**Heme Oxygenase-1 Induction Prevents Autoimmune Diabetes in Association With Pancreatic Recruitment of M2-Like Macrophages, Mesenchymal Cells, and Fibrocytes**

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Immunoregulatory and regenerative processes are activated in the pancreas during the development of type 1 diabetes (T1D) but are insufficient to prevent the disease. We hypothesized that the induction of cytoprotective heme oxygenase-1 (HO-1) by cobalt protophyryn (CoPP) would prevent T1D by promoting anti-inflammatory and pro-repair processes. Diabetes-prone BioBreeding rats received ip CoPP or saline twice per week for 3 weeks, starting at 30 days and were monitored for T1D. Immunohistochemistry, confocal microscopy, quantitative RT-PCR, and microarrays were used to evaluate postinjection pancreatic changes at 51 days, when islet inflammation is first visible. T1D was prevented in CoPP-treated rats (29% vs 73%). Pancreatic Hmox1 was up-regulated along with islet-associated CD68/HO-1 cells, which were also observed in a striking peri-lobular interstitial infiltrate. Most interstitial cells expressed the mesenchymal marker vimentin and the hematopoietic marker CD34. Spindle-shaped, CD34/vimentin cells coexpressed collagen V, characteristic of fibrocytes. M2 macrophage factors Krüppel-like factor 4, CD163, and CD206 were expressed by interstitial cells, consistent with pancreatic upregulation of several M2-associated genes. CoPP upregulated islet-regenerating REG genes and increased neogenic REG3\(\alpha\) and insulin clusters. Thus, short-term induction of HO-1 promoted a protective M2-like milieu in the pancreas and recruited mesenchymal cells, M2 macrophages, and fibrocytes that imparted immunoregulatory and pro-repair effects, preventing T1D. (*Endocrinology* 156: 3937–3949, 2015)

Type 1 diabetes (T1D) is an autoimmune disease characterized by an immune attack on insulin-producing \(\beta\)-cells in the pancreas (1, 2). During the course of \(\beta\)-cell destruction, cells with host defense potential are also recruited to the pancreas in a futile attempt to alter the balance between effector and regulatory processes (3). However, there is little information on these protective mechanisms and whether they can be harnessed to arrest disease progression. To investigate these questions, we used the diabetes-prone BioBreeding (BBdp) rat, a useful translational model of T1D that has accurately predicted outcomes in human trials of cyclosporin A, nicotinamide, insulin, and dietary antigens (4, 5).

Heme oxygenase-1 (HO-1) is a stress-inducible protein that breaks down heme to carbon monoxide, Fe\(^{2+}\), biliverdin, and bilirubin, which have antioxidant and anti...
inflammatory properties (6). Induction of HO-1 has been reported to prevent or ameliorate T1D in the nonobese diabetic (NOD) mouse model of autoimmune diabetes, dampening pancreas dendritic cell infiltration, inhibiting apoptosis, and upregulating antioxidant defenses (7–10). The HO-1 pathway is associated with the CD163 receptor on the surface of M2 macrophages (11). Free hemoglobin binds to haptoglobin, forming complexes (12) that are taken up via the CD163 receptor (13) and broken down by HO-1 to cytoprotective molecules. More recently it has been proposed that HO-1 modulates the immune system by a shift toward anti-inflammatory M2 macrophages (14).

M1 macrophages are the first inflammatory cells to enter the islets of BBdp rats and administering macrophage inhibitors prevents T1D (15), suggesting an imbalance of pro-inflammatory M1/anti-inflammatory M2 cells. M2 macrophages have important regulatory functions and promote wound healing. We previously described an increased number of intestinal CD163+ M2 macrophages in diet-protected hydrolyzed casein (HC)-fed BBdp rats (16). In addition, the highest intestinal expression of the gene encoding HO-1 (Hmox1) was observed in germ-free BBdp rats fed a low-antigen HC diet, environmental conditions associated with an almost complete inhibition of spontaneous T1D (16).

Interestingly, some markers and functional attributes of M2 macrophages have been described in other cell types. For example, fibrocytes are bone marrow-derived, spindle-shaped mesenchymal cells that produce collagen and express either CD34 or CD45. Mounting evidence supports fibrocyte involvement in wound healing and inflammatory diseases (17), but involvement in T1D has not been reported.

In the present study, we hypothesized that HO-1 induction by cobalt protoporphyrin (CoPP) treatment of young BBdp rats could prevent T1D by enhancing immunoregulatory mechanisms and pancreatic regenerative processes. Our data suggest that HO-1 induction prevented T1D in association with pancreatic recruitment of M2-like cells, some of which displayed characteristics of mesenchymal cells, anti-inflammatory macrophages and fibrocytes. Some of these cells were observed in association with islets, and the most striking feature was a multicellular M2-like infiltrate throughout the pancreatic interstitium, contributing to an anti-inflammatory, pro-repair milieu that afforded protection from T1D.

Materials and Methods

Animals

Animal studies were approved by the University of Ottawa/Ottawa Hospital Research Institute Animal Care Committee.

Sixty-four BBdp rats were weaned onto a γ-irradiated Teklad Global 18% protein rodent diet (Harlan Laboratories) and housed under specific pathogen-free conditions. CoPP (Frontier Scientific, Inc) was dissolved in 10 mmol/L Tris base at pH 7.8. Equal numbers of male and female rats from five litters were randomly assigned to either CoPP or saline groups. Beginning at 30 days, BBdp rats received ip injections of CoPP (6.5 mg/kg, n = 33) or saline (n = 31) twice per week at 2- to 3-day intervals for 3 weeks. At 51 days, subsets of saline- (n = 16) and CoPP-treated (n = 16) rats were killed and pancreata were harvested and snap frozen in liquid nitrogen or fixed in Bouin’s fixative. The remaining rats (n = 15–17 per group) were monitored for weight loss and glucosuria biweekly until 120 days. Animals with glucosuria were fasted overnight and those with fasting blood glucose of 11.1 mmol/L or greater were defined as diabetic. Animals were killed within 48 hours of diabetes onset. Those that remained normoglycemic until 120 days were defined as asymptomatic.

Immunohistochemistry and morphometric analyses

Paraffin-embedded pancreas tissue was cut into 5 μm sections. Insulitis was evaluated on hematoxylin and eosin (H&E)-stained sections at ×400 magnification using a ScanScope CS eSlide capture device and analyzed using ImageScope software (Aperio). We qualified islets as healthy if they displayed normal morphology of endocrine cells and contained four or fewer mononuclear cells in the islet core on H&E-stained sections. The extent of insulitis was determined by giving a score per islet and obtaining a mean score per animal. Data are presented as a percentage of healthy (noninflamed) islets.

For immunohistochemistry, antibodies to the following proteins were used: HO-1, CD68, CD163, CD206, Krüppel-like factor 4 (KLF4), CD34, vimentin, collagen V, desmin, S100, major histocompatibility complex (MHC) class II, CD14, glial fibrillary acidic protein (GFAP), fibroblast activating protein-2 (FAP-2), regenerating protein 3 (REG3)-β, and insulin (see Supplemental Table 1 for a complete list of antibodies and antigen retrieval methods). Sections were stained using the avidin-biotin-peroxidase method as described previously (18). Slides were analyzed using an Axioplan 2 light microscope (Zeiss) at ×400 magnification and Northern Eclipse software (Empix Imaging Inc) or an Aperio ScanScope with ImageScope software.

Islet-associated (peri- and intra-islet) HO-1+ cells and CD68+ cells were quantified separately and normalized to total islet area. For islet morphometric analyses, measurements consisted of the sum of positive cells quantified within the islet core, the peri-islet area, and areas immediately adjacent to the islet periphery (within a two cell distance from the islet boundary); we qualified these cells as islet-associated (CD68+ and/or HO-1+) cells normalized to square millimeters of islet area. The islet area used for normalization corresponded to the area within the islet boundary. REG3β− area fraction was determined by quantifying the proportion of intra-islet REG3β− area normalized to total islet area. REG3β− clusters and insulin− extra-islet clusters (EICs) were quantified and normalized to total pancreatic area. Extra-islet REG3β− or insulin− clusters consisted of three or fewer positive cells grouped together including instances of single positive cells. Data were expressed as the number of clusters per square millimeter of pancreas area. One analysis focused on acinar-associated (nonductal) clusters that were dispersed in acinar tissue; a second analysis focused on duct-associated clusters com-

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prising cells clearly localized within the ductal epithelium. For both analyses, data were normalized to total pancreas area. Most of these clusters coexpressed REG3β and insulin.

For immunofluorescence, primary antibodies were applied at 4°C overnight or 2 hours at room temperature, and sections were incubated with Cy3-, Alexa488-, or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch); nuclei were counterstained with Hoechst. Fluorescence was visualized using a Zeiss 510 Meta confocal microscope, and image processing was performed using ZEN LE 2009 (Zeiss) and Northern Eclipse. In some instances, pseudocoloring was used for clarity as specified in the figure legends. Double-positive islet-associated CD68+/CD68- cells were quantified and normalized to total islet area. CD68+, HO-1+, vimentin+, and KLF4+ cells were quantified in the expanded interstitium of CoPP-treated BBdp rats. The number of cells positive for various markers was expressed as a percentage of total cells present in the interstitium.

Gene expression studies

Approximately 30 mg of tissue from the splenic side of the pancreas was harvested from 51 day saline- and CoPP-treated BBdp rats (n = 8/group). Total pancreatic RNA was isolated and quantitative PCR analysis was performed as described previously (16) using TaqMan gene expression assays (Life Technologies) and the ABI Prism 7000 sequence detection system (Applied Biosystems). For microarray studies, samples from each group of eight were pooled at a final concentration of 100 ng/μL. Global gene expression was analyzed using Affymetrix Rat Gene 1.0 ST microarrays (OHRI StemCore Laboratories). CEL files were processed using AltAnalyze (University of California, San Francisco, San Francisco, California) and the Database for Annotation, Visualization, and Integrated Discovery online software (DAVID, National Institutes of Health; david.abcc.ncifcrf.gov) for the enrichment analysis of genes upregulated more than 5-fold to obtain functional interpretation of the microarray data (19, 20).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 4.0 (GraphPad Software). Survival curves were generated using the Kaplan-Meier method; a survival analysis was performed using the log-rank test. Means were calculated and differences were compared with a Student’s t test or the Mann-Whitney U test when the data were not normally distributed. Values of P < .05 were considered significant. Data are presented as mean ± SD.

Results

HO-1 induction by CoPP prevents T1D in BBdp rats

We observed HO-1 expression in islet-infiltrating macrophages in untreated BBdp rats but not in control, nondiabetes-prone BB control (BBc) animals (Figure 1A). When we chemically induced HO-1, diabetes was prevented in CoPP-treated animals (CoPP 29% vs saline 73% incidence, P = .01, log-rank, Figure 1B). At 51 and 120 days, insulin+ area fraction and β-cell mass were not different in asymptomatic animals (data not shown). However, the percentage of healthy (noninflamed) islets was increased in CoPP-treated rats at both 51 days and 120 days (Figure 1C), consistent with protection from
T1D. The expanded interstitial cell population observed in 51-day, CoPP-treated rats was markedly contracted in rats aged 120 days, indicating that the infiltrate subsided over time (data not shown). Residual CD34+, vimentin+ cells persisted in the interstitium to 120 days in asymptomatic rats and were associated with decreased T1D in CoPP-treated animals. The presence of the infiltrate did not have a detrimental effect on islet function as most CoPP-treated rats remained normoglycemic, even up to 120 days. Subsequent analyses focused on animals aged 51 days, when spontaneous pancreas inflammation is just beginning and immediately after the final CoPP injections.

Infiltration of HO-1+, CD68+, and HO-1+CD68+ cells in the pancreas after CoPP treatment

A previous report described the recruitment of HO-1+ macrophages to the pancreas after an ip injection of another inducer of HO-1, hemin (21). We evaluated whether HO-1+ macrophages were similarly present in the pancreas of CoPP-treated rats. In saline-injected rats, HO-1+ (Figure 2A, left panel) and CD68+ (Figure 2B, left panel) cells were rare around islets. In contrast, HO-1+ and CD68+ cells were abundant in peri-islet areas of CoPP-treated rats (Figures 2, A and B, middle panels). The numbers of islet-associated (peri- and intra-islet) HO-1+ cells (Figure 2A, right panel) and CD68+ cells (Figure 2B, right panel) were markedly increased, and there were approximately 22-fold more CD68+HO-1+ cells in CoPP-treated pancreata (Figure 2C).

Interstitial aggregate in pancreas of CoPP-treated rats

A striking aggregate of fibroblast-like cells was present throughout the pancreatic peri-lobular interstitium of CoPP-treated rats (Figure 3, A–C, right panels). This infiltrate was absent and the interstitium was not expanded in saline-injected rats (Figure 3, A–C, left panels). Similar to peri-islet areas, CD68+HO-1+ cells were present in the expanded interstitium of CoPP-treated rats (Figure 3D), representing approximately 32% of the total cells in this area (Figure 3E). CD68+HO-1+ cells represented 17% of the interstitial cells, whereas CD68+HO-1+ cells comprised only 4%. Other cell types that expressed neither CD68 nor HO-1 represented 47% of aggregate cells, indicating the heterogeneous nature of the cellular infiltrate.

Most cells in the interstitium of CoPP-treated rats are M2-like mesenchymal cells

To further characterize the phenotype of the aggregate cells in CoPP-treated rats, we immunostained for CD163 (M2 macrophages), MHC class II (antigen presenting cells), CD14 (monocyte/macrophage marker), KLF4 (a regulator of M2 macrophage polarization [22, 23] and epithelial-to-mesenchymal transition [24]), S100 (expressed in neural cells and also in macrophages, fibrocytes, and monocytes but absent in fibroblasts [25]), α-smooth muscle actin (α-SMA; a myofibroblast marker [28]), vimentin (a mesenchymal and fibroblast marker [27]), and desmin (a marker of myofibroblasts and stellate cells [28]). As shown in Supplemental Figure 1A, MHC class II+ cells comprised only a small proportion of the interstitial aggregate. CD163 was expressed by a subset of aggregate cells (Supplemental Figure 1B). KLF4+ cells were also observed (Supplemental Figure 1C), suggesting a subset of the aggregate cells was undergoing an active transition process; S100+ cells were less frequent (data not shown). Vimentin is a type III intermediate filament protein expressed in mesenchymal cells. Large numbers of vimentin+ cells were identified in the pancreata of CoPP-treated rats (Figure 4A, right panel). Indeed, the predominant population within the interstitium was the vimentin+HO-1+ subset (65%, Figure 4C); some of these cells (~8%) were triple-positive vimentin+HO-1+KLF4+, possibly M2-like mesenchymal cells (Figure 4, B and C). Myofibroblasts are contractile mesenchymal cells expressing cytoskeletal proteins such as vimentin, desmin, and α-SMA (28). Although some desmin+ cells were present in the interstitium (Supplemental Figure 1D), α-SMA+ cells were absent (data not shown), indicating that these vimentin+ cells were not myofibroblasts. FAP-2 is involved in the peri-cellular proteolysis of the extracellular matrix. It plays a role in tissue remodeling during development and wound healing and is specifically expressed on the cell membrane of fibroblasts. We observed only a few FAP-2+ cells in the peri-lobular aggregates, suggesting most of the aggregate cells were not fibroblasts.

CD34+, vimentin+, and collagen V+ cells with M2 markers in interstitium of CoPP-injected rats

In healthy pancreata, CD34+ cells are distributed in the endothelial cells of small blood vessels but are rarely observed in peri-lobular connective tissue. In striking contrast, we observed abundant CD34+ cells in the interstitial aggregates as well as ducts and blood vessels of CoPP-treated rats (Supplemental Figures 1E and 5, A and B). CD34 and vimentin were predominant in the aggregates and were largely colocalized (Figure 5A). Fibrocytes are spindle shaped, express the hematopoietic progenitor markers CD34 and/or CD45 on the cell surface, and pro-
duce collagen (27). Collagen V+ cells were abundant (Supplemental Figure 1F), whereas cells expressing collagen I, II, or III were not observed (data not shown). A small proportion of collagen V+ cells colocalized with both CD34 and KLF4 (Figure 5B), suggesting they were M2-like mesenchymal cells with a fibrocyte phenotype. Many of these interstitial cells expressed M2 macrophage-associated markers. For example, several CD206+ cells colocalized with vimentin and KLF4 (Figure 6A). In addition, a subset of CD163+ cells was also observed and these all coexpressed HO-1+ (Figure 6B).

Pancreatic expression of M2-associated genes increased after CoPP treatment

As expected, pancreatic Hmox1 expression was increased in CoPP-treated rats (Figure 7A), whereas the Hmox1 suppressor Bach1 was decreased (Figure 7B). The M2 macrophage-associated genes Mrc1 (CD206, Figure 7C), Msr1 (CD204, Figure 7D), and Cx3cr1 (Figure 7E) were increased. Two immunoregulatory cytokine genes, Il10 (Figure 7F) and Tgfb1 (Figure 7G), were increased after HO-1 induction as was the pro-inflammatory cytokine Il1b (IL-1β) (Figure 7H).
Figure 3. Pronounced peri-lobular interstitial infiltration consisting of HO-1\(^+\) cells and CD68\(^+\) cells in pancreata of CoPP-treated BBdp rats. A, Representative H&E-stained images of the pancreatic interstitium of saline- and CoPP-injected 51 day BBdp rats. Bars, 50 μm. Note the dramatic interstitial expansion and increased cellularity of this region in the CoPP group. Representative images of HO-1 (B) and CD68 (C) expression in pancreata of saline- and CoPP-injected 51-day BBdp rats are shown; insets display magnified interstitial areas. D, Representative confocal image showing CD68 (red) and HO-1 (pseudocolored green) expression in pancreatic interstitium of a CoPP-injected animal; insets display magnified areas containing double-positive cells. Bars, 20 μm. HO-1 was labeled using a Cy3-conjugated secondary antibody and pseudocolored green for clearer demonstration. E, Characterization of the cellular composition of the interstitium of CoPP-treated rats on the basis of HO-1 and CD68 expression (n = 7 CoPP treated rats). Most CD68\(^+\) cells coexpressed HO-1 and CD68\(^+\)HO-1\(^+\) cells represented a large component of the total peri-lobular infiltrate.
Microarray analysis was used to identify cellular mechanisms and pathways (Supplemental Table 2). Top upregulated candidate factors included lipid-related genes encoding intestinal fatty acid binding protein (Fabp2; upregulated ~300-fold) and gastrotropin (Fabp6, upregulated ~250-fold). Consistent with quantitative RT-PCR data (Figure 7A), Hmox1 expression was increased approximately 43-fold in the microarray analysis. Genes encoding members of the regenerative REG protein family were up-regulated in the pancreata of the CoPP-treated rats (Supplemental Table 2). Reg3g, Reg3b, and Reg3a were up-regulated (56-, 27- and 26-fold, respectively). The DAVID bioinformatics software (National Institutes of Health) analysis revealed the top three functional enrichment groups contained several genes encoding REG proteins, α-defensins, and chemokines, including chemokine ligand (CCL)-2 (monocyte chemoattractant protein-1) and CCL25 (Supplemental Table 2).

**HO-1 induction by CoPP is associated with islet regeneration**

HO-1 induction has been associated with improved islet function (10). A significant increase in pancreatic Reg3b gene expression was observed in the pancreata of CoPP-treated rats (Figure 8A), validating the microarray finding (see Supplemental Table 3). The REG3β islet area fraction was increased (Figure 8A) as were the numbers of duct- and acinar-associated REG3β clusters (Figure 8B). In addition, the number of duct-associated and acinar-associated insulin+ EICs was increased in the CoPP-treated rats (Figure 8B), and insulin and REG3β colocalized in both islets and EICs (Figure 8C).

**Discussion**

HO-1+ macrophages are present in inflamed islets from untreated BBdp rats but absent in controls. Administration of CoPP increased pancreatic Hmox1 gene expression, HO-1+ cells and prevented T1D in BBdp rats, consistent with other reports (7, 8). By studying BBdp rats at an age when pancreas inflammation is beginning and just after the final CoPP injections, we demonstrate a novel process whereby islet-associated anti-inflammatory M2-like macrophages and a prominent interstitial M2-like cellular infiltrate of CD68+HO-1+ M2 macrophages, mesenchymal cells, and fibrocytes was observed in association with signs of islet re-
generative processes. Remarkably, this short-term induction of HO-1 recruited anti-inflammatory innate immune cells specifically to the islets and the perilobular interstitium.

A striking infiltrate was observed in the interstitium of CoPP-treated rats consisting of cells expressing one or more of the following: HO-1, CD34, vimentin, collagen V, CD68, and KLF4; conversely, CD14 and MHC class II expression was rare. These findings support identification of mesenchymal cells with an M2-like phenotype rather than M1 macrophages, which express MHC class II and CD14. A major fibroblast marker, α-SMA, was not expressed in the cells comprising the M2-like aggregates, suggesting they were not differentiated myofibroblasts, and the lack of FAP-2 labeling further suggested few of the cells were fibroblasts. Some desmin⁺ cells were present, but other stellate cell markers such as GFAP, nestin, collagen II/III, and α-SMA were infrequent or absent, suggesting that the aggregates contained few if any stellate cells. These findings indicate that the HO-1-induced cells migrated from the circulation and represent a transitional mixture of M2-like macrophages, mesenchymal cells, and fibrocytes.

Neutrophils and CD3⁺ lymphocytes were rarely observed in the peri-lobular aggregate indicating a subacute inflammation. CD11b, CD103, CD83, and forkhead box P3 (Foxp3) were absent or infrequent in the aggregates, indicating that dendritic cells and T regulatory cells were rare, consistent with a macrophage-driven process. Keratin⁺ cells were also rare and cytokeratin 19 and 20 staining was negative, suggesting that the expanded connective tissue was not due to a process of epithelial to mesenchymal transition. Compared with acinar cells, mitotic figures were frequent, staining for proliferating cell nuclear antigen (PCNA) was more intense, and staining for the cell cycle inhibitor, p16, was weak, consistent with active cell proliferation in the aggregates (see Table 1).

Vimentin⁺ HO-1⁺ cells were the most frequent subset in the interstitium and most vimentin⁺ cells coexpressed...
CD34, suggesting a bone marrow-derived population. Vimentin, an intermediate filament, is a major cytoskeletal component. In the adult, vimentin expression is enriched in mesenchymal cells and is induced after injury (29). Vimentin-deficient adult mice displayed impaired wound healing (30). Interestingly, the induction of pancreatic regeneration by periostin injection resulted in the proliferation of vimentin\(^+\) cells (31).

Figure 7. Anti-inflammatory M2 macrophage gene signature in pancreata of CoPP-treated BBdp rats. Gene expression analyses of Hmox1 (A), Bach1 (B), Mrc1 (CD206) (C), Msr1 (CD204) (D), Cx3cr1 (E), Il10 (F), Tgfb1 (G), and Il1b (H) were performed by quantitative RT-PCR. Results are shown as relative amounts 2\(^{-ΔΔCt}\); genes of interest were normalized to β-actin expression. Data represent mean ± SD (n = 7–8 per group. (An outlier was identified for graph [E] and omitted from the data set analysis.)

\[ p = 0.0002 \]

\[ p = 0.01 \]

\[ p = 0.03 \]

\[ p = 0.001 \]

\[ p = 0.04 \]

\[ p = 0.03 \]

\[ p = 0.01 \]
tubular complexes in the pancreas of BBdp rats contained many vimentin+ cells (18), suggesting a role for these cells in tissue repair/regenerative processes. CD34, a membrane-associated glycoprotein, is a well-characterized marker of hematopoietic stem cells, and its expression is recognized on other cell types, including interstitial cells, mesenchymal stromal cells, and fibrocytes (32).

Myeloid-derived suppressor cells have also been shown to differentiate into fibrocytes (33, 34). A deficiency of KLF4, an important regulator of M2 macrophage polarization (23), resulted in decreased accumulation of myeloid-derived suppressor cells and fibrocytes along with impaired wound healing (34, 35). Thus, KLF4 expression promotes fibrocyte differentiation and acquisition of an anti-inflammatory M2 macrophage identity in myeloid-derived cells that contribute to tissue repair. Taken together, we have identified abundant infiltrating CD34+vimentin+collagen V+ cells in the pancreatic interstitium of CoPP-treated rats, some of which displayed a spindle-like morphology characteristic of fibrocytes. Fibrocytes are commonly accepted to be derived from circulating bone marrow-derived monocytes (36). Based on the prominent CD34 expression, we speculate that the aggregate cells originated in part from circulating, bone marrow-derived cells. This interpretation is supported by a study of streptozotocin-induced T1D, in which bone marrow-derived macrophages and fibroblasts provided protection from T1D (37). Nonetheless, we cannot exclude the possibility that these aggregate cells originated from a pancreas-resident macrophage or mesenchymal precursor population (38–40).

We hypothesize that HO-1 induction promoted pancreatic infiltration of circulating precursors that subsequently adopted mesenchymal features and underwent a shift toward an M2-biased anti-inflammatory phenotype. A similar sequence of events has been described after sterile inflammation in muscle tissue (41). In support of this concept, it has been shown that adoptively transferred bone marrow-derived mesenchymal stem cells migrated to the pancreas of streptozotocin-diabetic mice and recruited M2 macrophages that in turn induced β-cell expansion and corrected hyperglycemia (42). Also, M2 macrophages stimulated β-cell expansion after partial duct ligation, which promotes islet neogenesis (43). Furthermore, Tessem et al (44) demonstrated that bone marrow-derived M2-like macrophages were required for diabetes prevention, maintenance of islet mass, and vasculature during pancreatitis. We conclude that these pancreatic interstitial aggregates of M2-like cells supported islet homeostasis.
In this study, the number of neogenic insulin + EICs was increased in CoPP-treated animals. The microarray analysis also revealed the up-regulation of the gene encoding REG3, a C-type lectin (45), in the pancreata of CoPP-treated rats. The increased number of acinar- and duct-associated REG3β+ clusters is consistent with the finding of increased neogenic insulin + EICs and has not been reported previously to be linked with increased HO-1 expression. Short-term daily injections of human Reg3α/β peptide into nondiabetic mice increased the number of small extra-islet insulin + clusters and induced the expression of the endocrine pancreas progenitor markers Ngn3, Sox9, and Ins (46). REG3β has been shown to exert anti-inflammatory effects and display anti-apoptotic activity (47, 48). REG3β can polarize macrophages toward an M2 phenotype in vitro, and peritoneal macrophages from Reg3b knockout mice display a pro-inflammatory M1 phenotype (49). Therefore, the protection afforded by HO-1 induction was likely attributable in part to the promotion of an anti-inflammatory, prorepair milieu in the interstitium and peri-islet areas.

Microarray analyses of islets from long-term protected NOD mice demonstrated preferential expression of M2-associated macrophage genes, notably those of the scavenger receptor family, including macrophage scavenger receptor 1 (Msr1) and class B scavenger receptor (Scarb1) (50); in contrast, islets from diabetic NOD mice displayed an increased expression of M1-associated genes, including Stat1 and chemokine (C-C motif) ligand 5 (Ccl5) (50). We observed an increased expression of Msr1 and Mrc1 in the pancreas after CoPP treatment along with several other M2-associated genes, including the chemokine receptor, Cx3cr1, which may promote islet function (51).

There is additional support for the protective capacity of M2 macrophages in the islet microenvironment. For example, isolated islet-resident macrophages from diabetes-resistant NOD mice (NOD.Ncf1m/y) gradually displayed increased anti-inflammatory M2-biased gene expression in parallel with decreased M1-associated transcription during the period of diabetes progression compared with normal NOD mice (52). In addition, the seminal study by Parsa et al (26) demonstrated the protective effect of transplanted M2 macrophages, which migrated to the inflamed pancreas to inhibit T1D. Thus, the enhancement of pancreatic M2 macrophages affords protection from spontaneous autoimmune diabetes in at least two rodent species.

In conclusion, we have shown that the upregulation of the enzyme HO-1 in vivo prevented T1D in BBdp rats and enhanced several immune dampening and regenerative processes. Importantly, protection was associated with formation of aggregates consisting of M2-like macrophages, mesenchymal cells, and fibrocytes, which induced a prosurvival M2 environment during early stage insulitis.

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### Table 1. Characteristics of Peri-lobular Infiltration in the Pancreas of CoPP-Treated Rats

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<th>Marker</th>
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<td>Collagen I/II/III</td>
<td>–</td>
</tr>
<tr>
<td>Collagen V</td>
<td>+ +</td>
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<tr>
<td>Cell proliferation markers</td>
<td></td>
</tr>
<tr>
<td>P16</td>
<td>–</td>
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<tr>
<td>PCNA</td>
<td>++</td>
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<tr>
<td>Epithelial markers</td>
<td></td>
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<tr>
<td>Keratin</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin 19</td>
<td>–</td>
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<tr>
<td>Cytokeratin 20</td>
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</table>

In this study, the number of neogenic insulin + EICs was increased in CoPP-treated animals. The microarray analysis also revealed the up-regulation of the gene encoding REG3, a C-type lectin (45), in the pancreata of CoPP-treated rats. The increased number of acinar- and duct-associated REG3β+ clusters is consistent with the finding of increased neogenic insulin + EICs and has not been reported previously to be linked with increased HO-1 expression. Short-term daily injections of human Reg3α/β peptide into nondiabetic mice increased the number of small extra-islet insulin + clusters and induced the expression of the endocrine pancreas progenitor markers Ngn3, Sox9, and Ins (46). REG3β has been shown to exert anti-inflammatory effects and display anti-apoptotic activity (47, 48). REG3β can polarize macrophages toward an M2 phenotype in vitro, and peritoneal macrophages from Reg3b knockout mice display a pro-inflammatory M1 phenotype (49). Therefore, the protection afforded by HO-1 induction was likely attributable in part to the promotion of an anti-inflammatory, prorepair milieu in the interstitium and peri-islet areas.

Microarray analyses of islets from long-term protected NOD mice demonstrated preferential expression of M2-associated macrophage genes, notably those of the scavenger receptor family, including macrophage scavenger receptor 1 (Msr1) and class B scavenger receptor (Scarb1) (50); in contrast, islets from diabetic NOD mice displayed an increased expression of M1-associated genes, including Stat1 and chemokine (C-C motif) ligand 5 (Ccl5) (50). We observed an increased expression of Msr1 and Mrc1 in the pancreas after CoPP treatment along with several other M2-associated genes, including the chemokine receptor, Cx3cr1, which may promote islet function (51).

There is additional support for the protective capacity of M2 macrophages in the islet microenvironment. For example, isolated islet-resident macrophages from diabetes-resistant NOD mice (NOD.Ncf1m/y) gradually displayed increased anti-inflammatory M2-biased gene expression in parallel with decreased M1-associated transcription during the period of diabetes progression compared with normal NOD mice (52). In addition, the seminal study by Parsa et al (26) demonstrated the protective effect of transplanted M2 macrophages, which migrated to the inflamed pancreas to inhibit T1D. Thus, the enhancement of pancreatic M2 macrophages affords protection from spontaneous autoimmune diabetes in at least two rodent species.

In conclusion, we have shown that the upregulation of the enzyme HO-1 in vivo prevented T1D in BBdp rats and enhanced several immune dampening and regenerative processes. Importantly, protection was associated with formation of aggregates consisting of M2-like macrophages, mesenchymal cells, and fibrocytes, which induced a prosurvival M2 environment during early stage insulitis.

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M.H., G.-S.W., and F.W.S. designed the study. M.H., G.-S.W., J.A.C., A.N., A.S., and C.P. performed the experiments and analyzed the data. M.H., G.-S.W., C.P., A.J.M., and F.W.S. wrote the manuscript. The authors declare there are no competing interests. These studies were supported by the Canadian Institutes of Health Research (CIHR), Canadian Diabetes Association and Cure Diabetes (Ottawa). F.W.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Disclosure Summary: The authors have nothing to disclose.

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