure 2I). However, clodronate liposome treatment does not result in a decrease in pancreatic T cells (Figure 4C). Thus, we were able to assess the macrophage-specific effects on CTGF-mediated β-cell proliferation.

CTGF induction elicits β-cell regeneration by increasing β-cell proliferation [2]. Thus, we assessed whether the increase of macrophages after β-cell ablation was required for increased β-cell proliferation. Depletion of macrophages during CTGF-mediated β-cell regeneration decreased the percentage of proliferating β-cells to control cohort levels (Figure 4D; 2 vs. 4). Thus, macrophages are essential for CTGF-mediated increases in β-cell proliferation.

Since 50% β-cell ablation promotes a more immature β-cell phenotype, which is further heightened upon CTGF induction [2], we also assessed the requirement of macrophages for this effect. Macrophage depletion decreased the percentage of immature (MafA−) β-cells as compared to the Ablation + CTGF control cohort (Figure 4E; 2 vs 4). However, it must be noted that "Ablation + CTGF + Clodrinate" islets are still more phenotypically immature as compared to control, non-ablated islets (Figure 4E 1,3 vs 4), [2]). We also assessed β-cell immaturity via MafB immunolabeling. We observed no significant difference in the percentage of immature (MafB+) β-cells in our Ablation + CTGF + Clodrinate cohort as compared to the Ablation

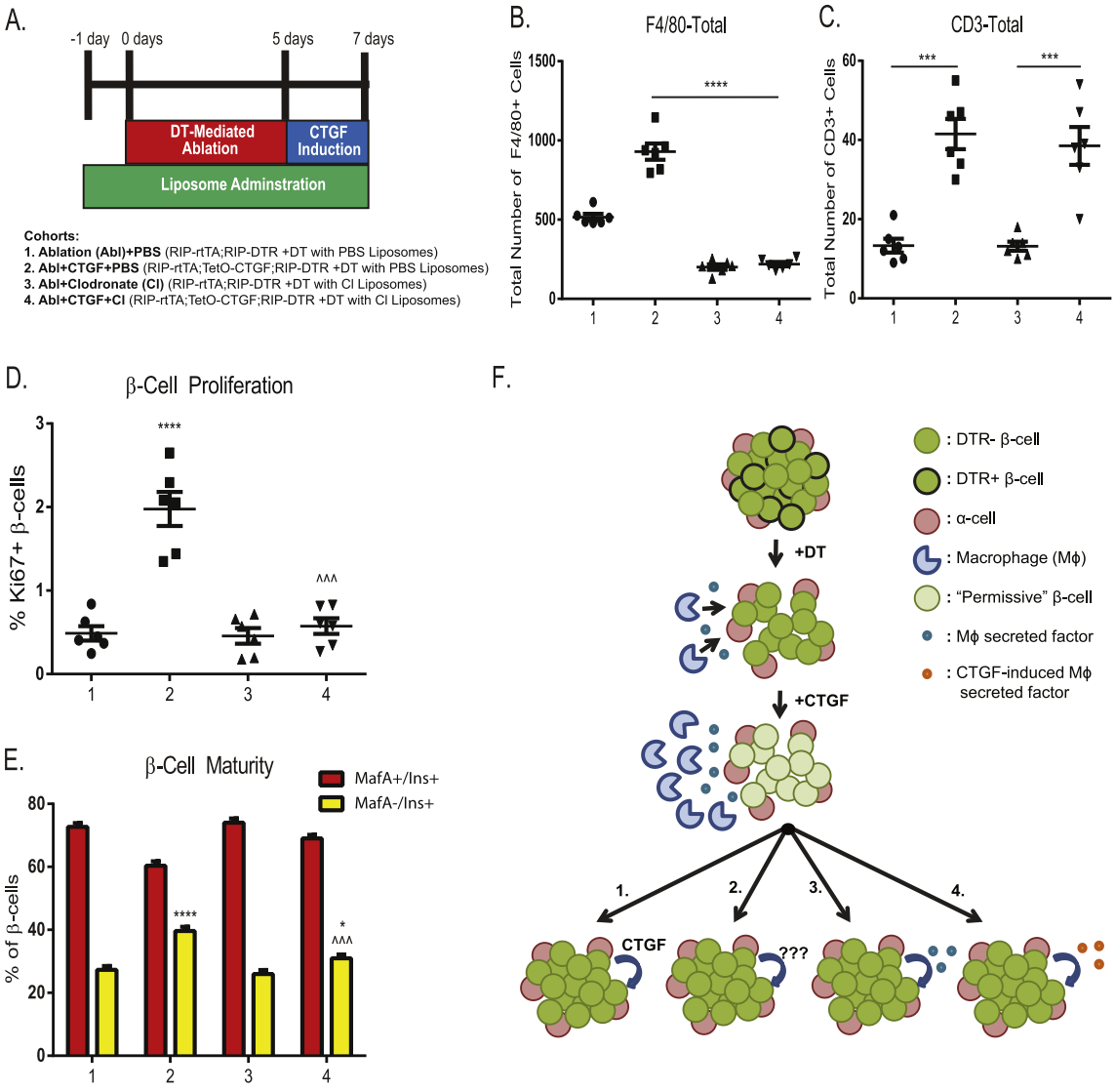

Figure 4: CTGF-mediated β-cell regeneration is dependent on macrophages. **(A)** Experimental outline and cohorts. Total number of **(B)** F4/80- and **(C)** CD3-positive cells. **(D)** β-cell proliferation. **(E)** Proportion of MafA+ (red bars) or MafA− (yellow bars) β-cells. **(F)** Model of potential mechanisms of CTGF mediated β-cell mass regeneration. After 50% β-cell ablation, macrophages enter the islet to remove dead β-cells. Upon CTGF induction, more macrophages are recruited to the islet, elevating the levels of a secreted factor that promotes β-cell permissiveness to the proliferative factor. The β-cell proliferative factor is: 1. CTGF itself, 2. other CTGF-induced proliferative factors, such as 5-HT, Integrin β1, or HGF, 3. elevated levels of a macrophage-derived factor, or 4. upon CTGF induction, the character of recruited macrophages is altered by CTGF (i.e. polarization, chemokine profile etc.) resulting in the secretion of CTGF-induced macrophage factor. n = 6 (B–C) ***$p < 0.001$, ****$p < 0.0001$. (D) ****$p < 0.0001$ comparing 2 vs 1,3. $^{\#\#\#}p < 0.0001$ comparing 4 vs 2. (E) *$p < 0.05$ comparing 4 vs 1,3. ****$p < 0.0001$ comparing 2 vs 1,3. $^{\wedge\wedge\wedge}p < 0.001$ comparing 4 vs 2.

cohorts (Data not shown). Thus, macrophages are required for both the β-cell proliferative and maturity state alterations mediated by CTGF after 50% β-cell ablation.

4. DISCUSSION

In other pancreatic injury models, CTGF recruits immune cells to the site of injury [7]. Here we investigated the potential role of the immune system in CTGF-mediated β-cell proliferation after partial β-cell destruction. Importantly, blood glucose homeostasis is unaffected by partial β-cell ablation or CTGF induction. β-cell destruction and CTGF induction in the setting of DT-mediated β-cell ablation increased the total number of macrophages and T cells in the pancreas and heightened the number of macrophages specifically within the endocrine compartment. In fact, macrophages comprised the vast majority of immune cells within the pancreas. Our findings are consistent with studies from other groups who have shown that macrophages are involved in β-cell mass regeneration following injury [9,10]. However, our study is the first to our knowledge, to utilize a β-cell-specific injury model and is the first to link a specific β-cell proliferative factor to increased macrophage numbers and enhanced β-cell proliferation.

We considered the possibility that a fraction of proliferating insulin-positive cells were actually proliferating macrophages that had engulfed β-cells. However, we failed to detect any proliferating macrophages nor did we observe any cells co-labeled with insulin and F4/80, arguing against this idea. Additionally, gene expression analysis showed that CTGF induction specifically increased expression of several

macrophage markers and macrophage chemoattractant genes. Several genes (*CD86*, *Ccl19*, *IL-12b*) associated with the pro-inflammatory M1 macrophage polarization phenotype were increased in the setting of CTGF induction after β-cell destruction. Likewise, preliminary qRT-PCR data showed a highly significant decrease in *Arg1* expression, a gene associated with M2 macrophage polarization, in islets from Ablation + CTGF mice compared to Control islets (not shown). *Mgl1* and *Chil3*, two other genes associated with M2 macrophage polarization, also displayed a trend towards decreased expression in Ablation + CTGF samples compared to Ablation alone (not shown). Further studies are needed to fully characterize the macrophage polarization phenotype in this model of β-cell regeneration.

The increase in T cells specifically in the setting of CTGF induction after β-cell destruction was not unexpected, as studies have proposed that T cells promote β-cell mass regeneration in the setting of diabetes mellitus [8,11]. Also, CTGF promotes T cell recruitment in the kidney [6]. Gene expression analysis on whole islets corroborated our immunofluorescence findings as upregulation of several T cell markers and chemoattractant genes was observed in the Ablation + CTGF cohort. While several of the T cell marker genes pointed towards a T helper cell population, further studies are needed to determine the specific T cell sub-population(s) involved in CTGF-mediated β-cell proliferation. Our current analysis cannot distinguish whether CTGF induction after β-cell ablation recruits T cells from outside the pancreas to the pancreas/islets or if CTGF promotes proliferation of pre-existing resident pancreatic T cells. Regardless, our data demonstrate that T cells are not required for CTGF-mediated β-cell proliferation in this model.

Through clodronate liposome-based macrophage depletion techniques, we observed that macrophages are absolutely essential for CTGF-mediated increases in β-cell proliferation in the setting of β-cell destruction. Additionally, macrophages may play a role in β-cells adapting a more immature phenotype after β-cell ablation, as removal of macrophages in the presence of Ablation + CTGF appears to prevent the decline in MafA+ β-cells we observed in Ablation + CTGF alone. We propose a potential model on how CTGF promotes β-cell mass expansion (Figure 4F). An increase in macrophages is observed following β-cell destruction. However, these macrophages alone are unable to promote β-cell proliferation. We hypothesize that CTGF induction increases the number of islet-associated macrophages, thus elevating the levels of an as yet unidentified factor above a required threshold to allow for β-cell responsiveness (Figure 4F). CTGF-mediated macrophage recruitment alters the islet microenvironment to become more responsive to proliferative stimuli. We propose four potential candidates for this proliferative stimulus (Figure 4): 1) CTGF itself, 2) other proliferative factors induced by CTGF (i.e. Hepatocyte Growth Factor (HGF), Serotonin (5-HT), Integrin β1, [2]), 3) an endogenous macrophage-derived factor or 4) a novel macrophage-derived factor induced by CTGF. In other words, CTGF could affect the concentration of a factor or the type of factor being produced.

The relevance of CTGF to human patients with diabetes and diabetic animal models remains unknown. A gene expression analysis reported by Keller et al., found a reduction in *Ctgf* expression in islets from 4 week old but not 10 week old non-diabetic ob/ob animals compared with controls [28]. The relationship between the difference in CTGF expression and the impact it had on β-cell proliferation is not clear.

CTGF mediated β-cell mass proliferation involves the modification of several β-cell intrinsic characteristics. We now show that the immune system, specifically macrophages, is also required for effective CTGF-mediated β-cell mass expansion following injury. We have identified a novel interaction between CTGF and the immune system to promote β-cell proliferation and thus, regeneration. As all forms of diabetes are demarked by both inflammation and insufficient functional β-cell mass, our studies highlight the significance of understanding the role of the immune system in promoting β-cell regeneration.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.05.002

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