Immune Cell–Derived C3 Is Required for Autoimmune Diabetes Induced by Multiple Low Doses of Streptozotocin

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OBJECTIVE—The complement system contributes to autoimmune injury, but its involvement in promoting the development of autoimmune diabetes is unknown. In this study, our goal was to ascertain the role of complement C3 in autoimmune diabetes.

RESEARCH DESIGN AND METHODS—Susceptibility to diabetes development after multiple low-dose streptozotocin treatment in wild-type (WT) and C3-deficient mice was analyzed. Bone marrow chimeras, luminex, and quantitative reverse transcription PCR assays were performed to evaluate the phenotypic and immunologic impact of C3 in the development of this diabetes model.

RESULTS—Coincident with the induced elevations in blood glucose levels, we documented alternative pathway complement component gene expression within the islets of the diabetic WT mice. When we repeated the experiments with C3-deficient mice, we observed complete resistance to disease, as assessed by the absence of histologic insulitis and the absence of T-cell reactivity to islet antigens. Studies of WT chimeras bearing C3-deficient bone marrow cells showed that bone marrow cell–derived C3, and not serum C3, is involved in the induction of diabetes in this model.

CONCLUSIONS—The data reveal a key role for immune cell–derived C3 in the pathogenesis of murine multiple low-dose streptozotocin-induced diabetes and support the concept that immune cell mediated diabetes is in part complement-dependent.
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Type 1 diabetes is a T-cell–dependent autoimmune disease in which islet antigens are presented by antigen-presenting cells (APCs) to autoreactive T cells, breaking self tolerance (1,2). After attraction to the pancreas, the autoreactive CD4 T cells cause β-cell injury in part through secreting proinflammatory cytokines that directly act on the islet cells (3), as well as by activating macrophages that amplify injury (4).

In previous work, we showed that during cognate T cell/APC interactions, immune cell–derived complement activates locally, yielding C3a and C5a that bind to C3a/C5a receptors (C3aR/C5aR) on both partners (5). The resultant G-protein–coupled receptor (GPCR) signaling further activates the APCs (upregulating costimulatory molecule expression and innate cytokine production) and directly induces survival and proliferation of the responding T cells. These concepts apply to in vivo immunity as T-cell responses to autoantigens (6–8), transplant antigens (9–12), and viruses (5,13) are diminished in mice in which immune cells are deficient in C3 or C3aR/C5aR, whereas T-cell immunity is enhanced in mice in which immune cells are deficient in the cell surface complement regulatory protein decay-accelerating factor (DAF, CD55) (8,10).

These results, along with a multitude of reports documenting that complement contributes to autoimmune injury (14–16), prompt the question of the possible involvement of the complement effectors in promoting the development of T-cell–mediated diabetes. This gap in the understanding of the function of complement in type 1 diabetes is unexpected, given that complement effectors, in particular C3a and C5a, are potent proinflammatory mediators and that inflammation has long been linked in the pathogenesis of type 1 diabetes.

To test the role of complement C3 on the development of T-cell–mediated diabetes, we employed an established model using multiple low-dose streptozotocin (MLDS) treatment. We chose the MLDS model over the NOD model because C3 and the diabetes susceptibility genes in the NOD strain are closely linked on chromosome 17 (17,18), thus impairing our ability to produce C3-deficient NOD animals. Streptozotocin (STZ), a toxin that binds to the GLUT2 receptor on pancreatic β-cells, has been used for decades to induce diabetes in rodent models (19). When administered at a single high dose (Hi-STZ, 180 mg/kg), it induces necrosis of the β-cells without leukocytic infiltrate. Collapsed islets and elevated serum glucose levels are detectable within 2–3 days (20). In contrast, when STZ is administered as multiple low doses (MLDS, 40 mg/kg daily for 5 days), it induces distortion of the islet architecture in conjunction with mononuclear cell infiltration. Although elevated serum glucose can be detected as early as day 7, typically 2 to 3 weeks are required for sustained diabetes (19). Rather than necrosis, apoptosis is the underlying mechanism of islet cell death, documented by findings that animals deficient in islet-associated caspase-3 are resistant to STZ effects (21). Current concepts are that apoptosis provides an environment in which islet autoantigens can be processed and presented by infiltrating APCs. Immune cell mediated injury by autoreactive T cells that have escaped thymic deletion is the dominant pathogenic mechanism (22). Consistent with this hypothesis,

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studies in the early 1980s demonstrated that T-cell–depleted or –deficient (nude) animals are resistant to MLDS-induced diabetes (23–25), and that T cells from animals with MLDS-induced disease can transfer diabetes to naïve mice (26,27).

Herein we report that immune cell C3 is required for MLDS-induced diabetes, and strikingly, that the C3 must derive from immune cells rather than from the serum. Our results suggest that further studies are warranted in autoimmune diabetes in humans.

RESEARCH DESIGN AND METHODS

Reagents and antibodies. Anti-mouse CD45.1-PE, CD45.2-PerCP-Cy5.5, anti-mouse IFN-γ and biotinylated anti-IFN-γ mAb, anti-Annexin *V-PE* (BD Biosciences; San Jose, CA); anti-mouse C3-FITC (MP Biomedicals; Solon, OH); alkaline phosphatase-conjugated antibiotin antibody (Vector Laboratories; Burlingame, CA); streptavidin-HRP conjugate (Dako; Carpinteria, CA); collagenase P (Roche; Mannheim, Germany); zymosan A (Sigma Aldrich; St. Louis, MO); streptozotocin (Alexis Biochemicals; Farmingdale, NY).

Mice. BALB/cJ (H-2^d), C57BL/6 (H-2^b), B6.SJL-Ptprc^a Pepc^b/BoyJ (CD45.1), B6.C3$^{-/-}$, and RAG-1$^{-/-}$ (B6.129S7-Rag1$^{tm1Mom/J}$), nude (B6.Cg-Foxn1^{nu}/J) male mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6.C3$^{-/-}$ mice were backcrossed (>10 generations) to BALB/c to obtain BALB/c.C3$^{-/-}$. C3 deficiency was confirmed via zymosan A C3-binding assay (11). Male mice were used at 6 to 10 wks of age, housed under specific-pathogen–free conditions, and treated in strict compliance with regulations established by the Institutional Animal Care and Use Committee.

Diabetic model, islet isolation, and islet transplantation. To induce diabetes, male mice (6–10 weeks of age) were injected intraperitoneally for 5 consecutive days with streptozotocin (40 mg/kg) dissolved in cold 0.1 mol/l citrate buffer pH 4.5 as previously described (26). Tail-vein glucose was measured between 10 A.M. and 12:00 P.M., and mice were considered diabetic when blood glucose levels were >200 mg/dl in two consecutive measurements on the OneTouch Ultra Blood Glucose Meter (LifeScan; Milpitas, CA). In some experiments, mice were treated with a single 180-mg/kg body weight intraperitoneal injection of STZ. Islet isolation and transplantation were previously described (28). Isolated islets from male B6 mice were cultured overnight and incubated with STZ (0.5 mg/ml) for 1 h, washed, and transplanted beneath the renal capsule of diabetic male B6 recipients (29). Islets were transplanted 10 days after initiating MLDS or 5 days after Hi-STZ treatment in the recipient mice. Intraperitoneal glucose tolerance testing was performed on day 7 after transplantation and the area under the curve (AUC) was calculated.

Generation of bone marrow chimeric mice. Bone marrow (BM) cells were collected from male WT or C3$^{-/-}$ mice of B6 background. Recipient male B6 mice had been lethally irradiated with 900 rads (2 doses of 450 rads with a 3-h resting period) from a cesium source using a Mark I Model 137Cs irradiator (JL Shepherd & Associates; San Fernando, CA). Six hours after irradiation, recipient irradiated mice received 8×10^6 BM cells via the tail vein. Chimerism of >90% donor origin was confirmed at week 8 by staining for CD45.1 versus CD45.2, and systemic C3 was assayed by flow cytometry via zymosan binding followed by staining (11).

Adoptive cell transfer. Splenocytes from male WT or C3$^{-/-}$ mice of B6 background were obtained by gently grinding moistened spleen through a 70-μm filter and washing the cells. Erythrocytes were lysed with ACK Lysis Buffer (Invitrogen; Carlsbad, CA). Splenocytes were resuspended in sterile PBS at a concentration of 3×10^6/200 μl for intraperitoneal transfer into male B6.C3$^{-/-}$ mice. Twenty-four hours after adoptive transfer, recipient B6.C3$^{-/-}$ mice were treated with MLDS.

Annexin V staining. Isolated islets were cultured overnight with STZ (0.5 mg/ml) at 37°C in humidified air and 5% CO_2. Islets were disrupted into a single-cell suspension and Annexin V staining and analysis was performed as manufacturer instructed.

Flow cytometry. All flow cytometry experiments were performed using a BD FACSCanto II (BD Biosciences; San Jose, CA) and data were analyzed on FlowJo software (TreeStar; Ashland, OR).

Quantitative real-time PCR. Total RNA was extracted from the distal portion of the pancreas devoid of lymphoid tissue using TriZol solution (Life Technologies; Carlsbad, CA) and cDNA was generated with oligo(dT) primers. PCR was performed on a CFX96 Real Time System (Bio-Rad; Hercules, CA) with the FastStart QuantiTect SYBR Green PCR kit (Qiagen; Valencia, CA) as described (28). Quantitative real-time PCR (qRT-PCR) data were normalized to cyclophylin. Primer sequences are available on request.

Histopathology and insulitis evaluation. Islet grafts were harvested and fixed in optimal cutting temperature compound. Frozen sections were cut into 5-μm–thick sections and islet morphology and leukocyte infiltration were assessed by H&E staining. Islets were graded by blinded investigators for severity of insulitis on a scale of 0–4: islets devoid of mononuclear cells = 0; minimum focal islet infiltrate = 1+; peri-islet infiltrate of <25% of islet circumference = 2+; peri-islet infiltration and <50% intra-islet infiltrate = 3+; intraislet infiltration >50% of islet area = 4+. The insulitis score (%) for each group was calculated as: sum of (1× number of islets with 1+; 2 × number of islets with 2+; 3 × number of islets with 3+; 4 × number of islets with 4+) divided by 4 × total number of islets scored (30). The calculated ratio represents the insulitis score percentage and was expressed as the mean ± SEM. Each study group included 3 mice with a minimum of 10 islets scored.

Luminex assay. Splenocytes were harvested on experimental day 0, 10, or 19 from MLDS-treated male B6 and B6.C3$^{-/-}$ mice. Dilutions of spleen cells were plated in HL-1 media stimulated in the presence or absence of purified islet cells (5×10^5/well) and cultured at 37°C in 5% CO_2 for 24 h. Supernatants from cultures were collected for quantification of cytokines by Bio-Plex Pro Mouse Cytokine TH1/TH2 assay. Assays were conducted according to the manufacturer's instructions and analyzed on a Bio-Plex 200 System (Bio-Rad; Hercules, CA).

Statistics. Results are expressed as mean ± SEM, unless stated otherwise. Differences in gene expression were calculated using the nonparametric Mann-Whitney *U* tests. $P < 0.05$ was considered statistically significant. Statistical analysis was performed with the SPSS Version 16.0 software package (SPSS; Chicago, IL).

RESULTS

MLDS induces T-cell–mediated autoimmune diabetes. To verify that MLDS is T-cell dependent in our pathogen-free colony [contrasting with previous work done in the 1980s in which experiments were not done in a specific-pathogen–free environment (25,31)] we injected WT, RAG1$^{-/-}$, and nude B6 mice with MLDS. In all WT mice, we detected progressively elevated serum glucose levels beginning on experimental day 7 and all became diabetic by experimental day 17. In contrast, we found that none of the RAG1$^{-/-}$ and none of the nude mice developed diabetes (Fig. 1*A*). In control experiments, we observed that Hi-STZ (which directly destroys islet tissue) induced diabetes by experimental day 7 comparably in WT and RAG1$^{-/-}$ mice (Fig. 1*A*). On H&E-stained pancreas tissues obtained on experimental day 19, we found significant intra- and peri-islet mononuclear infiltration in the pancreas of all WTs with a mean insulitis score of 50.4 ± 6.5%, whereas we noted intact islets with no mononuclear cell infiltrates in all RAG1$^{-/-}$ mice (Fig. 1*B*).

To test whether the MLDS protocol induced islet-reactive T-cell autoimmunity, we reasoned that after syngeneic islet transplantation, the primed islet-reactive, cellular immune response would rapidly destroy the transplanted tissue and induce recurrent diabetes. To test this hypothesis, we isolated islets from WT B6 mice, pretreated them in vitro with STZ to facilitate neoantigen expression (29), and then transplanted 500 islets under the kidney capsules of syngeneic MLDS-induced diabetic B6 mice. We injected identically-treated islets into Hi-STZ diabetic B6 recipient mice as controls. We observed that after transplantation, all animals rendered diabetic by either MLDS or Hi-STZ initially significantly lowered their serum glucose values by day 2 after transplantation, demonstrating that the transplanted islets were functional (Fig. 1*C* and *D*). Subsequently, the serum glucose of all of the transplanted MLDS-treated animals increased to pretransplant values within 1 week after transplant (Fig. 1*C*). In contrast, in the Hi-STZ treated mice, we found that islet transplantation markedly reduced and stabilized lower serum glucose in all animals and fully normalized serum glucose in 6 of 8 mice (Fig. 1*D*). On day 7 after transplantation, the MLDS recipients had a significantly impaired insulin response after intraperitoneal glucose load compared with the Hi-STZ–treated recipients (Fig. 1*E*). When we examined the

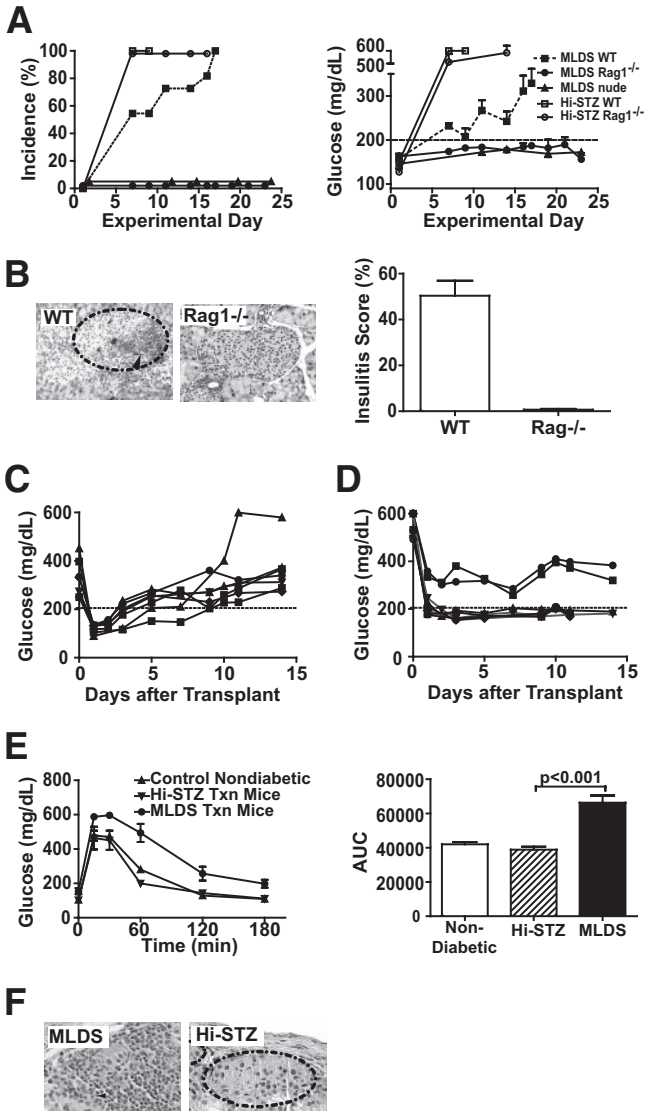

FIG. 1. MLDS-induced diabetes is T-cell dependent. *A*: Cumulative diabetes incidence and mean blood glucose levels in MLDS WT (n = 11), MLDS Rag1$^{-/-}$ (n = 7), MLDS nude (n = 5), Hi-STZ WT (n = 7), and Hi-STZ Rag1$^{-/-}$ (n = 4) mice. *B*: Histopathology of pancreatic sections from WT and Rag1$^{-/-}$ mice stained with H&E from experimental day 19 MLDS mice reveal infiltration present in WT, but absent in Rag1$^{-/-}$ mice. *Arrowhead* indicates areas of infiltration; original magnification ×200. Five hundred islets were pretreated with 0.5 mg/ml STZ for 1 h, then transplanted into (*C*) MLDS (n = 7) and (*D*) Hi-STZ–treated (n = 8) syngeneic recipients. Individual blood glucose levels are shown. *E*: AUC was calculated from glucose tolerance test performed on day 7 after islet transplantation in addition to control nondiabetic mice (n = 6). *F*: Histopathology of transplanted islets from MLDS and Hi-STZ–treated mice stained with H&E day 15 after transplantation. Heavy infiltration (*arrowhead*) was present in the transplanted islets from MLDS, but not Hi-STZ mice; original magnification ×200.

pancreas tissue, we found mononuclear cell infiltration in the transplanted islets from the MLDS-treated mice, but not in the Hi-STZ–treated animals (Fig. 1*F*). Control experiments with islets that were not treated in vitro with STZ prior to transplantation into MLDS mice resulted in prolonged euglycemia (n = 3; data not shown). Together these experiments support the concept that MLDS induces autoimmune diabetes, whereas Hi-STZ induces diabetes through an islet-toxic mechanism.

C3 is required for MLDS-induced diabetes. Because we (8,32,33) and others (34–36) have shown that immune

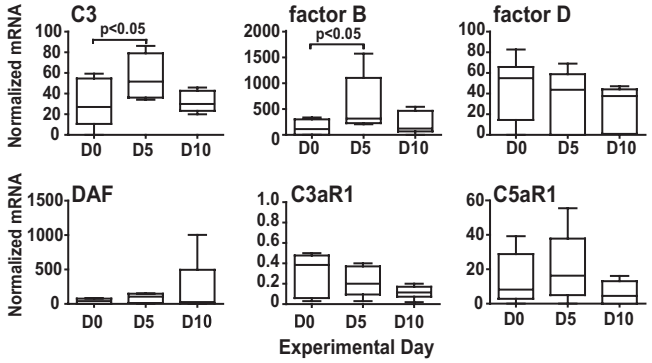

FIG. 2. MLDS induces the differential expression of complement genes. Total pancreata were isolated from MLDS-treated WT mice on experimental day 0, 5, and 10, and complement genes were analyzed by qRT-PCR (n = 6 per time point). C3 and factor B mRNA expression levels (normalized to cyclophilin) increased significantly after MLDS, whereas factor D, C3aR1, and C5aR1 remained unchanged. DAF expression showed an increasing trend. Box and whisker blots show the medians and the percentile values (10, 25, 75, and 90) for normalized mRNA.

cell-produced complement exerts control over T-cell autoimmunity in other models, we assessed the kinetics of complement component gene expression in total pancreatic tissue after MLDS administration using qRT-PCR. We detected gene transcripts for complement components C3 and factor B in pancreatic tissue obtained on experimental day 5 of MLDS. We noted that other complement components, receptors, and regulators, including factor D, C3aR1, C5aR1, and decay-accelerating factor (DAF) were detectable, but were not increased during the same time period (Fig. 2). We did not detect C3 or factor B transcripts from purified islets obtained from untreated animals or from mice treated with MLDS on days 5 or 10 (data not shown), suggesting that the pancreatic C3-derived from peri-islet immune cell infiltrates rather than from islet cells.

To test whether C3 is required to induce diabetes after MLDS, we administered MLDS to C3$^{-/-}$ mice on both C57BL/6 (n = 12) and BALB/c (n = 7) background. In contrast to the WTs (C57BL/6 n = 11, BALB/c n = 11) in which blood sugars exceeded 200 mg/dl at experimental day 11 in all mice, none of the C3$^{-/-}$ mice from either background developed hyperglycemia (Fig. 3*A*). Although we found histologic evidence of insulitis on experimental day 19 after MLDS in WT mice, we did not observe mononuclear infiltration within the islets of C3$^{-/-}$ animals (Fig. 3*B*). The mean insulitis score was 50.4 ± 6.5% in WT versus 5.3 ± 2.7% in C3$^{-/-}$ mice ($P < 0.01$) (Fig. 3*B*). In control experiments to test whether islets lacking C3 limits susceptibility to the effects of STZ, we cocultured WT and C3$^{-/-}$ islets overnight with 0.5 mg/ml STZ. When we then tested the cultured islets for apoptosis, we found similar levels of Annexin V staining in WT islets and C3$^{-/-}$ islets, 50.1% vs. 56.3%, respectively (Fig. S1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0044/DC1). In confirmation of equivalent susceptibility to STZ, all Hi-STZ treated C3$^{-/-}$ mice (n = 4) and WT mice (n = 7) developed diabetes with identical kinetics to the WT controls (Fig. 3*A*).

Because mononuclear cell chemoattractants CCL2 and CCL3, in addition to T-cell–derived IFN-γ and the innate cytokine IL-6, are implicated as mediators of insulitis, we measured their gene transcripts in pancreatic tissue of MLDS-treated WT and C3$^{-/-}$ mice. Compared with WT

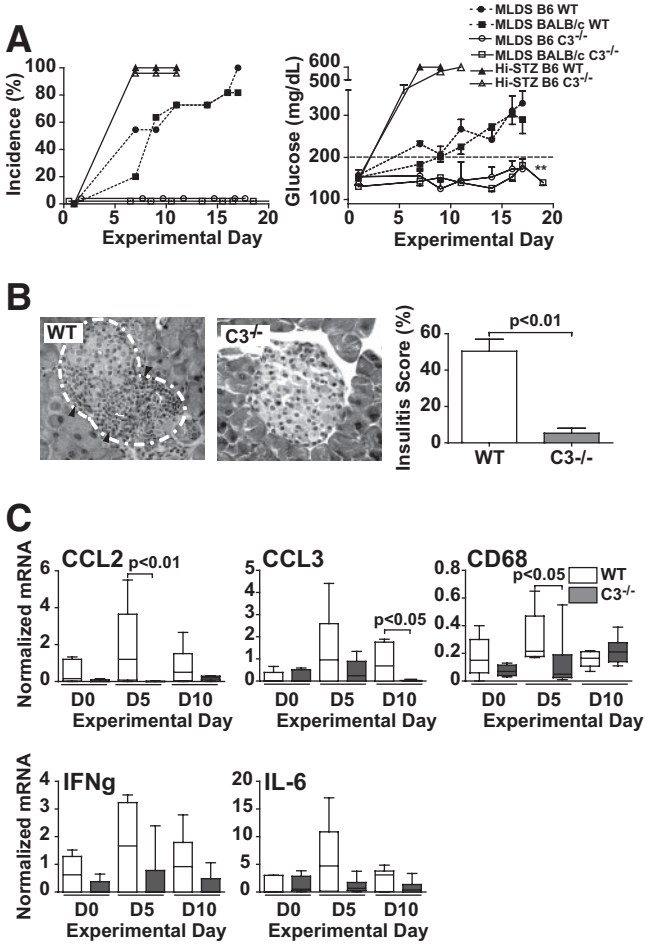

FIG. 3. C3 deficiency protects from MLDS-induced diabetes. *A*: Cumulative diabetes incidence and average blood glucose levels of WT or $C3^{-/-}$ mice on both the B6 and Balb/c background after administration of MLDS (n = 7–11 per group) or Hi-STZ (n = 3–4); $^{**}P < 0.01$ (comparing blood glucose level of WT versus $C3^{-/-}$ on day 17). *B*: Histopathology of pancreata on experimental day 19 of MLDS showing mononuclear cell infiltration in WT, but not in $C3^{-/-}$ mice. *Arrowheads* indicate areas of infiltration; original magnification ×200. Mean insulitis scores (% ± SEM) of mice on experimental day 19 were obtained from 3 mice for each group. $P < 0.01$ (*C*) qRT-PCR of CCL2, CCL3, IFN-γ, IL-6, and macrophages (CD68) from total pancreata (normalized to cyclophilin) on experimental days 0, 5, and 10 after initiation of MLDS (n = 6 per group and time point) with $C3^{-/-}$ mice with lower expression compared with WT mice. Box and whisker blots show the medians and percentile values (10, 25, 75, and 90) for normalized mRNA.

mice, C3 deficiency was linked with lower expression of these genes, with CCL2 and CCL3 achieving statistical significance. Notably, we found that these differences were associated with lowered expression of the macrophage marker CD68 within the pancreas (Fig. 3*C*).

Diminished T-cell immunity to islet antigens in $C3^{-/-}$ mice. To test the effect of C3 deficiency on the islet-reactive T-cell response, we cultured spleen cells from MLDS-treated WT and $C3^{-/-}$ mice (and from untreated controls) in the presence or absence of purified islet cells and measured cytokine production in culture supernatants 24 h later (Fig. 4). These analyses revealed islet-induced production of TNFα (experimental day 10) and IFN-γ (experimental day 19) in MLDS-treated WT mice. Splenocytes obtained from untreated mice did not respond to islet stimulation, confirming that the noted cytokine production was induced by MLDS. In contrast to the results in WT mice, we did not detect cytokines in cultures of day-19

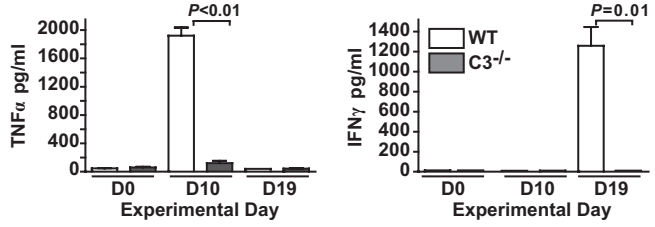

FIG. 4. Presence of C3 is required in T-cell priming and activation. Cytokines produced by splenocytes isolated from MLDS-treated mice and then stimulated with islet cells for 24 h. $C3^{-/-}$ cells had significantly lower production of TNFα (experimental day 10) and IFN-γ (experimental day 19) compared with WT. Supernatants were analyzed in duplicates by Luminex and the data shown are representative of three independent experiments.

splenocytes obtained from MLDS-treated $C3^{-/-}$ mice. Other cytokines including IL-17 and IL-10 were barely detectable and not different between groups (data not shown).

MLDS-induced diabetes requires bone marrow cell-derived C3. Because immune cell–derived complement plays an integral role in adaptive T responses (13,37), we transplanted $C3^{-/-}$ (CD45.2) BM into lethally irradiated CD45.1 WT B6 mice ($C3^{-/-}$ BM→WT) to produce animals that contained serum C3, but possessed $C3^{-/-}$ BM cells. Conversely, we transplanted WT B6 CD45.1 BM into lethally irradiated CD45.2 $C3^{-/-}$ mice (WT BM→$C3^{-/-}$) to produce animals deficient in serum C3, but with C3+ BM–derived cells. WT BM→WT chimeras on the B6 background were produced as controls. Staining peripheral blood for CD45.1/CD45.2 and analyzing the cells by flow cytometry (Fig. 5*A*) verified that the immune cells were >90% donor BM-derived. Zymosan C3 uptake assays (Fig. 5*B*) validated the presence or absence of C3 in the serum of each animal. We did not detect C3 in the sera of any WT BM→$C3^{-/-}$ chimeras (same as $C3^{-/-}$ controls), but we observed that all sera from $C3^{-/-}$ BM→WT and WT BM→WT chimeras was C3+ comparable to WT controls (Fig. 5*B*). When we administered MLDS to the chimeric animals, we found that only 2 of 8 $C3^{-/-}$ BM→WT chimeras developed diabetes, whereas all of the WT BM→$C3^{-/-}$ chimeras and all of the control WT BM→WT chimeras developed sustained hyperglycemia (Fig. 5*C*).

In separate "add back" experiments to test the requirement for spleen cell–derived C3 in MLDS- induced diabetes, we adoptively transferred 3×10^6 WT or control $C3^{-/-}$ spleen cells into $C3^{-/-}$ mice, and then treated all animals with MLDS. Although 2 of 3 $C3^{-/-}$ mice that received WT spleen cells became diabetic, none of 4 animals that received $C3^{-/-}$ spleen cells developed hyperglycemia (Fig. S2 in the online appendix).

DISCUSSION

Taken together, our findings indicate that immune cell–derived C3 is required for the development of diabetes in the MLDS model. We showed that in WT animals, MLDS-induced hyperglycemia and islet inflammation are associated with complement gene upregulation (Fig. 2). We then documented that C3-deficient mice from two different genetic backgrounds are resistant to MLDS-induced diabetes (Fig. 3). This protective phenotype occurs in the absence of islet inflammation (Fig. 3) and in association with diminished islet antigen-induced spleen cell-derived IFN-γ and TNFα production (Fig. 4). Using a bone marrow chimera strategy (and confirmed by spleen cell adoptive

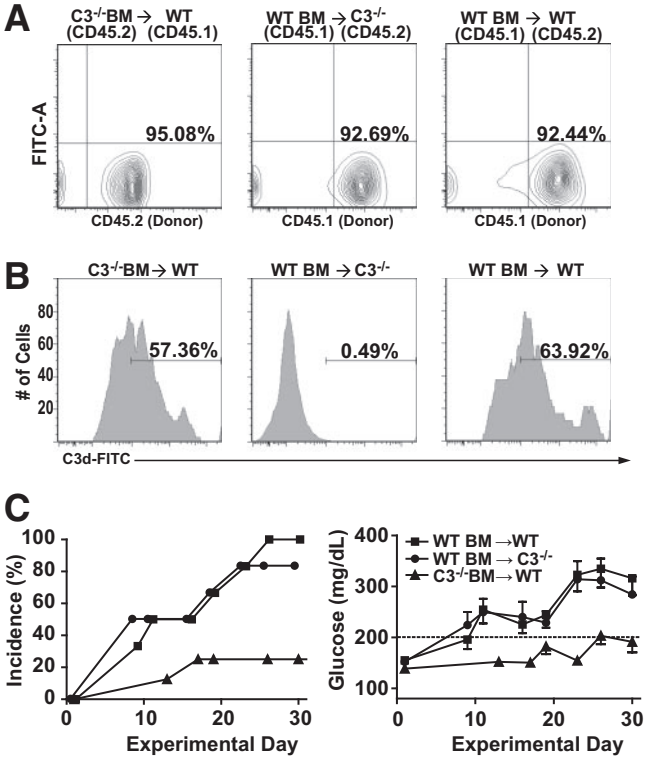

FIG. 5. Functional C3 on BM-derived cells, and not systemic C3, is required for development of MLDS-induced diabetes. *A*: $C3^{-/-}$ BM→WT (n = 8), WT BM→$C3^{-/-}$(n = 6), and WT BM→WT (n = 6) chimeric mice were stained for CD45.2 and CD45.1 and analyzed by flow cytometry to verify lethal irradiation and bone marrow engraftment on week 8. *B*: Zymosan C3 binding assay confirming presence of systemic C3 in WT and absence in $C3^{-/-}$ mice. *C*: Cumulative diabetes incidence and average blood glucose levels after administration of MLDS in chimeric mice. Results represent two independent experiments.

transfers), we showed that BM-derived C3 and not serum C3 is critical for the development of autoimmune diabetes in this model (Fig. 5).

Previous mechanistic work performed by our group (5) demonstrated that cognate T-cell/APC interactions result in release and activation of alternative pathway complement yielding locally produced C3a and C5a. These intermediaries bind to their respective receptors, C3aR and C5aR expressed directly on T cells, and through regulating AKT phosphorylation, augment T-cell proliferation and prevent T-cell apoptosis (5,11). The C3a and C5a also bind to their receptors on APCs, upregulating cytokine production and costimulatory molecule expression, which further amplify the T-cell response (5). In the absence of either C3 or C3aR/C5aR, T-cell proliferation is prevented, T-cell apoptosis is enhanced, and APC-produced cytokines and costimulatory molecule expression are reduced (5,11), together resulting in diminished cellular immune responses. Our new findings that islet-reactive T-cell immunity and graft inflammation are diminished in MLDS treated $C3^{-/-}$ mice without diabetes are consistent with these mechanisms. In further support of the role for these C3aR and C5aR signaling as an underlying mechanism for our observations, we found that only 1 of 5 mice deficient in both C3aR and C5aR developed diabetes within 30 days after initiation of MLDS treatment (Fig. S3).

As others have demonstrated a key role for immune cell–derived complement as an opsonin (38,39), limited antigen processing and presentation of autoantigens may contribute to the protective phenotype of the C3-deficient mice (40). Macrophage-derived C3, a key regulator of macrophage activation (41), may also be important in facilitating macrophage mediated islet injury (4,15). These, among other effects of C3 on regulating innate and adaptive immunity, require additional study.

It is notable that NOD mice develop spontaneous autoimmune diabetes despite being C5 deficient (42), indicating that C5, C5a, and the membrane attack complex (43) are not required in the pathogenesis of diabetes in that model system. Whether C3 and/or its activation cleavage products, C3a, C3b, or C3dg are involved in the pathogenesis of diabetes in NOD mice is an issue that remains to be tested. However, the generation of $C3^{-/-}$ NOD mice is improbable because both C3 and the diabetes-susceptible H-$2K^d$ genes are located on chromosome 17 (17,18).

Our data support the interpretation that MLDS-induced diabetes is, in part, an autoreactive, T-cell–mediated process. We showed that both T-cell–deficient nude mice and T- and B-cell–deficient $Rag1^{-/-}$ mice do not develop hyperglycemia despite administration of MLDS that is pathogenic in WT animals (Fig. 1). Our documentation of recurrent hyperglycemia after syngeneic islet transplantation of MLDS-induced diabetic animals, but not diabetic mice induced by Hi-STZ (Fig. 1), supports this interpretation.

In summary, this work demonstrates an unanticipated key role for immune cell–derived C3 in the pathogenesis of murine autoimmune diabetes. These results argue that studies testing the function of immune cell–derived complement in human diabetes are warranted.

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M.L. researched data and wrote the manuscript. N.Y. and S.S. researched data. B.M. and M.E.M. reviewed/edited the manuscript. P.S.H. and B.S. contributed to discussion and reviewed/edited the manuscript.

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