Activated Macrophages Create Lineage-Specific Microenvironments for Pancreatic Acinar- and \( \beta \)-Cell Regeneration in Mice

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**BACKGROUND & AIMS:** Although the cells that contribute to pancreatic regeneration have been widely studied, little is known about the mediators of this process. During tissue regeneration, infiltrating macrophages debride the site of injury and coordinate the repair response. We investigated the role of macrophages in pancreatic regeneration in mice. **METHODS:** We used a saporin-conjugated antibody against CD11b to reduce the number of macrophages in mice following diphtheria toxin receptor-mediated cell ablation of pancreatic cells, and evaluated the effects on pancreatic regeneration. We analyzed expression patterns of infiltrating macrophages after cell-specific injury or from the pancreas of nonobese diabetic mice. We developed an in vitro culture system to study the ability of macrophages to induce cell-specific regeneration. **RESULTS:** Depletion of macrophages impaired pancreatic regeneration. Macrophage polarization, as assessed by expression of tumor necrosis factor–\( \alpha \), interleukin 6, interleukin 10, and CD206, depended on the type of injury. The signals provided by polarized macrophages promoted lineage-specific generation of acinar or endocrine cells. Macrophage from nonobese diabetic mice failed to provide signals necessary for \( \beta \)-cell generation. **CONCLUSIONS:** Macrophages produce cell type-specific signals required for pancreatic regeneration in mice. Additional study of these processes and signals might lead to new approaches for treating type 1 diabetes or pancreatitis.

**Keywords:** Macrophage Polarization; Tissue Damage; Pancreatitis; Diabetes; NOD.

Macrophages are monocyte-derived myeloid cells that develop from a common myeloid progenitor cell residing within the bone marrow of adult mammals.\(^3\) Upon tissue damage or infection, monocytes are rapidly recruited to the injured tissue, where they differentiate into macrophages.\(^1\) Infiltrating macrophages exist across an M1—M2 polarization state in which M1 cells are implicated in initiating and sustaining inflammation through production of high levels of pro-inflammatory cytokines, reactive nitrogen, and oxygen intermediates, while the more heterogeneous M2 cells are characterized by alternative arginine metabolism, exhibit a different chemokine expression profile, and are associated with resolution or smoldering chronic inflammation.\(^2,3\) Accumulating data suggest that macrophages produce different mediators during normal development or regeneration after injury in several organs, such as liver, kidney, and heart.\(^4-14\) In addition, recent reports indicate that in the mouse pancreas, macrophages might be involved in inducing acinar-to-ductal metaplasia (ADM), as well as promoting \( \beta \)-cell regeneration.\(^15-17\)

In a previous work we used diphtheria toxin receptor (DTR)—mediated conditional and targeted cell ablation to study acinar and endocrine regeneration in the absence of autoimmunity.\(^18\) In these models, the internalization of DT leads to rapid apoptotic cell death of the targeted cells, followed by massive infiltration of macrophages. This led us to speculate that macrophages might contribute to pancreatic regeneration by providing the proper signals to the ductal cell progenitor niche.

Here we first examine the role of macrophages following different cell type-specific ablation models in the absence of autoimmunity. Specifically, we show with both in vivo and in vitro approaches that uptake of apoptotic cells by macrophages determines a progressive switch in macrophage phenotype in an injury-specific manner. Using a saporin-conjugated monoclonal antibody to kinetically deplete macrophages in the pancreas, we then demonstrate that polarized macrophages are required for proper pancreatic regeneration. Likewise, we find that the decreased ability to regenerate observed in old animals is also related to insufficient polarization. Finally, we show that this pro-regenerative function is impaired in nonobese diabetic (NOD) mice, strongly suggesting that this aspect should be addressed in the search of novel therapies for patients with type 1 diabetes (T1D).

**Abbreviations used in this paper:** ADM, acinar-to-ductal metaplasia; DTR, diphtheria toxin receptor; Fizz1, found in inflammatory zone 1; IL, interleukin; Macf1-SAP, saporin-conjugated anti-Macf1 antibody; NOD, non-obese diabetic; T1D, type 1 diabetes; Tgf, transforming growth factor; TNF, tumor necrosis factor.
Methods

Mice

Mice used in this study were maintained according to protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Description of additional methods and details for reagents are provided in Supplementary Materials and Methods.

Results

Infiltrating Macrophages Polarize to an M2-Phenotype in a Successful Nonautoimmune Model of Global Pancreatic Regeneration

We have recently reported that after extensive ablation of both acinar and endocrine cells in adult PdxCre;R26DTR mice, epithelial cells within the ductal network are capable of contributing to organ regeneration through recapitulation of the embryonic pancreatic developmental program.19 In this model, during the first week after injury (early stage), the pancreas is massively infiltrated by macrophages engulfing the apoptotic cells (Figure 1A–C).

As the regenerative process proceeds (mid stage), the frequency of apoptosis among the epithelial cells, as well as the number of macrophages, drops significantly (Figure 1D) and becomes almost nonexistent during the late stage.18

It is known that phagocytosis of apoptotic cells regulates macrophage immune response by shifting the cellular phenotype from classical M1 to alternative M2 activation after a critical number of cells have contributed to an overall change in the microenvironment.19–21 To confirm the dynamic change in macrophage activation in the regenerating pancreas, we analyzed F4/80-positive macrophages (pan-macrophage marker) for expression of the M2-marker Fizz1.8 Macrophages present during the early stage were mainly Fizz1+ (Figure 1E). By contrast, during mid stage, the number of F4/80+/Fizz1+ cells in the pancreas progressively increased (Figure 1E). To further characterize phenotypically the macrophage infiltrates, we isolated F4/80+/CD11b+ cells (Figure 2A) from the pancreas of DT-treated PdxCre;R26DTR mice. Flow cytometry analysis confirmed that macrophages progressively switch from a classic tumor necrosis factor (TNF)α−/interleukin (IL)6+ M1 to an alternative IL10+ /CD206+ M2 phenotype during the transition from early to mid stage (Figure 2C).

Polarized Macrophages Exhibit a Different Cytokine Phenotype After Cell-Specific Injury

M2 is a generic name for various forms of macrophage activation other than the classic M1, expressing heterogeneous repertoires of cytokines.2 To investigate whether cellspecific ablation (rather than global) induces a different polarization profile, F4/80+/CD11b+ cells were isolated from PdxCreERT;R26DTR mice (apoptotic β-cells), or ElaCreERT2;R26DTR mice (apoptotic acinar cells) at different time points and analyzed for M1 (TNFα or IL6) and M2 (IL10 or CD206) markers (Figure 2B–E). Interestingly, we found that macrophages obtained from DT-treated PdxCreERT;R26DTR mice displayed initially a M1 signature (TNFα+/IL6+); however, they did not switch completely to a classic M2 signature and instead remained in a M1/M2 (TNFα+/IL6+/IL10+) state (Figure 2D). On the other hand, phagocytosis of apoptotic acinar cells after acinar-specific ablation in ElaCreERT2;R26DTR mice, induced a prominent M1/M2 phenotype (TNFα+/IL6+/IL10+), which persisted throughout the regenerative process (Figure 2E).

Macrophages Are Required for Both Acinar and β-Cell Regeneration

The existence of different macrophage activation states implies distinct roles during the phases of the immunologic response, ie, inflammation vs resolution and tissue remodeling. We speculated that the initial function of macrophages would be that of debriding the massive apoptotic cell residue, while their persistence during active regeneration could be related to their role as mediators of regenerative signals. To unequivocally assess the role of macrophages in vivo during tissue repair in our DT/DTR-mediated cell ablation models, we used a saporin-conjugated antibody against CD11b/Mac1 (Mac1-SAP) to deplete infiltrating pancreatic macrophages during regeneration. Compared with clodronate, which mainly depletes circulating monocytes, this antibody is effective to deplete tissue macrophages in both the spleen and the injured pancreas 48 hours after administration (Figure 3A). To evaluate the effect of macrophage depletion on pancreatic regeneration, we first allowed the macrophages to debride the injured pancreas after DT treatment, and subsequently administered Mac1-SAP on day 7 post-DT, when the M2 polarization peak happens (Figure 3C). The mice were then sacrificed either at mid stage (Figure 3B–D) or at late stage (Figure 3E). We have previously shown that pancreatic regeneration in the PdxCre;R26DTR model is associated with re-expression of Pdx1, and apparent reactivation of the embryonic program in the ducts.18 As expected, in the control DT-treated mice, the vast majority of the regenerative duct cells were SOX9+/PDX1+ (Figure 3D). However, macrophage ablation on day 7 post DT led to a significant reduction in the number of PDX1-positive cells during the regenerative process (Figure 3D) and ultimately caused failed regeneration at later stages (Figure 3E).

To evaluate the role of macrophages specifically in β-cell regeneration, PdxCreERT;R26DTR mice were injected with Mac1-SAP antibody on the last day of DT treatment, and were subsequently sacrificed 3 days later (Figure 4A). The adult mouse pancreas contains few (if any) resident macrophages, however, after β-cell ablation, the islets are specifically infiltrated by F4/80+ cells (Figure 4B). Mac1-SAP–mediated macrophage depletion was confirmed by a substantial decline in the number of F4/80+ cells within the islets of DT-treated PdxCreERT;R26DTR mice (Figure 4B). Interestingly, β-cell proliferation after β-cell ablation decreased significantly in macrophage-depleted mice (Figure 4C and D).

Next, we depleted macrophages in DT-treated ElaCreERT2;R26DTR mice (Figure 4E and F). As expected,18 the acinar compartment in DT-treated ElaCreERT2;R26DTR mice was recovered on day 7 after injury. However,
Figure 1. Macrophage polarization is required for pancreatic regeneration. (A–C) Fluorescent staining of sections obtained from early-stage DT-treated PdxCre;R26<sup>DTR</sup> pancreas using anti-F4/80 to label macrophages and E-cadherin (Ecad) to label epithelial cells shows abundant macrophage infiltration within the apoptotic acinar and endocrine compartments. (B) Note the absence of macrophages in the areas next to the surviving ducts. (C) Phagocytosis of the apoptotic epithelial cells by macrophages (asterisks). (D) Flow cytometry analysis highlights how CD11b<sup>+</sup> macrophages decline in number during the transition from early- to mid-stage in the regenerating PdxCre;R26<sup>DTR</sup> pancreata. (E) F4/80/Fizz1 double-staining of early and mid-stage pancreata reveals that macrophage polarization coincides with the peak of the regenerative process. Scale bars = 20 μm.
ElaCreERT;R26DTR mice that had been treated with DT and Mac1-Sap antibody displayed impaired acinar regeneration (Figure 4F).

Macrophages Induce Cell Type-Specific Differentiation In Vitro

To determine whether macrophages play only a permissive or rather an inductive role in pancreatic regeneration, we established an in vitro system where we co-cultured embryonic pancreatic epithelium with bone marrow-derived macrophages previously exposed to different types of apoptotic cells (Supplementary Figure 1). The embryonic pancreas is receptive to cues necessary for normal pancreatic differentiation and was therefore used to evaluate whether macrophages are able to provide regenerative signals. We first generated bone marrow-derived macrophages from 8- to 12-week-old C57Bl/6 mice, which

Figure 2. Macrophages polarize in a cell type specific injury manner. (A) Flow cytometry analysis confirms that the isolated F4/80⁺ macrophages are CD11b⁺. (B) Gating strategy. Cells obtained after digestion of the pancreas are stained for CD11b and intracellular cytokines. Then the percentage of cytokine⁺ cells is calculated on gated CD11b⁺ cells. (C–E) Gated CD11b⁺ cells isolated from early- or mid-stage regenerating pancreas of PdxCre;R26DTR (C), PdxCreERT;R26DTR (D), or ElaCreERT2;R26DTR mice (E) were stained for M1 (TNFα, IL6) or M2 markers (IL10, CD206).
we subsequently exposed overnight to apoptotic acinar cells, endocrine cells, or a mix of both. On the following day, we removed the cell debris, added fresh media, and started the co-culture with E11.5 pancreatic epithelium on a Transwell system to avoid any direct contact between the macrophages and the explant. As expected, the intact embryonic buds (with mesenchyme) grew, underwent branching, and differentiated into both endocrine and acinar cells (Figure 5A and F), and dorsal epithelium depleted of mesenchyme gave rise mainly to endocrine cells (Figure 5A and F). Similarly, the isolated epithelium cultured with noninduced macrophages (ie, nonexposed overnight to apoptotic cells) also gave rise almost entirely to endocrine cells (Figure 5B and F). Interestingly, the explants where the epithelium was co-cultured with macrophages that had been previously fed with apoptotic acinar
Figure 4. Macrophages are necessary for $\beta$- and acinar cell regeneration. (A) Schematic outline of DT and Mac1-SAP treatment of PdxCreERT;R26$^{DTR}$ mice. (B, C) Comparative analyses of tissues obtained from PdxCreERT or DT-treated PdxCreERT; R26$^{DTR}$ mice with or without Mac1-SAP injection, and harvested 3 days post DT demonstrate that proliferation among the surviving $\beta$-cells is correlated with the presence of macrophages. Note the specific homing of the macrophages to the islets. Bromodeoxyuridine (BrdU) was given via the drinking water 18 hours before the harvest. (D) Quantification of the proliferation rate among $\beta$-cells. (E) Schematic outline of DT and Mac1-SAP treatment of ElaCreERT2;R26$^{DTR}$ mice. (F) Comparative analysis of tissues obtained from DT-treated ElaCreERT2; R26$^{DTR}$ mice with or without Mac1-SAP injection, and harvested on day 7 post-DT shows that macrophage depletion results in impaired acinar regeneration, with persistence of Sox9$^+/DBA^+$ structures. INS: Insulin. Scale bars = 20 $\mu$m.

(Figure 5C and F) or islet cells (Figure 5D and F) showed an increase in the number of acinar or endocrine cells, respectively. Consistently, the epithelium co-cultured with macrophages that had been fed with apoptotic endocrine and acinar cells, promoted differentiation of both cell types (Figure 5E and F). In addition, the ability to mediate signals is likely to be independent of the type of cell death, as similar results were obtained from macrophages that had been fed with necrotic pancreatic cells (Supplementary Figure 2).

Macrophages From Old Mice Display Defective Polarization and Impaired Regeneration In Vivo But Can Induce Cell-Specific Differentiation In Vitro

Macrophages from old mice and humans display an age-related systemic chronic pro-inflammatory status, which includes defects in polarization, a process known as inflamma-ageing. This led us to investigate pancreatic regeneration in older animals in vivo. We noticed an overall decline in the regenerative capacity in the 8- to 12-month-old
PdxCre;R26\textsuperscript{DTR} mice, as assessed by impaired tissue regeneration (Supplementary Figure 3\textit{A}). Interestingly, this was not associated with a decreased number of infiltrating macrophages in the early stage, indicating the recruitment was not affected (Supplementary Figure 3\textit{B} and \textit{C}). However, the infiltrating macrophages in older mice failed to acquire the M2 phenotype during mid stage, as shown by absence of Fizz1\textsuperscript{+} cells (Supplementary Figure 3\textit{D}). These findings were also consistent with a significant reduction in PDX1\textsuperscript{+}/SOX9\textsuperscript{+} double positive cells within the ductal structures, confirming
that the impaired macrophage polarization affects the microenvironment of the epithelial progenitor niche (Supplementary Figure 3E and F).

Next, we asked whether the defective polarization observed in vivo in older PdxCre:R26DTR mice was due to intrinsic macrophage deficiency or rather to an aging environment. To remove the macrophages from the aged milieu, we generated bone marrow-derived macrophages from 8- to 12-month-old mice and repeated the in vitro co-culture system described previously. As shown in Supplementary Figure 4, macrophages originating from older mice were able to convert injury signals into proper regenerative signals in vitro, suggesting that their inability to do so in vivo is likely the result of the aging environment.

**Nonobese Diabetic Macrophages Have an Inherent Inability to Provide Appropriate Signals for β-Cell Generation Both In Vivo and In Vitro**

Macrophages derived from autoimmune-susceptible rodent strains or T1D patients produce abnormally high levels of cytokines, which is thought to contribute to persistence of the inflammation, instead of its resolution. This exacerbated M1 profile plays a prominent role in the pathogenesis of islet autoimmunity by promoting inefficient clearance of apoptotic cells, epitope-spreading and progressive destruction of β-cells. To explore whether this inherent macrophage defect is associated with additional inability to provide proper regenerative signals, we analyzed the expression profile of macrophages isolated from NOD pancreas at the onset of diabetes. As expected, NOD macrophages display a persistent M1-signature, confirming their inability to contract the inflammation response and transition to a regenerative state (Figure 6A). Transforming growth factor (Tgf) – β1/Smad signaling prevents β-cell proliferation, which can be blocked through activation of epidermal growth factor receptor (EGFR) pathway. Macrophages isolated from diabetic NOD mice express high levels of Tgfβ1, while lacking epidermal growth factor expression (EGF) (Figure 6B).

The expression profile of NOD macrophages might be heavily influenced by the cytokine milieu generated by other inflammatory cells, primarily autoreactive T cells. To specifically study the ability of NOD macrophages to produce regenerative signals outside the autoimmune setting, we performed co-culture experiments with bone marrow-derived macrophages harvested from young nondiabetic NOD mice, and fed them with apoptotic acinar, islets, or a mixture of acinar and endocrine cells (Figure 6C). Surprisingly, we found that when co-cultured with embryonic E11 pancreatic epithelium, the NOD macrophages promoted acinar differentiation, regardless of the type of apoptotic cells they had been exposed to (Figure 6C and D).

**Macrophages Exhibit Different Expression Profiles in Response to Cell-Specific Injury In Vivo**

Macrophages promote regeneration and tissue repair by producing numerous growth factors and Wnt-ligands. Based on these premises and our results showing that polarized macrophages exhibit a different cytokine phenotype after cell-specific injury, we investigated whether the phagocytosis of different apoptotic cell types in vivo leads to distinct expression profiles. To do so, CD11b+ cells were isolated from the pancreas of diabetic NOD mice or DT-treated PdxCre:R26DTR, ElaCreERT;R26DTR, or PdxCreERT; R26DTR mice. The isolated macrophages were then analyzed for the expression of selected growth factors or Wnt-ligands (Figure 7A). The growth factors and Wnt-ligands were chosen based on their expression or function in the developing mouse pancreas. Quantitative reverse transcription polymerase chain reaction (QRTPCR) showed macrophages exhibit a differential expression profile, depending on the type of injury. In addition, ligands and factors expressed by the macrophages display a dynamic expression pattern during different phases of the same regenerative process. The QRTPCR data were confirmed by Western blot and immunostaining analyses (Figure 7B, Supplementary Figure 5). Accordingly, isolated duct cells from the conditions mentioned show a different expression profile for selected Wnt-target genes, indicating an active cross-talk between the polarized macrophages providing the regenerative signals and the ductal cells receiving them (Figure 7C).

NOD-derived macrophages express high levels of TNFa and IL6 (Figure 6A). Interestingly, this is associated with lack of hepatocyte growth factor (HGF) up-regulation (Figure 7A). We have previously shown that hepatocyte growth factor suppresses the production of the pro-inflammatory cytokine IL6 in lipopolysaccharide-stimulated (LPS) macrophages from C57Bl/6 mice, at the same time allowing for the production of anti-inflammatory IL10. Here, we show that LPS NOD macrophages not only secrete significantly higher basal amount of the pro-inflammatory cytokine TNFa (Figure 7D), but also fail to down-regulate its synthesis after treatment with HGF (Figure 7D). These results are consistent with our in vitro findings showing how NOD-derived macrophages possess a more prominent M1 signature during the culture period compared with their nonautoimmune counterparts (Supplementary Figure 6). Compared with macrophages isolated from DT-treated PdxCreERT;R26DTR (non-autoimmune β-cell–specific injury), the NOD-derived macrophages displayed significantly lower expression of Wnt7b, and expressed higher levels of Tgfα and vascular endothelial growth factor–a (VEGFa), although they shared similar expression profile for fibroblast growth factor–10 (FGF10), Wnt3a, and Wnt5a (Figure 7A).

**Discussion**

It is well known that macrophages respond to environmental cues with the acquisition of distinct functional phenotypes, undergoing classical M1 or alternative M2 activation, where the pro-inflammatory M1 macrophages act as scavengers, and the anti-inflammatory M2 macrophages facilitate repair and regenerative activities. This property has been a recent object of intense study as the search for clinical strategies that improve the body’s
endogenous repair mechanisms are topics of major public health concern. However, the molecular mechanisms underlying tissue repair or its failure are not completely understood. This is particularly important for the pancreas and T1D, as the question whether β-cells can regenerate even when autoimmunity is abrogated is still under debate.34,35

We have previously shown that following selective ablation of acinar tissue in the ElaCreERT;R26DTR model, tissue regeneration occurred by direct reprogramming of ductal cells into acinar cells, as opposite to recapitulation of the embryonic pancreatic program observed in the PdxCre;R26DTR mouse when both acinar and endocrine cells were ablated.18 Here, and consistent with previous reports,36,37 we show that in the PdxCreERT;R26DTR model, the mechanism for β-cell regeneration is self-duplication of surviving β-cells. In this study, we sought to determine how these
Figure 7. Macrophages exhibit a different profile in response to injury in vivo. (A) Macrophages isolated from the pancreas of NOD mice, or the DT-treated PdxCre;R26\textsuperscript{DTR}, ElaCreERT2;R26\textsuperscript{DTR}, or PdxCreERT;R26\textsuperscript{DTR} transgenic animals display different messenger RNA expression profiles for selected growth factors and Wnt-ligands. Bars represent relative gene expression/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mean ± SE). n = 5 independent experiments; for each experiment macrophages were isolated from 5 pooled pancreata (for a total of 25 animals per time point). P value is calculated by comparing each bar with spleen macrophages of C57Bl/6 (WT) mice. *P ≤ .05; **P ≤ .01; ***P ≤ .001. (B) Western blot analyses for Wnt5a, Wnt7b, and β-actin on protein lysates extracted from spleen macrophages of C57Bl/6 (WT) mice, or infiltrating macrophages isolated from early- or mid stage of PdxCre;R26\textsuperscript{DTR} pancreata (PD early, PD mid). (C) Duct cells isolated from the pancreas of wild-type or DT-treated PdxCre;R26\textsuperscript{DTR}, ElaCreERT2; R26\textsuperscript{DTR}, or PdxCreERT;R26\textsuperscript{DTR} mice display different expression profiles with respect to Wnt-target genes. Bars represent relative gene expression/GAPDH (mean ± SE). Mouse embryonic pancreas (E14) was used as control. n = 5 independent experiments; for each experiment macrophages were isolated from 5 pooled pancreata (for a total of 25 animals per time point). (D) Compared with WT (C57BL/6) controls, bone marrow–derived macrophages isolated from NOD mice secrete higher basal levels of TNFα in response to lipopolysaccharide (LPS) and fail to suppress it when pretreated with hepatocyte growth factor (HGF). (E) Schematic representation of macrophage polarization in different injury models.
seemingly different regenerative processes are regulated. Mounting evidence suggests that macrophages are highly involved in orchestrating tissue regeneration in different organs.\textsuperscript{4,5,8,12,14–17} Our data confirm that regardless of the ablation model, the injured pancreas is infiltrated by macrophages. However, the subsequent phenotypes that these infiltrating macrophages develop seem to be heavily dependent on the apoptotic cell types they phagocyte. As summarized in Figure 7E, we could detect a gradual but clear switch from an M1 to M2 phenotype during the transition from early to mid stage in the PdxCre;R26\textsuperscript{DTR} pancreas, but not in PdxCreERT;R26\textsuperscript{DTR} mice, where the initial M1 macrophages later acquired a M1/M2 phenotype. In contrast to the PdxCre;R26\textsuperscript{DTR} or PdxCreERT;R26\textsuperscript{DTR} mice, macrophages isolated from DT-treated ElaCreERT2;R26\textsuperscript{DTR} pancreas maintained a M1/M2 signature throughout regeneration. Additionally, our analysis revealed that macrophages with the same cytokine phenotype but obtained from different ablation models, show different growth factor and wnt ligand expression profiles (compare M1 macrophages isolated from PdxCre;R26\textsuperscript{DTR} and PdxCreERT;R26\textsuperscript{DTR} in Figure 7). Likewise, macrophages originating from the same injury model, but isolated at different regenerative stages, despite sharing the same polarization signature, express a different set of genes (compare M1/M2 macrophages isolated from early- or mid-stage ElaCreERT2;R26\textsuperscript{DTR} mice in Figure 7). Our results highlight the necessity of a revision in how we define different macrophage polarization phenotypes, at least in studies exploring the regenerative properties of macrophages.

The data presented here on DT-mediated injury models indicate that macrophages undergo a phenotype switch during regeneration. This is in line with a recent report in which it was shown that tissue-specific signals control a reversible program of functional polarization of macrophages.\textsuperscript{12} Our conclusion is based on the facts that i) high percentage of cells positive for IL6, IL10, and CD206, especially in the PdxCre;R26\textsuperscript{DTR} model; ii) it is unlikely that a putative second wave of macrophages would display differentially expression profiles, depending on the apoptotic cell type that the initially infiltrating macrophages have engulfed; and iii) in our in vitro co-culture system there is no influx of newly infiltrating macrophages.

In this study, we demonstrate in several independent experiments how macrophage polarization is required for pancreatic regeneration. First, we show that depletion of macrophages after β-cell, acinar cell, or both endocrine and acinar cell ablation leads to impaired tissue regeneration (Figures 3 and 4). Secondly, we report that the impaired pancreatic regeneration in older PdxCre;R26\textsuperscript{DTR} mice is associated with the absence of M2 macrophages (Supplementary Figure 3). Finally, our in vitro co-culture system confirm that macrophages are able to translate injury signals into cell-type-specific regenerative signals (Figure 5). Collectively, these data demonstrate that proper pancreatic regeneration is dependent on precise temporally regulated macrophage polarization.

It has been reported that defective function of macrophages is a characteristic of T1D-prone individuals and animals.\textsuperscript{27,28,36} Here, we show that macrophages from NOD mice have a persistent pro-inflammatory M1 phenotype and inefficient ability to produce several molecular mediators involved in pancreatic regeneration (Figure 6 and Supplementary Figure 6). Accordingly, in vitro, they exhibit intrinsic deficiency in providing the proper signals for β-cell generation, suggesting that their functional impairment might be a key factor concurring to failure of regeneration in the setting of autoimmunity. It was recently reported that the inhibitory effect of Tgf1β/Smad-signaling on β-cell proliferation could be blocked through activation of the EGFR pathway.\textsuperscript{17} Interestingly, NOD-derived macrophages express significantly high levels of TGFβ1, and no EGF (Figure 6). It is tempting to speculate that the excessive presence of TGFβ1, produced by macrophages may prevent any attempt on β-cell proliferation in the diabetic NOD pancreas.

Pancreatic regeneration is the result of the coordinated effects of regenerative signals on responding cells. Interestingly, we could detect an overall higher reactivation of selected Wnt-target genes among ductal cells in the injury models compared with duct cells isolated from normal pancreas (Figure 7), which could be explained by Wnt-ligands secreted by macrophages. Other components of regeneration that should be considered are the nature of cell death (apoptosis vs necrosis) and also whether the damage triggers significant inflammatory response (other than macrophages). It is likely that the DT approach, which leads to a apoptotic cell death, may be one of the reasons for the robust regeneration that is observed in these ablation models. The data presented in this study indicate that macrophages play an essential role in mediating regenerative signals. To what extent macrophages play a similar role in acinar regeneration in pancreatitis remains to be revealed. Macrophages secrete RANTES, TNFα, and IL6 during pancreatitis, which in turn initiate ADM in acinar cells.\textsuperscript{15} Our data show that macrophages isolated from DT-treated ElaCreERT2;R26\textsuperscript{DTR} mice maintain high level of TNFα and IL6 expression throughout acinar regeneration (Figure 2). Although, the regenerative mechanism in our acinar ablation model does not entail ADM, it suggests that phagocytosis of acinar cells may promote expression of these cytokines. Similarly, NOD-derived macrophages display high levels of baseline TNFα and IL6 expression and secretion (Figures 6 and 7), which might explain why acinar differentiation was primarily promoted in the explants co-cultured with NOD macrophages (Figure 6). ADM is considered as an early step in acinar regeneration,\textsuperscript{39} therefore, it is possible that macrophages support acinar regeneration in pancreatitis by producing factors that induce ADM.

In summary, we show here for the first time that the spatiotemporal secretion of signals arising from macrophages is necessary for proper pancreatic regeneration. This process is reminiscent of their role in wound healing, where macrophages regulate the resolution of inflammation and promote tissue repair.\textsuperscript{8} In the absence of this temporarily defined activity, such as after macrophage depletion, as well as in the context of an “aged” environment, the regeneration process is impaired. The inability of NOD-derived macrophages to specifically promote β-cell generation strongly
suggests a potential, but as yet unexplored, link between defective macrophage polarization and impaired β-cell regeneration in the pathogenesis of T1D. Although more detailed studies are required to identify the specific factors that are necessary for cell type–specific regeneration, our findings provide a framework with which to test these key questions. The identification of the factors that regulate and promote the macrophage-derived environment could be successfully exploited for macrophage-centered therapeutically approaches.

Supplementary Material
Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2014.08.008.

References


35. Herold KC, Bluestone JA. Type 1 diabetes immunotherapy: is the glass half empty or half full? Sci Transl Med 2011;3:95fs1.


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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Material

Mice, Tamoxifen, and Diphtheria Toxin Treatment

The Rosa26DTR and NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The PdxCre1.2 mice were obtained from the Mouse Models of Human Cancer Consortium. The ElaCreERT2- and PdxCreERT strains were generated in the laboratories of Drs Craig Logsdon (University of Texas MD Anderson Cancer Center) and Douglas Melton (Harvard Medical School). Mice were treated with tamoxifen and DT, as described previously. Briefly, ElaCreERT2; R26DTR and PdxCreERT; R26DTR mice were first injected intraperitoneally with 2 mg tamoxifen for 2 consecutive days to activate Cre. All animals were then treated intraperitoneally with 0.5 ng/g body weight of DT for 5 consecutive days and killed at different time points. In the PdxCre; R26DTR model, the early stage of regeneration refers to the first week post DT injections, when the pancreas sustains a massive loss of the epithelial cell population and is infiltrated by macrophages. Mid and late stages of regeneration correspond approximately to days 7–21 and 14–35, when the actual regeneration process starts and is completed, respectively.

In the ElaCreERT2; R26DTR and PdxCreERT; R26DTR models, early corresponds to days 4–5 of DT injections and mid corresponds to day-3 post-DT. PdxCre; R26DTR, ElaCreERT2; R26DTR, and PdxCreERT; R26DTR mice described as “young” throughout the article were sacrificed at 8–10 weeks of age. PdxCre; R26DTR mice described as “old” were sacrificed at 8–12 months of age. Diabetic NOD mice at onset are females about 12–16 weeks of age with blood glucose ≥16.5 mmol/L in 2 consecutive measurements. For the in vivo experiments, n = 5 represents 5 independent experiments, where in each experiment pooled samples from macrophages isolated from several mice (similar conditions) have been used.

For the in vitro experiments, NOD mice were sacrificed at 8–10 weeks for bone marrow harvesting (nondiabetic). Eight- to 10-week-old C57Bl/J6 mice were used as control animals for the transgenic strains in all experiments, included in vivo macrophage isolation and in vitro generation of bone marrow–derived macrophages.

Macrophage Depletion

To specifically deplete infiltrating pancreatic macrophages, we administered, by tail vein injection 20 μg saporin-conjugated antibody against the pan-macrophage surface marker Mac1/CD11b (Mac-1-SAP) mouse/human; Advanced Targeting Systems, San Diego, CA; cat. no. IT-06-25).

Targeted SAP conjugates are powerful and specific lesioning agents used in the technique known as molecular surgery. The ribosome-inactivating protein saporin is bound to a targeting agent (in this case anti-Mac1 antibody). The requirement for the target is that it should be on the cell surface and also be able to get internalized. The targeted conjugate is administered to cells and binds to its target on the cell surface, and gets internalized. Upon internalization, saporin breaks away from the targeting agent, inactivates the ribosomes, which causes protein synthesis inhibition and, ultimately, leads to cell death. Mac1 (also known as CD11b) is specifically expressed in the macrophage lineage and is localized to the cell surface.

PdxCre; R26DTR model. To test the efficacy of macrophage depletion in the injured pancreas, Mac-1-SAP was administered on day 5 of DT injections, and then the mice were harvested on day 2 post-DT (see Figure 3A). To deplete macrophages at the beginning of the mid stage, Mac-1-SAP was administered on day 7 post-DT injections, and then the mice were harvested on days 14 and 35 post DT injections (see Figure 3B), respectively.

ElaCreERT2; R26DTR and PdxCreERT; R26DTR models. To deplete macrophages during the regenerative phase, Mac-1-SAP was administered on day 5 of DT injections, and mice were then sacrificed on day 7 post-DT (ElaCreERT2; R26DTR) or day 3 post-DT (PdxCreERT; R26DTR), respectively (see Figure 4A and E). For the evaluation of β-cell proliferation in the PdxCreERT; R26DTR model, bromodeoxyuridine (BrdU) was provided in the drinking water for 18 hours before sacrifice (see Figure 4A), as described previously.

Isolation of Macrophages and Ductal Cells From Injured Pancreata

After harvesting, pancreata were finely minced and put into a 50-mL conical tube in a solution containing 2 mg/mL Collagenase type V (Sigma-Aldrich, St Louis, MO; cat. no. C9263) in Hank's balanced salt solution with Ca2+ and Mg2+. Digestion was carried out in a shaking water bath at 37°C for 8–12 minutes and stopped with ice-cold Hank's balanced salt solution without Ca2+ and Mg2+. The digested tissue was subsequently filtered through a 40-μm pre-wet cell strainer in order to remove large acinar clumps and islets. Cells were then centrifuged for 5 minutes at 1300 rpm and filtered into polystyrene round-bottom tubes with 35-μm cell strainer caps. If necessary, red cell lysis was performed (Red Blood Cell Lysis Buffer Hybri-Max, Sigma-Aldrich; cat. no. R7757) according to the manufacturer's instructions. Cells were then washed, counted, and resuspended at 107 cells/100 μL Separation/Sorting Buffer (phosphate-buffered saline without Ca2+ and Mg2+, 0.5% bovine serum albumin and 2 mM EDTA) and subjected to magnetic sorting in order to isolate macrophages or ductal cells. Briefly, cells were first treated with 10 μL FeC Block Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany; cat. no. 130-092-575) for 10 minutes at 4°C, and then incubated for 20 minutes at 4°C in the dark with the primary antibody. For macrophage isolation, cells were incubated with eFluor660-conjugated ant-mouse F4/80 antibody (eBioscience, San Diego, CA; cat. no. 50-4801-82). After washing, cells were magnetically labeled and sorted with anti-Cy5/anti-Alexa Fluor 647 Microbeads (Miltenyi Biotec; cat. no. 130-091-395), according to the manufacturer's instructions. Alternatively, macrophages were isolated with CD11b microbeads (Miltenyi Biotec; cat. no. 130-049-601), according to the manufacturer’s instructions. For ductal cell
isolation, cells were incubated with a biotinylated dolichos biflorus agglutinin lectin (DBA) and then magnetically sorted with anti-biotin microbeads (Miltenyi Biotec; cat. no. 130-090-485). Cell purity (≥90%) after magnetic sorting was confirmed by flow cytometry. Isolated macrophages and ductal cells were then subsequently used for downstream analyses. Antibodies and dilutions used are listed in Supplementary Table 1.

**Flow Cytometry Analysis**

For surface staining, cells were treated with 10 μL FcR Blocking Reagent (Miltenyi Biotec; cat. no. 130-092-575) for 10 minutes at 4°C, and then incubated with primary conjugated antibodies for 20 minutes at 4°C in the dark. For intracellular cytokine staining, BD Cytofix/Cytoperm Fixation/Permeabilization solution Kit (BD Bioscience, San Jose, CA; cat. no. 554714) was used according to the manufacturer's instructions. Cells were then incubated with conjugated intracellular antibodies for 20 minutes at 4°C in the dark. Data were acquired with a BD FACSAria Cell Sorter, analyzed with FACSDiva Software (BD Bioscience). Gating strategy is shown in Figure 2A. Antibodies and dilutions used are listed in Supplementary Table 1.

**Generation of Bone Marrow–Derived Macrophages**

Femurs and tibiae were harvested from 8- to 12-week-old C57Bl/6 (young), 8- to 12-month-old C57Bl/6 (OLD) or 8-week-old NOD/LtJ mice and the bones were flushed with Complete Macrophage Media using a 26-gauge needle into a 50-mL conical tube. The collected bone marrow was centrifuged at 1100 RPM for 7 minutes at 4°C. The pellet was resuspended in 10 mL Complete Macrophage Media (high-glucose Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.1% 2-mercaptoethanol) and filtered into a new tube. The cells were seeded at 2 x 10^6 per well in 24-well plates and cultured in a 37°C humid CO2 (5%) incubator. The cells were fed with fresh media every other day for 7 days. On day 7, the cells were replenished with L929-free media and were ready for subsequent assays.

**Co-Culture of Bone Marrow–Derived Macrophages With explants**

Islets and acinar cells were isolated from 8- to 12-week-old C57Bl/6 mice as described previously. For induction of apoptosis, freshly isolated hand-picked islets and acinar cells were resuspended in phosphate-buffered saline and irradiated in ultra low-attachment plates with a dose of 160 kV, 19 mA for 6 minutes (XRAD-160, Precision X-ray Inc., North Branford, CT). Necrosis was achieved by subjecting cells to multiple cycles of freezing/thawing. Bone marrow–derived macrophages obtained at day 7 were then incubated overnight with apoptotic or necrotic acinar cells, islets, or a mixture of islets and acinar cells. Each 24-well received 20 islets/well, 40 μL acinar cell suspension/well, or 20 islets + 20 μL acinar cell suspension, resuspended in 500 μL Pancreatic Culture Media (M199 media supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin) (Supplementary Figure 1).

After 18–24 hours, the dead cells were removed and fresh Pancreatic Culture Media was added to the wells containing the macrophages. At the same time, E11.5 dorsal pancreatic epithelium (depleted of the mesenchyme) was dissected and put on top of Transwell inserts and cultured in 700 μL Pancreatic Culture Media, as described previously. To ensure the constant presence of the factors produced and released by the macrophages during the 7-day culture period, 100 μL fresh media was added every other day to compensate for the evaporation. Control E11.5 dorsal buds, with or without mesenchyme, were cultured in the absence of macrophages or with unfed macrophages.

Minimum number of cells counted for each condition was: intact buds: 1200 cells, epithelium: 300 cells, epithelium with noninduced wt macrophages: 300 cells, epithelium with wt macrophages fed with apoptotic acinar cells: 800 cells, epithelium with wt macrophages fed with apoptotic islets: 380 cells, epithelium with wt macrophages fed with a mixture of apoptotic acinar cells and islets: 750 cells, epithelium with wt macrophages fed with apoptotic acinar cells: 780 cells, epithelium with wt macrophages fed with apoptotic islets: 700 cells, epithelium with wt macrophages fed with a mixture of apoptotic acinar cells and islets: 700 cells.

**Quantitative Real-Time Polymerase Chain Reaction and Western Blotting**

Quantitative real-time polymerase chain reaction and Western Blotting were carried out on in vivo infiltrating macrophages isolated from injured pancreata or on bone marrow–derived macrophages cultured in vitro in different conditions. For quantitative reverse transcription polymerase chain reaction, messenger RNA isolation and subsequent complementary DNA synthesis were performed using μMACS One-step cDNA kit (Miltenyi Biotec, Cat. No. 130-091-902) according to the manufacturer’s instructions. Polymerase chain reaction primers were purchased from Qiagen (QuantiTect Primer Assays, Qiagen) and are listed in Supplementary Table 2. Reactions were performed with PerfeCTa SYBR Green SuperMix for IQ (Quanta Biosciences, Cat. No. 95053) on a BioRad IQ5 Instrument (Biorad). Reactions were performed at least in triplicates and specificity of the amplified products was determined by melting peak analysis. Quantification for each gene of interest was performed with the 2^-ΔΔCt method. Quantified values were normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, which proved to be stable across the samples. Primers are listed in Supplementary Table 2. For Western Blotting, cells were lysated in Complete Lysis-M Reagent supplemented with protease inhibitors (Roche, Cat. No. 04719964001). Protein quantification was performed with Pierce BCA protein...
Assay Kit (Thermo Scientific, Cat. No. 23227). Protein lysates were separated on 4%–20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Mini-PROTEAN TGX Gels, BioRad, Cat. No. 456-1094) and transferred onto 0.45-μm charged polyvinylidene difluoride membranes using a Mini Trans-Blot electrophoretic apparatus (Bio-Rad). All buffers were purchased from BioRad. Antibodies and dilutions used are listed in Supplementary Table 1.

Immunofluorescence and Quantification Analysis
Tissue processing, immunostaining, and quantification analysis were performed as described previously. Antibodies and dilutions used are listed in Supplementary Table 1.

Enzyme-Linked Immunosorbent Assay
Bone marrow–derived macrophages from both C57Bl/6 and NOD mice were treated with 10 ng/mL recombinant mouse hepatocyte growth factor (R&D Systems) for 24 hours at 37°C before stimulation with 100 ng/mL lipopolysaccharide from Escherichia coli (055:B5) (Sigma Aldrich) for 6 hours. Only lipopolysaccharide treatment was performed in controls. Supernatants were collected from triplicate wells and TNFα was measured by enzyme-linked immunosorbent assay using Mouse TNF-alpha DuoSet (R&D Systems).

References

Author names in bold designate shared co-first authorship.
**Supplementary Table 1. List of Antibodies Used in this Study**

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**NOTE.** All secondary antibodies used for immunostaining were purchased from Jackson ImmunoResearch Laboratories: biotin-conjugated anti-rabbit (1:500), biotin-conjugated anti-rat (1:500), biotin-conjugated anti-guinea pig (1:500), biotin-conjugated anti-goat (1:250); Cy2-conjugated streptavidin 1:500; Cy3-conjugated streptavidin 1:500; Cy5-conjugated streptavidin 1:100; and Cy2- and Cy3-conjugated donkey anti-guinea pig, anti-rabbit, anti-rat, anti-goat (all 1:300). All isotypes for flow cytometry (Flow) were purchased from BD Pharmingen. Secondary antibodies for Western blotting (WB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used 1:2000 for 1 hour at room temperature. APC, allophycocyanin; Ck, cytokeratin; DBA, dolichos biflorus agglutinin; FITC, fluorescein isothiocyanate; Fizz1, Found in inflammatory zone 1; Pdx1, pancreatic and duodenal homeobox 1; PE, phycoerythrin; Sox9, SRY (sex determining region Y)-box 9; TGF, transforming growth factor; IF, immunofluorescence.
## Supplementary Table 2. List of Primers Used in This Study

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Arg1, arginase 1; EGF, epidermal growth factor; FGF10, fibroblast growth factor 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; iNOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor.
Supplementary Figure 1. Schematic outline of the co-culture experiments. Full description has been provided in the Materials and Methods section. Aci, acinar; Apo, apoptotic; BM, bone marrow; CSF-1, colony-stimulating factor 1; Isl, islet; Nec, necrotic.
Supplementary Figure 2. Macrophages induce cell-specific differentiation in vitro independent of the type of cell death. E11.5 dorsal pancreatic bud depleted of mesenchyme (Epi) were co-cultured with macrophages that had been “fed” with necrotic acinar (Nec Aci) (A), islet (Nec Isl) (B), or a mixture of acinar and islet cells (Nec Mix) (C). The day-7 explants were analyzed for the expression of amylase (AMY) and E-cadherin (Ecad), or insulin (INS), glucagon (GCG), and E-cadherin, and quantified (D). n = 5 for each condition, *P ≤ .05; **P ≤ .01. Scale bar = 20 μm.
Supplementary Figure 3. Impaired pancreatic regeneration in older mice is associated with defective macrophage polarization in vivo. (A) The late stage pancreas in old PdxCre;R26\textsuperscript{DTR} mice after DT treatment does not recover and consists entirely of Ck19\textsuperscript{+}/Ecadherin\textsuperscript{+} ductal structures. (B) The number of macrophages in the pancreas of DT-treated old PdxCre;R26\textsuperscript{DTR} mice is not reduced during early stage, indicating that the recruitment of macrophages is normal. (C, D) Older mice lack proper macrophage polarization during the mid stage of regeneration. Tissues harvested at early (C) or mid stage (D) of DT-treated old PdxCre;R26\textsuperscript{DTR} pancreata show decreased number of F/80/Fizz1 double-positive cells. (E, F) The reactivation of embryonic program associated with regeneration in the DT-treated PdxCre;R26\textsuperscript{DTR} pancreas does not occur in the old mice. Tissues obtained from DT-treated old PdxCre;R26\textsuperscript{DTR} pancreas lack PDX1 re-expression in SOX9\textsuperscript{+} ductal cells. Ck19, cytokeratin 19, Ecad, Ecadherin. Scale bars = 20 \mu m.
Supplementary Figure 4. Macrophages isolated from old mice are capable of inducing cell-specific differentiation in vitro. E11.5 dorsal pancreatic bud depleted of mesenchyme (Epi) were co-cultured with noninduced macrophages (A), or with macrophages that had been “fed” with apoptotic acinar (Apo Aci) (B), islet (Apo Isl) (C), or a mixture of acinar and islet cells (Apo Mix) (D). The day 7 explants were analyzed for the expression of amylase (AMY) and E-cadherin (Ecad), or insulin (INS), glucagon (GCG) and E-cadherin, and quantified (E). n = 5 for each condition, *P ≤ .05; **P ≤ .01. Scale bar = 20 μm. Aci, acinar; Apo, apoptotic; Isl, islet; epi, epithelium.
Supplementary Figure 5. WNT7b expression in macrophages. Tissues obtained from DT-treated PdxCre;R26<sup>DTR</sup> (A) or diabetic NOD mice (B) were stained for Wnt7b, Ecadherin (Ecad), and F4/80. WNT7b is detected in a subset of F4/80<sup>+</sup> macrophages in the regenerating PdxCre;R26<sup>DTR</sup> pancreas, but it is hardly detected in NOD macrophages at the onset of diabetes. Arrows and asterisk highlight the ducts or perinsulitis, respectively. Scale bars = 20 μm.
Supplementary Figure 6. NOD-derived macrophages display a different growth factor profile when fed with apoptotic cells in vitro. Quantitative reverse transcription polymerase chain reaction analysis of bone marrow-derived macrophages originating from C57Bl/6 control or NOD mice that had been fed with apoptotic acinar (Apo Acin), islets (Apo Isl), or a mixture of endocrine and acinar cells (Apo Mix) and harvested on days 2 or 7 in culture. The NOD-derived macrophages exhibit an exacerbated M1-signature, and express different expression profile than their control counterparts. Bars represent relative gene expression/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mean ± SE) expressed as fold over non-induced C57Bl/6 control macrophages. n = 5. *P ≤ .05; **P ≤ .01; ***P ≤ .001.