Macrophages are early islet-infiltrating cells seen in type 1 diabetes (T1D). While proinflammatory M1 macrophages induce T1D, M2 macrophages have been shown to delay this autoimmune disease in nonobese diabetic (NOD) mice, but the environmental cues that govern macrophage polarization and differentiation remain unresolved. We previously demonstrated the importance of reactive oxygen species (ROS) in T1D, as NOD mice deficient in NADPH oxidase (NOX)-derived superoxide (Ncf1m1J) were protected against T1D partly because of blunted Toll-like receptor-dependent macrophage responses. We provide evidence that NOX-derived ROS contribute to macrophage differentiation in T1D. During spontaneous diabetes progression, T1D-resistant NOD.Ncf1m1J islet-resident macrophages displayed a dampened M1 and increased M2 phenotype. The transfer of diabeticogenic T cells into NOX-deficient NOD.Rag.Ncf1m1J recipients resulted in decreased TNF-α* and IL-1β* islet-infiltrating M1 macrophages and a concomitant enhancement in arginase-1* M2 macrophages. Mechanistic analysis of superoxide-deficient bone marrow-derived macrophages revealed a marked diminution in a proinflammatory M1 phenotype due to decreased P-STAT1 (Y701) and interferon regulatory factor 5 compared with NOD mice. We have therefore defined a novel mechanistic link between NOX-derived ROS and macrophage phenotypes, and implicated superoxide as an important factor in macrophage differentiation. Thus, targeting macrophage redox status may represent a promising therapy in halting human T1D.
Antibody depletion of macrophages or inhibition of macrophage trafficking into the islets prevents the onset of T1D (11,12).

While M1 macrophages promote T1D, murine studies have demonstrated that M2 macrophages protect against T1D. A recent report demonstrated that a single transfer of alternatively activated macrophages delayed T1D in NOD mice just prior to clinical onset with protection lasting up to 3 months (13). Neutralization of IFN-γ in NOD.scid mice receiving diabetogenic BDC-2.5 CD4 T cells, which induce pancreatic β-cell death by recruiting M1 macrophages (6,8), were protected against T1D because of an influx of islet-infiltrating M2 macrophages (14). Previous studies have demonstrated that redox status modulates macrophage phenotypes, as the antioxidant protein thioredoxin skewed murine peritoneal macrophages from a proinflammatory M1 to an immunosuppressive M2 phenotype, ameliorating atherosclerotic lesions (15). Macrophages deficient in p47phox, a scaffolding protein of the NADPH oxidase (NOX) complex, displayed an elevated M2 macrophage signature (16). We recently demonstrated that NOX-deficient NOD (NOD.Ncf1m1J) mice were protected against autoimmune T1D (11,17) due, in part, to decreased activation of Toll-like receptor 3 and Toll-like receptor 4 signaling pathways in macrophages, and, subsequently, a robust diminution in IL-12p70, TNF-α, IL-1β, and type I IFN synthesis (11,17,18). Because oxidative stress is tightly linked with inflammation (19,20), and ROS synthesis and inducible nitric oxide synthase (iNOS) are characteristic M1 markers (1), we investigated the role of NOX-derived superoxide as a contributing proinflammatory molecule involved in M1 macrophage differentiation in T1D.

**RESEARCH DESIGN AND METHODS**

**Mice**

NOD/ShiLtJ, NOD.Ncf1m1J, NOD.BDC-2.5, NOD.Rag, and NOD.Rag.Ncf1m1J mice were bred and housed under pathogen-free conditions at the Research Support Building animal facility at the University of Alabama at Birmingham. Female mice between 8 and 16 weeks of age were used. NOD.BDC-2.5 mice were obtained from Dr. Kathryn Haskins (National Jewish Hospital, Denver, CO). NOD.Rag.Ncf1m1J mice were generated by backcrossing NOD.Rag with NOD.Ncf1m1J (17) mice for at least eight generations and were genotyped for the Ncf1m1J mutation (21). Mice received standard chow and acidified water weekly except for NOD.Rag.Ncf1m1J mice, which were supplemented with 80 mg trimethoprim/sulfamethoxazole (Hi-Tech Pharmacal) in drinking water. All animal studies were performed in accordance with the University of Alabama at Birmingham Institutional Animal Use and Care Committee, in compliance with the laws of the U.S.

**NOD and NOD.Ncf1m1J Islet Isolation, Quantitative RT-PCR, ELISA, and Western Blotting**

Islets were isolated from age- and sex-matched mice via a collagenase inflation method and were handpicked in sterile Hanks’ balanced salt solution (HBSS) (22). Islets were stored in RNalater (Ambion) or were lysed for Western blotting (17). The following mRNAs were isolated via the RNeasy Kit (Qiagen), reverse transcribed using SuperScript III (Invitrogen), and amplified with TaqMan Gene Expression Assays (Applied Biosystems): Arg1 (Mm00475988_m1), Retnla (Mm00335109_m1), Ccl17 (Mm00516136_m1), Mrc1 (Mm00485248_m1), Stat6 (Mm01160477_m1), Cxcl10 (Mm00445235_m1), Ccl5 (Mm01024282_m1), Nos2 (Mm00440502_m1), Ifng (Mm01168134_m1), Tnfα (Mm00443258_m1) Stat1 (Mm00439531_m1), Emr1 (Mm00802529_m1), and Gapdh (Mm99999915_g1). Relative gene expression levels were calculated using the 2ΔΔCT method; Gapdh or Emr1 was set to 1. Serum levels of chemokine (C-C motif) ligand (CCL) 5 were quantified using a mouse Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore), while CCL17 production was measured using a DuoSet ELISA kit (R&D Systems) (18). For Western blotting, whole-cell lysates were probed with antibodies against iNOS (Novus Biologicals), Arg-1, STAT6 (Cell Signaling), P-STAT6 (Y641) (BD Bioscience), or β-actin (Sigma-Aldrich) as described (18).

**Bone Marrow–Derived Macrophage Polarization to M1 and M2 Phenotypes**

NOD and NOD.Ncf1m1J bone marrow–derived macrophages (BM-Mφs) were generated (18) and polarized to an M1 phenotype, by treating them with 20 ng/mL recombinant human (r-h) IFN-γ (PeproTech) and 100 ng/mL LPS (Sigma), or to an M2 phenotype by treating them with 20 ng/mL r-mIL-4 (PeproTech) (16). Nitrite production was measured by Griess assay (23), while IL-1β, TNF-α, IL-12p70, CCL5, and CCL17 were measured using a DuoSet ELISA kit (R&D Systems) (18). For Western blotting, whole-cell lysates were probed with antibodies against P-STAT1 (Y701), STAT1, and IFN regulatory factor 5 (IRF5) (Cell Signaling Technology), and β-actin (Sigma-Aldrich).

**CD4 T-Cell Isolation, Expansion, and Adoptive Transfer of T1D**

CD4 T cells were purified by negative selection according to the manufacturer’s instructions via the RoboSep CD4 T-cell isolation kit (STEMCELL Technologies) with >90% purity (17). T cells were stimulated with plate-bound anti-CD3ε and anti-CD28 (1 μg/mL) (BD Biosciences) with 100 units/mL r-mIL-2 for 3 days at 37°C with 5% CO2 and expanded with 100 units/mL IL-2 for 3 days. T cells (5 × 10^6) were transferred in a 0.1-mL volume in HBSS via intraperitoneal injection. Mice were treated intraperitoneally with MnTE-2-PyP (MnP), a superoxide dismutase mimetic (24), or HBSS prior to T-cell injection and daily thereafter.

**Ex Vivo Analysis of Macrophages Within the Pancreata and Intracellular Staining**

Macrophages were isolated from pancreata of NOD.Rag and NOD.Rag.Ncf1m1J recipient mice at 7 days after diabetogenic CD4 T-cell adoptive transfer and plated onto
a 24-well plate for a 5-h incubation at 37°C with 5% CO₂. Pancreata from three to four recipient mice were pooled together and surface stained with anti-F4/80, anti-CD11b, or anti-I-A<sup>e</sup> antibodies as described previously (8,25). Intracellular staining was performed with anti-TNF-α, anti-IL-1β, and anti-Arg-1 antibodies, and isotype controls (R&D Biosciences) (8). Cells were read on a FACSCalibur Flow Cytometer (BD Biosciences) with at least 300,000 events collected for each sample, according to the gating scheme shown in Supplementary Figure 1, and analyzed with FlowJo version 9.6.1 software (Tree Star Inc.).

Statistical Analysis
Data were analyzed using GraphPad Prism version 5.0 statistical software. The difference between mean values and the SEM was assessed using the two-tailed Student t test, with P < 0.05 considered to be significant. Kaplan-Meier survival analysis was used to evaluate diabetes onset.

RESULTS

NOD.Ncf1<sup>m1J</sup> Mice Display an Increase in M2 Chemokines and a Concomitant Decrease in M1 Chemokines During Spontaneous T1D Resistance

Superoxide-deficient NOD.Ncf1<sup>m1J</sup> mice are significantly protected against spontaneous and adoptive transfer of T1D (11,17). Our incidence study (Fig. 1A) indicated that 70% of NOD mice were hyperglycemic by 30 weeks of age (n = 21) in comparison with 15% of NOD.Ncf1<sup>m1J</sup> mice (n = 9) with the onset of diabetes occurring as early as 14 weeks of age. To assess whether this protection was partly due to a diminished M1 macrophage phenotype, systemic analysis of the chemokine ligand CCL5, a marker of proinflammatory M1 macrophages (1), was examined within the sera of NOD and NOD.Ncf1<sup>m1J</sup> mice during progression to T1D. No difference in CCL5 serum levels was observed at 8 weeks of age; however, at 12 weeks and immediately after the onset of diabetes at 16 weeks, serum CCL5 levels in NOD mice were elevated in comparison with NOD.Ncf1<sup>m1J</sup>, which remained below the limit of detection (Fig. 1B). To determine whether this protection was associated with an enhanced M2 macrophage phenotype, the chemokine ligand CCL17, a marker of alternatively activated macrophages (1), was examined within the sera of NOD and NOD.Ncf1<sup>m1J</sup> mice. At 8 weeks of age, CCL17 levels were similar (Fig. 1C). However, at 12 weeks of age, CCL17 levels in NOD.Ncf1<sup>m1J</sup> mice were enhanced approximately twofold compared with NOD mice, and at 16 weeks serum-specific production of this M2 chemokine ligand was elevated fivefold (Fig. 1C), indicating that T1D protection elicited by superoxide deficiency may involve altered macrophage phenotypes during autoimmune T1D.

NOD.Ncf1<sup>m1J</sup> Islets Display Diminished M1 and Enhanced M2 Macrophage Markers

To determine whether the delay in T1D resistance within NOD.Ncf1<sup>m1J</sup> mice was due to a skewed proinflammatory microenvironment within the islet, the islet transcriptional profiles of female NOD and NOD.Ncf1<sup>m1J</sup> mice were compared at 8 (Fig. 2A and B), 12 (Fig. 2C and D), and 16 (Fig. 2E and F) weeks of age during spontaneous autoimmune diabetes. Quantitative RT-PCR revealed a pronounced diminution in mRNA accumulation of M1-associated proinflammatory effector molecules from 8-week-old NOD.Ncf1<sup>m1J</sup> islets in contrast to NOD islets, including decreases in Cxcl10 (5-fold), Ccl5 (18-fold), Nos2 (1.6-fold), Tnfa (1.1-fold), and Ifng (11-fold) (Fig. 2A). The transcription factor responsible for M1 macrophage differentiation, Stat1 (26), was reduced threefold in NOD.Ncf1<sup>m1J</sup> mice compared with NOD mice (Fig. 2A). Analysis of M2 macrophage transcripts revealed an almost fourfold upregulation in Ccl17; however, additional M2 markers, including Arg1, Retnla, and Cd206, were reduced by superoxide-deficient islets compared with those in NOD islets (Fig. 2B). mRNA accumulation of Stat6, a marker of M2 lineage commitment (26) was similarly expressed within NOD and NOD.Ncf1<sup>m1J</sup> islets (Fig. 2B). A decreased M1 macrophage profile was observed at 12 weeks of age by NOD.Ncf1<sup>m1J</sup> islets with reductions in Cxcl10 (2-fold), Ccl5 (2-fold), Nos2 (3-fold), Tnfa (1.3-fold), Ifng (2.6-fold), and Stat1 (1.6-fold) (Fig. 2C). An M2 macrophage signature was observed at 12 weeks of age, as NOD.Ncf1<sup>m1J</sup> islets displayed 2- and 11-fold increases in Ccl17 and Retnla mRNA, respectively, in addition to...
a 1.5-fold elevation in the M2 transcription factor Stat6. However, NOD.Ncf1m1J islet–resident macrophages were not fully differentiated into an M2 phenotype, as a 1.6-fold decrease in Arg1 and a 7-fold reduction in Cd206 were observed (Fig. 2D).

Transcriptional analysis of islets at 16 weeks of age when 20% of NOD mice have progressed to diabetes (Fig. 1A) revealed a blunted M1 macrophage profile with decreased accumulation of Cxcl10 (3.8-fold), Ccl5 (5-fold), Nos2 (eightfold), Tnfa (6.5-fold) Ifng (6.5-fold) and Stat1 (1.7-fold) by NOD.Ncf1m1J islets compared with NOD islets (Fig. 2E). In contrast to 8- and 12-week-old islet macrophages, 16-week-old NOD.Ncf1m1J islet macrophages were more M2 biased, with elevated Ccl17 (1.2-fold), Arg1 (1.5-fold), Retnla (17-fold), and Cd206 (2.3-fold) (Fig. 2F). This skewed islet-specific macrophage phenotype at 16 weeks was also observed with Emr1 (F4/80) normalization, in which transcript accumulation of M1 genes, including Cxcl10 (2.4-fold), Ccl5 (1.3-fold), Nos2 (2.5-fold), Tnfa (1.2-fold), and Stat1 (1.1-fold), was blunted by NOD.Ncf1m1J islets (Fig. 3A) with elevations in the M2 markers Ccl17, Retnla, and Stat6 compared with NOD islets (Fig. 3B).

To confirm the elevated M2 transcriptional profile of NOD.Ncf1m1J islet–resident macrophages, P-STAT6 (Y641), iNOS, and Arg-1 were analyzed in NOD and NOD.Ncf1m1J islet whole-cell lysates via Western blot. Analysis of P-STAT6 (Y641) at 16 weeks revealed a 1.5-fold enhancement by NOD.Ncf1m1J (Fig. 4A), reafﬁrming the M2 macrophage bias with spontaneous T1D onset. Total STAT6 protein level was also elevated in superoxide-deﬁcient NOD islets (Fig. 4A). At 16 weeks, iNOS, a proinflammatory M1 macrophage marker, was decreased 1.3-fold in NOD.Ncf1m1J islets compared with NOD islets (Fig. 4B), while Arg-1 was enhanced 2.5-fold (Fig. 4C).
gradual enhancement in M2 macrophage mRNA within skewed toward an M2-like phenotype with an accompanied mice progressing to T1D display a macrophage pro

data indicate that, in the absence of superoxide, NOD was not detected.

...that the expression of the M2 marker Arg-1 by islet-infiltrating CD11b+, MHC II* (I-A^d^) cells within NOD.Rag.Ncf1^m1J^ recipients, recapitulating the M2-bias observed in NOD.Ncf1^m1J^ islet-resident macrophages with progression to autoimmune disease (Fig. 2B, D, and F). The specificity of TNF-α, IL-1β, and Arg-1 synthesis by NOD.Rag and NOD.Rag.Ncf1^m1J^ pancreas-infiltrating macrophages was verified by isotype control staining (Supplementary Figs. 2A–C).

To mirror the skewed macrophage phenotypes within NOD.Rag.Ncf1^m1J^ transferred recipient mice, NOD.Rag mice were treated with the ROS scavenger MnP (24) after adoptive transfer with diabetogenic BDC-2.5 CD4 T cells. MnP induced a 1.2-fold reduction in TNF-α levels by islet-infiltrating macrophages of NOD.Rag mice compared with HBSS (Fig. 6A). In contrast to treatment with TNF-α, MnP treatment did not lessen IL-1β expression; therefore, this proinflammatory cytokine may not be modulated by this superoxide dismutase mimetic (Fig. 6B).

Interestingly, this ROS scavenger induced a 1.7-fold enhancement in Arg-1 compared with HBSS (Fig. 6C), recapitulating the elevation in this M2 marker in superoxide-deficient NOD.Rag recipients (Fig. 5C). Specific expression of TNF-α, IL-1β, and Arg-1 by pancreas-infiltrating macrophages from NOD.Rag recipients treated with HBSS or MnP was verified by isotype control staining (Supplementary Figs. 3A–C).

Superoxide-Deficient NOD.Rag Mice Display a Reduced M1 and Elevated M2 Signature Within the Pancreas After Diabetogenic CD4 T-Cell Transfer

As β-cells express STAT6 in response to cytokines to enhance viability (27), we used the BDC-2.5 adoptive transfer model of diabetogenic CD4 T cells to confirm that recruited NOX-deficient macrophages possessed an M2 bias. We previously demonstrated that adoptive transfer of BDC-2.5 CD4 T cells, which recruit proinflammatory M1 macrophages to mediate β-cell destruction (6,8), was protective in NOD.Ncf1^m1J^ mice, but induced T1D in 100% of NOD recipients (11). In addition, treatment of NOD.scid mice with a superoxide dismutase dismutase mimetic (MnP) to scavenge ROS upon adoptive transfer with BDC-2.5 was efficacious in delaying T1D (28). To dissect the role of redox status on macrophage phenotypes in T1D, we generated the lymphocyte-deficient NOD.Rag.Ncf1^m1J^ strain, containing antigen-presenting cells unable to generate NOX-derived superoxide. Ex vivo analysis of macrophage infiltration within the pancreata at 7 days post-transfer with preactivated BDC-2.5 CD4 T cells revealed a 1.7-fold decrease in TNF-α synthesis by F4/80^+^, MHC II^+^ (I-A^d^) macrophages within NOD.Rag.Ncf1^m1J^ recipients compared with NOD.Rag recipients (Fig. 5A). Synthesis of IL-1β–producing macrophages was decreased twofold by NOD.Rag.Ncf1^m1J^ recipients in contrast to NOD.Rag recipients (Fig. 5B). The expression of the M2 marker Arg-1 at 7 days post-transfer revealed a 1.6-fold increase by islet-infiltrating CD11b^+, MHC II^+^ (I-A^d^) cells within NOD.Rag.Ncf1^m1J^ recipients, recapitulating the M2-bias observed in NOD.Ncf1^m1J^ islet-resident macrophages with progression to autoimmune disease (Fig. 2B, D, and F). The specificity of TNF-α, IL-1β, and Arg-1 synthesis by NOD.Rag and NOD.Rag.Ncf1^m1J^ pancreas-infiltrating macrophages was verified by isotype control staining (Supplementary Figs. 2A–C).

These results reaffirm the decrease in M1-associated proinflammatory genes (Figs. 2E and 3A) and a concomitant gradual enhancement in M2 macrophage mRNA within NOD.Ncf1^m1J^ islets (Figs. 2F and 3B). Collectively, our data indicate that, in the absence of superoxide, NOD mice progressing to T1D display a macrophage profile skewed toward an M2-like phenotype with an accompanied decrease in a proinflammatory M1 phenotype. This elevation in M2 macrophages within the islet may explain, in part, the unique resistance of NOD.Ncf1^m1J^ mice to spontaneous disease onset and further demonstrates that alterations in redox status can influence macrophage differentiation (15,16). These studies also demonstrate that NOX-derived superoxide is a key signal to promote proinflammatory M1 macrophage differentiation.

**Superoxide-Deficient NOD.Rag Mice Display a Reduced M1 and Elevated M2 Signature**

**Within the Pancreas After Diabetogenic CD4 T-Cell Transfer**

To determine the mechanistic effects of superoxide deficiency in the absence of NOX-derived superoxide, NOD and NOD.Ncf1^m1J^ BM-Mφs were generated and polarized to either an M1 or M2 phenotype. Recapitulating the reductions in Nos2 transcripts within the islet (Figs. 2A, C, and E, and 3A) and decreased iNOS protein (Fig.
(4B), NOD.Ncf1<sup>m1J</sup> BM-Mφs exhibited diminished nitric oxide synthase activity as nitrite levels were reduced almost twofold, a hallmark of M1 macrophages (1), under M1 polarizing conditions (Fig. 7A). Expression of the proinflammatory cytokine and M1 marker IL-1β was significantly reduced by NOD.Ncf1<sup>m1J</sup> mice and was below the limit of detection, in contrast to NOD mice (Fig. 7B). Synthesis of TNF-α was dampened twofold under M1 polarizing conditions, and, interestingly, this proinflammatory cytokine was markedly reduced by almost 13-fold by M2-polarized NOD.Ncf1<sup>m1J</sup> BM-Mφs compared with NOD mice (Fig. 7C). In addition to a diminution in the proinflammatory cytokines TNF-α and IL-1β, IL-12p70, produced in high abundance by M1-polarized macrophages (1), was reduced 1.3-fold by M1-polarized superoxide-deficient BM-Mφs compared with NOD (Fig. 7D). Mirroring the reduced CCL5 synthesis within the serum (Fig. 1B) and mRNA accumulation in islets (Figs. 2A, C, and E, and 3A) of NOD.Ncf1<sup>m1J</sup> mice, an almost threefold reduction in this M1 chemokines was observed in the absence of NOX-derived superoxide under M1 polarizing conditions (Fig. 7E). CCL5 also remained below the limit of detection by M2-polarized NOD.Ncf1<sup>m1J</sup> BM-Mφs, but was readily detected by NOD islets (Fig. 7E). Besides a reduction in M1 markers, superoxide-deficient NOD.Ncf1<sup>m1J</sup> BM-Mφs were more M2 like, as M2-polarized NOD.Ncf1<sup>m1J</sup> BM-Mφs exhibited a ninefold elevation in the M2 chemokine ligand CCL17 (Fig. 7F), mirroring the elevated serum-specific CCL17 (Fig. 1C) and enhanced Ccl17 transcript accumulation of NOD.Ncf1<sup>m1J</sup> islets compared with NOD islets (Figs. 2B, D, and F, and 3B).

To mechanistically explain the reduced M1 phenotype by superoxide-deficient BM-Mφs, P-STAT1 (Y701), a transcription factor for M1 lineage commitment, was analyzed by Western blot. Under M1 polarizing conditions, NOD.Ncf1<sup>m1J</sup> BM-Mφs exhibited a dramatic blunting in P-STAT1 (Y701) (Fig. 8A). As further demonstration of this reduced M1 phenotype, expression of IRF5, a key M1 macrophage marker (29), was severely reduced in the absence of NOX-derived superoxide (Fig. 8B). These
results indicate that ROS depletion modulates macrophage phenotypes by affecting STAT1 phosphorylation and downstream signaling molecules essential for M1 macrophage differentiation, such as IRF5.

**DISCUSSION**

We report that in the absence of NOX-derived superoxide, macrophages exhibit an alternatively activated M2 phenotype during T1D. This M2 bias in T1D-resistant NOD.Ncf1<sup>m1J</sup> mice may render islet-resident and islet-infiltrating macrophages defective in mediating β-cell destruction via diminished proinflammatory cytokine production and/or may impair antigen presentation. These data further demonstrate the significance of ROS as a proinflammatory third signal in synergizing innate with adaptive immune responses (24,30), an essential process in educating autoreactive lymphocytes to destroy β-cells in T1D (17).

The importance of M2 macrophages in halting T1D pathogenesis has been demonstrated, as a single administration of M2 macrophages to 16-week-old prediabetic female NOD mice provided long-term T1D protection (13). While M2 macrophages represent a promising cellular therapy for T1D, the developmental cues for macrophage polarization and differentiation require further investigation before their use as translational therapeutics. Our results demonstrate that redox status influences macrophage phenotypes. Islet-resident macrophages from T1D-resistant NOD.Ncf1<sup>m1J</sup> mice displayed an M2 bias by 16 weeks of age (Figs. 2F and 3B), when NOD mice within our colony have already progressed to T1D (Fig. 1A). As macrophages are sensitive to the microenvironment and NOX-derived...

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**Figure 5**—Pancreas-infiltrating macrophages from superoxide-deficient NOD.Rag mice display a decrease in TNF-α and IL-1β and a concomitant increase in Arg-1 synthesis after diabetogenic CD4 T-cell transfer. Dot plots of TNF-α<sup>+</sup> (A), IL-1β<sup>+</sup>, and Arg-1–expressing (B) macrophages in the pancreata of NOD.Rag and NOD.Rag.Ncf1<sup>m1J</sup> recipient mice at 7 days post-transfer with NOD.BDC-2.5 CD4 T cells treated with HBSS or MnP. Dot plots were generated by first gating on granulocytes and then gating on F4/80<sup>+</sup> or CD11b<sup>+</sup> cells via side scatter. Mean fluorescence intensity values of TNF-α and IL-1β were quantified by F4/80<sup>+</sup>, I-A<sup>q7</sup> cells, while those of Arg-1 were calculated from CD11b<sup>+</sup>, I-A<sup>q7</sup> cells. Data are representative of four independent experiments. (A high-quality color representation of this figure is available in the online issue.)

**Figure 6**—Pancreas-infiltrating macrophages from NOD.Rag mice treated with MnP display a decrease in TNF-α and a concomitant increase in Arg-1 synthesis after diabetogenic CD4 T-cell transfer. Dot plots of TNF-α<sup>+</sup> (A), IL-1β<sup>+</sup>, and Arg-1–expressing (B) macrophages in the pancreata of NOD.Rag recipient mice at 7 days post-transfer with NOD.BDC-2.5 CD4 T cells treated with HBSS or MnP. Dot plots were generated by first gating on granulocytes and then gating on F4/80<sup>+</sup> or CD11b<sup>+</sup> cells via side scatter. Mean fluorescence intensity values of TNF-α and IL-1β were quantified by F4/80<sup>+</sup>, I-A<sup>q7</sup> cells, while those of Arg-1 mean fluorescence intensity were calculated from CD11b<sup>+</sup>, I-A<sup>q7</sup> cells. For TNF-α and IL-1β, expression is shown by F4/80<sup>+</sup>, I-A<sup>q7</sup> cells, while Arg-1 expression by CD11b<sup>+</sup>, I-A<sup>q7</sup> cells is displayed. Data are representative of four independent experiments. (A high-quality color representation of this figure is available in the online issue.)
superoxide promotes inflammation (31,32), NOD.Ncf1m1J islet–resident macrophages may be unable to fully differentiate into an M1 phenotype and, as a consequence, may adopt an alternatively activated M2 signature. Besides a gradual increase in M2 markers by islet-resident macrophages of diabetes-resistant NOD.Ncf1m1J mice during T1D progression (Fig. 2B, D, and F), and enhanced CCL17 production by NOD.Ncf1m1J BM-Mφs (Fig. 7F), we observed elevations in P-STAT6 (Y641) (Fig. 4A), a key transcription factor involved in M2 differentiation within the islets of superoxide-deficient NOD mice. Concomitant to an observed increase in P-STAT6 (Y641) within the islets of NOD.Ncf1m1J mice, BM-Mφs displayed a severe diminution in P-STAT1 (Y701) activation (Fig. 8A), in addition to blunted IRF5 (Fig. 8B), indicating that superoxide synthesis is an important inducer of M1 macrophage differentiation. Forthcoming studies will determine how redox status influences the STAT1 and STAT6 signaling axes by determining the expression and activity of the cognate receptors Janus kinase 1 and Janus kinase 2 in the absence of NOX-derived superoxide. As superoxide deficiency promoted an M2 signature within the islet during spontaneous T1D progression (Figs. 2B, D, and F, and 3B) within the pancreas upon diabetogenic T-cell transfer (Figs. 5C and 6C), and with polarized BM-Mφs (Fig. 7F), future studies will determine whether NOX-deficient macrophages dampen T-helper cell type 1 T-cell responses involved in β-cell destruction and/or heal damaged β-cells. Our study provides evidence

Figure 7—Polarized NOD.Ncf1m1J BM-Mφs display reduced M1 and enhanced M2 macrophage markers. The synthesis of nitrite (A), IL-1β (B), TNF-α (C), IL-12p70 (D), and CCL5 (E) was analyzed using NOD and NOD.Ncf1m1J BM-Mφs incubated for 12 h under M1 or M2 polarization conditions, respectively. F: The production of CCL17 was analyzed using NOD and NOD.Ncf1m1J BM-Mφs incubated for 3 h with M1 and M2 polarization cocktails. Statistical analysis represents the average of three independent experiments. ***P < 0.001; *P < 0.05. ND, not detected; ns, not significant.
that therapies capable of modulating redox status, including use of a superoxide dismutase mimetic (Fig. 6) or thioredoxin (15), may be beneficial in the treatment of immune-mediated diseases, such as T1D, displaying a proinflammatory M1 macrophage response. Unfortunately, significant challenges still exist that need to be overcome before these immunotherapies can be used as viable cures, including the development of novel strategies and techniques to preferentially target islet-resident macrophages in T1D patients to promote an alternatively activated M2 macrophage phenotype.

Our findings of decreased Cxcl10 and Ccl5 mRNA accumulation by NOD.Ncf1m1J islets at 8 (Fig. 2A), 12 (Fig. 2C), and 16 weeks (Fig. 2E and G) of age, diminished CCL5-specific sera in NOD.Ncf1m1J islets at 12 and 16 weeks of age (Fig. 1B), and reduced CCL5 synthesis by NOD.Ncf1m1J BM-Mdfs (Fig. 7E) demonstrate that optimal synthesis of these proinflammatory chemokines is redox dependent. CXCL10 has been linked to human T1D (33,34), with one study reporting a significant elevation in the serum of subjects with recent-onset T1D (35), and polymorphisms in CCL5 mediate T1D protection (36). Our studies demonstrate that NOX-derived ROS promote T1D in NOD mice by inducing M1 macrophage differentiation and enhancing the synthesis of proinflammatory cytokines (TNF-α and IL-1β) (Fig. 7B and C) and chemokines (CXCL10 and CCL5) involved in β-cell destruction. Since islet-resident NOD.Ncf1m1J macrophages displayed blunted M1 chemokine synthesis and decreased insulin (17), fine-tuning the redox balance within the islet microenvironment may serve as a means to counter T1D pathogenesis. Future studies will dissect the role of NOX-derived ROS in modulating chemokine expression in T1D pathogenesis, and further define the synergism of oxidative stress and macrophage differentiation in T1D.

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