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

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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,
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-theria 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-

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; 1:100). 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.). Five random insulin-positive areas (4000² 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 (4000² 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

2.3. 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 QG 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 Pfaffi 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 (4000² 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 (4000² 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 ≤ 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 stained indicating 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...
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 Ccr2 expression, which regulates T cell development [25]. Overall, these findings align well with our observed increase in T cells in the Ablation + CTGF cohort, suggesting that CTGF induction promotes β-cell regeneration through macrophages and/or T cells.

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 2F,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 2J,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 1l–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).

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).
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 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.
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 2B). 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−/−/C0) 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 + CTGF + Clodrinate cohort.
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. 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-secreted factors.

In the setting of DT-mediated 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 secretions of CTGF-induced macrophage factors.

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 secretions of CTGF-induced macrophage factors: n = 6 (B–E) ***p < 0.001, ****p < 0.0001. (D) ****p < 0.0001 comparing 2 vs 1,3. **p < 0.01 comparing 4 vs 2. (E) *p < 0.05 comparing 4 vs 1,3. ****p < 0.0001 comparing 2 vs 1,3. ***p < 0.001 comparing 4 vs 2.
We propose a potential model on how CTGF promotes \( \beta \)-cell proliferation and thus, regeneration. As all forms of diabetes are demarked by both inflammation and insufficient functional \( \beta \)-cell mass, our studies highlight the significance of understanding the role of the immune system in promoting \( \beta \)-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

**REFERENCES**


Brief communication


