The American Journal of Pathology, Vol. 169, No. 6, December 2006

DOI: 10.2353/ajpath.2006.060539

Immunopathology and Infectious Disease

In $CD4^+$ T-Cell-Induced Diabetes, Macrophages Are the Final Effector Cells that Mediate Islet β-Cell Killing

Studies from an Acute Model

Boris Calderon, Anish Suri, and Emil R. Unanue

From the Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri

To understand better how diabetogenic $CD4^+$ T cells induce islet β-cell death and cause diabetes, a transfer model of acute diabetes using the diabetogenic $CD4^+$ BDC2.5 T-cell clone was established. Transfer of activated BDC T cells into NOD.scid mice resulted in diabetes within a week, characterized by strong inflammatory reaction. Electron micrographs of pancreas depicted macrophages in close contact with β cells that exhibited signs of apoptosis. Transfer into irradiated recipients inhibited inflammation and the development of diabetes, demonstrating an obligatory role for leukocytes. Selective depletion of neutrophils or natural killer cells had no effect on diabetes induced by BDC2.5 T cells. In contrast, *in vivo* depletion of phagocytic cells by injection of liposomes containing clodronate abolished diabetes, although inflammation remained present and was characterized mainly by neutrophil infiltration. Treatment with clodronate-liposomes did not affect the antigen-presenting cells within the pancreas. Last, activated macrophages isolated from infiltrated pancreas exhibited cytolytic activity toward primary islet β cells. Taken together, these results demonstrate that activated macrophages are the key cells mediating islet β-cell death induced by activated $CD4^+$ T cells. ***(Am J Pathol 2006, 169:2137–2147; DOI: 10.2353/ajpath.2006.060539)***

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder wherein the pancreatic islet β cells are destroyed by autoreactive T cells resulting in a state of persistent hyperglycemia. The nonobese diabetic (NOD) mouse and the bio breeding (BB) rat are two attractive animal models for T1DM that follow many characteristics of the human disease including the expression of the diabetes-susceptible class II major histocompatibility complex (MHC) alleles.[1–3] T1DM in both humans and rodents is characterized by distinct histopathological stages. The first stage, termed peri-insulitis, consists of an initial infiltration of leukocytes surrounding the islets without apparent effect on β cells; this is followed by an aggressive phase wherein the infiltrate actively invades the islets and kills the β cells, leading to diabetes. $CD4^+$ T cells are essential for development of diabetes by recognizing β-cell antigens in the context of the class II MHC I-A^{g7}. Involvement of $CD8^+$ T cells has also been extensively documented.[4–7] Various mechanisms for inducing β-cell death have been proposed including a role for Fas/FasL, perforin/granzyme pathway, Rae1-NKG2D interaction, and reactive oxygen species induced by proinflammatory cytokines.[8–12]

A major hurdle in understanding the role of various leukocytes in T1DM is the large and varied time span between peri-insulitis and onset of diabetes (in NOD mice it can be anywhere between 10 to 14 weeks). Moreover, the presence of both $CD4^+$ and $CD8^+$ T cells makes it difficult to dissect the effector pathways used by each to induce islet β-cell death. To this end, we have examined an accelerated model of T1DM using the diabetogenic $CD4^+$ T cell, BDC2.5, expressed as a T-cell receptor (TCR) transgene in NOD mice (from here on referred to as BDC T cells). BDC T cells recognize an unidentified islet β-cell antigen presented by the I-A^{g7} class II MHC molecule of NOD mice.[13] Activated BDC T cells transfer diabetes into NOD.scid recipients in a short period of time with reproducible kinetics and incidence.[14–16] This

Supported by the National Institutes of Health and the Kilo Diabetes and Vascular Research Foundation.

Accepted for publication September 6, 2006.

Address reprint requests to Emil R. Unanue, Washington University School of Medicine, Department of Pathology and Immunology, 660 S. Euclid Ave., St. Louis, MO 63110. E-mail: unanue@pathbox.wustl.edu.

model has several advantages: 1) the T cell inducing diabetes is a bona fide islet β-cell-reactive T cell initially isolated from islet-infiltrating leukocytes in NOD mice; 2) the time between injection of BDC T cells and onset of diabetes can be short—as early as a week depending on the number of cells transferred; and 3) BDC T cells induce diabetes on their own without the need for any other $CD4^+$ or $CD8^+$ T cell. In summary, this model offers an opportunity to analyze the role of various leukocytes (that form the insulitic infiltrate) in diabetes induced by $CD4^+$ T cells. Here we investigate how BDC T cells affect β-cell viability by selective depletion of leukocytes. We conclude that activated macrophages cause β-cell death in this model of acute diabetes.

Materials and Methods

Mice

The BDC2.5 TCR transgenic mice on the NOD background and B6.G7 congenic mice were established in our mouse colony at Washington University School of Medicine. NOD mice on the scid genetic background, NOD.CB17-*Prkdc*^{scid}/J or on the Rag-$1^{-/-}$, NOD.129S7(B6)-*Rag*1^{tm1Mom}/J were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in our mouse colony, and are referred to as NOD.scid and NOD.Rag mice, respectively. All experimental mice were 6 to 8 weeks old.

Adoptive Transfer

Splenocytes from BDC2.5 mice were cultured at 37°C in Dulbecco's minimal essential medium with 10% fetal calf serum and concanavalin A (5 μg/ml) (Sigma Chemical Co., St. Louis, MO) at a final cell concentration of 2×10^6 per ml. After 3 days of culture, cells were washed once with 4 ml of 0.2 mol/L methyl α-D-mannopyranoside (1:10) (Sigma Chemical Co.) and 36 ml of Dulbecco's minimal essential medium and then in 10 ml of Dulbecco's minimal essential medium. A dose range between 10^6 to 10^7 activated BDC2.5 cells were transferred intravenously via the tail vein in a 0.5-ml volume. The recipient mice were followed for diabetes incidence. Two consecutive readings of blood glucose of ≥250 mg/dl, measured by a glucometer (Bayer, Elkhart, IN), were indicative of diabetes. Control experiments established that the transfer was entirely dependent on the BDC T cells in the inoculum: transferring purified BDC T cells or the unpurified cells gave identical results. NOD.Rag and congenic B6.G7 mice received 650 and 750 rads, respectively, before the adoptive transfer of BDC T cells. In pilot experiments, we had established the doses required for sublethal irradiation and found that NOD strains were more sensitive to irradiation.

Histology and Immunohistochemistry

Mice were anesthetized with ketamine and sacrificed by cervical dislocation. The pancreata were fixed in 10% formalin. Slides were stained with hematoxylin and eosin (H&E). For detection of insulin, 5-μm serial sections were deparaffinized and stained with the HistoMouse-SP kit (AEC, Broad Spectrum, Bulk) (Invitrogen, Carlsbad, CA) using guinea pig anti-insulin polyclonal antibody (1:100) (Linco, St. Charles, MO). For insulin staining by immunofluorescence, sections were incubated overnight at 4°C with guinea pig anti-insulin polyclonal antibody (1:100) (Linco) followed by Texas Red-conjugated goat anti-guinea pig secondary antibody (1:100) (Jackson ImmunoResearch, West Grove, PA). All sections were counterstained with bis-benzamide (Sigma) for 5 minutes, washed in phosphate-buffered saline (PBS), quenched to reduce background autofluorescence with Sudan Black (Sigma) (Sudan black 0.5% in 70% ethanol) for 3 minutes, and finally rinsed in PBS. For antigen-presenting cell staining by immunofluorescence, serial 5-μm sections from embedded frozen pancreata (in Tissue-Tek compound; VWR International, West Chester, PA) were stained for 1 hour at room temperature with phycoerythrin-conjugated anti-mouse CD11b (1:100) and phycoerythrin-conjugated anti-mouse CD11c (1:100) (eBioscience, San Diego, CA).

Electron Microscopy

Pancreata from untreated and day 4 after adoptive cell transferred mice were removed and fixed in modified Karnovsky's fixative (2% paraformaldehyde, 3% glutaraldehyde in 0.1 mol/L cacodylate buffer) for 24 hours and then processed for electron microscopy analysis. Pancreatic endocrine granules of mice were determined based on their electron density, size, and shape reported previously by other groups.[17,18]

Selective Depletion in Vivo *by Antibodies*

For neutrophil depletion, NOD.scid mice received 500 μg of RB6-8C5 monoclonal antibody (mAb)[19] or isotype rat IgG (Sigma) intraperitoneally in 0.5 ml of PBS 1 day before and 2 days after cell transfer. We corroborated the high effectiveness of RB6-8C5 mAb depletion of neutrophils by three different approaches: 1) flow cytometry analysis of peripheral blood leukocytes, 2) examination of peripheral blood smears, and 3) direct neutrophil counts on H&E-stained slides from pancreata at the time of diabetes onset. This same batch of antibodies was used previously by us and our colleagues in various experimental situations.[19,20] Natural killer (NK) cell depletion in the NOD.scid mouse was performed by intravenous administration of 200 μg of anti-asialo GM1 (Wako Chemicals, Richmond, VA) or isotype rabbit IgG (Sigma) (days −1, 3, and 7).[21,22] NK cell depletion in B6.G7 congenic mice was achieved by injecting 500 μg of anti-NK1.1 monoclonal antibody or isotype mouse IgG2aκ(UPC-10) (Sigma) intraperitoneally on days −1, 3, and 7.[23,24]

Liposome Preparation and in Vivo *Application*

Multilamellar liposomes containing clodronic acid disodium salt (Cl_2MDP) (Sigma) in PBS (CLOD-LIP) were

prepared as described by van Rooijen's laboratory.[25,26] In brief, 86 mg of phosphatidyl choline (Sigma) and 8 mg of cholesterol (Sigma) were dissolved in 10 ml of chloroform and dried *in vacuo* in an evaporator to form a lipid film. The lipid was dispersed in 10 ml of PBS for preparation of PBS-containing liposomes (PBS-LIP). To enclose the Cl_2MDP, 2.5 g of Cl_2MDP was dissolved in 10 ml of deionized water (adjusted to pH 7.3 with NaOH) in which the lipid film was dispersed. The preparations were kept for 2 hours at room temperature, sonicated for 3 minutes, and then incubated overnight at 4°C. Nonencapsulated Cl_2MDP was removed by centrifugation and then washed three times in sterile PBS. Finally, the pellet was resuspended in 4 ml of sterile PBS. A volume of 0.1 ml of liposome suspension for every 10 g of body weight was injected intravenously for 4 consecutive days starting 24 hours after BDC T-cell transfer.

Isolation of Mouse Islets

Mouse islets were isolated as described before and dispersed by trypsinization.[27] In brief, usually approximately seven mice were sacrificed per islet preparation by cervical dislocation. Hanks' solution (Invitrogen) was injected into the common bile duct and then the pancreas was resected, cut into small evenly sized pieces, and digested in collagenase at 39°C in a shaking water bath for 5 minutes. The tissue suspension was then centrifuged through a Ficoll (Sigma) gradient of 23, 20.5, and 11% at 1800 rpm for 12 minutes at room temperature. Purified islets were removed from the top two interfaces and washed twice in Hanks' solution, then handpicked under a dissecting microscope. For cell dispersion, islets were gently digested with trypsin solution in a 37°C water bath for 3 minutes and then washed in CMRL medium 1066 (Invitrogen) several times. Infiltrating islet leukocytes were obtained from diabetic NOD.scid mice that received the BDC T cells. In brief, islets were processed as above except that all of the Ficoll interfaces were removed. The pooled Ficoll layers were then resuspended in a 50-ml conical tube with Hanks' solution and washed three times.

Flow Cytometry

Spleen cells and infiltrating islet leukocytes were stained for different cellular markers. Leukocytes were stained with a fluorescein isothiocyanate (FITC)-labeled anti-CD45(Ly-5) antibody; T cells were stained with either an FITC-labeled anti-$CD4^+$ (L3T4) or FITC-labeled anti-CD3ε chain antibody; neutrophils were stained with an FITC-labeled anti-Gr-1(RB6-8C5) antibody. Macrophages were analyzed with an FITC-labeled anti-F4/80 or FITC-labeled anti-CD11b antibody, whereas dendritic cells were analyzed with an FTIC-labeled anti-CD11c antibody. For NK cell analysis, we used a biotinylated anti-CD49b/Pan-NK and secondary stain was performed with streptavidin-allophycocyanin; NK cells were defined as CD3ε-negative and CD49b/Pan-NK-positive. All antibodies used were obtained from BD Biosciences, San Jose, CA. All FACS analysis was performed on a FACSCalibur and data analyzed using CellQuest software (BD Biosciences).

Cytotoxicity Assay

Cytotoxicity killing assay was performed using chromium-labeled YAC-1 cells and peritoneal exudate cells to corroborate effectiveness of NK cell depletion treatment following the Current Protocols in Immunology.[28] For assays testing the cytotoxicity of the macrophages for β cells, islet-infiltrating leukocytes from acute diabetic mice and β cells from NOD.scid mice were isolated and dispersed as described previously. Phagocytes were enriched by positive selection using magnetic cell sorting cell separation columns with mouse CD11b (Mac-1) and CD11c MicroBeads (Miltenyi Biotec, Auburn, CA). β cells were labeled with chromium-51 (50 μCi per 10^6 β cells) (MP Biomedicals, Irvine, CA) for 1.5 hours at 37°C, 5% CO_2, and then washed three times in Dulbecco's minimal essential medium-10% fetal calf serum. β cells were plated in a 96-well, round-bottom plate at a concentration of 10^4, and phagocytes were added at a 50:1 ratio (5×10^5). Supernatant was collected at different time points (0, 4, and 12 hours) and chromium released was measured using an automatic gamma counter (1272 Clinigamma LKB; Wallac, Tlurku, Finland). The percentage of specific chromium release (experimental release) was calculated from the maximum levels released by detergent lysis of β cells (maximal release) after subtracting the amount released spontaneously in cultures of β cells without macrophages (spontaneous release).[28]

Statistical Analysis

The Mann-Whitney *U*-test was used for determining the level of significant differences in sample means.

Results

BDC T-Cell-Induced Diabetes: Kinetics of Disease Transfer and Histological Evaluation of the Islet Infiltrates

BDC T cells isolated from spleens of transgenic mice were activated with concanavalin A and then transferred intravenously into NOD.scid recipient mice. As shown in Figure 1A, diabetes developed in a dose-dependent manner: in 100% of mice between days 6 to 9 when 3 to 10×10^6 T cells were injected, and in 80% at day 20 when only 10^6 BDC T cells were transferred. This condensed window of induction of diabetes by BDC T cells had several histological features. Infiltration with a variety of cells, particularly macrophages and neutrophils, were first noticed on the 3rd day, becoming more prominent in the next several days (Figure 1B). After day 4, the exudate progressively penetrated the islets and disrupted their normal architecture. By the 7th day, the inflammation

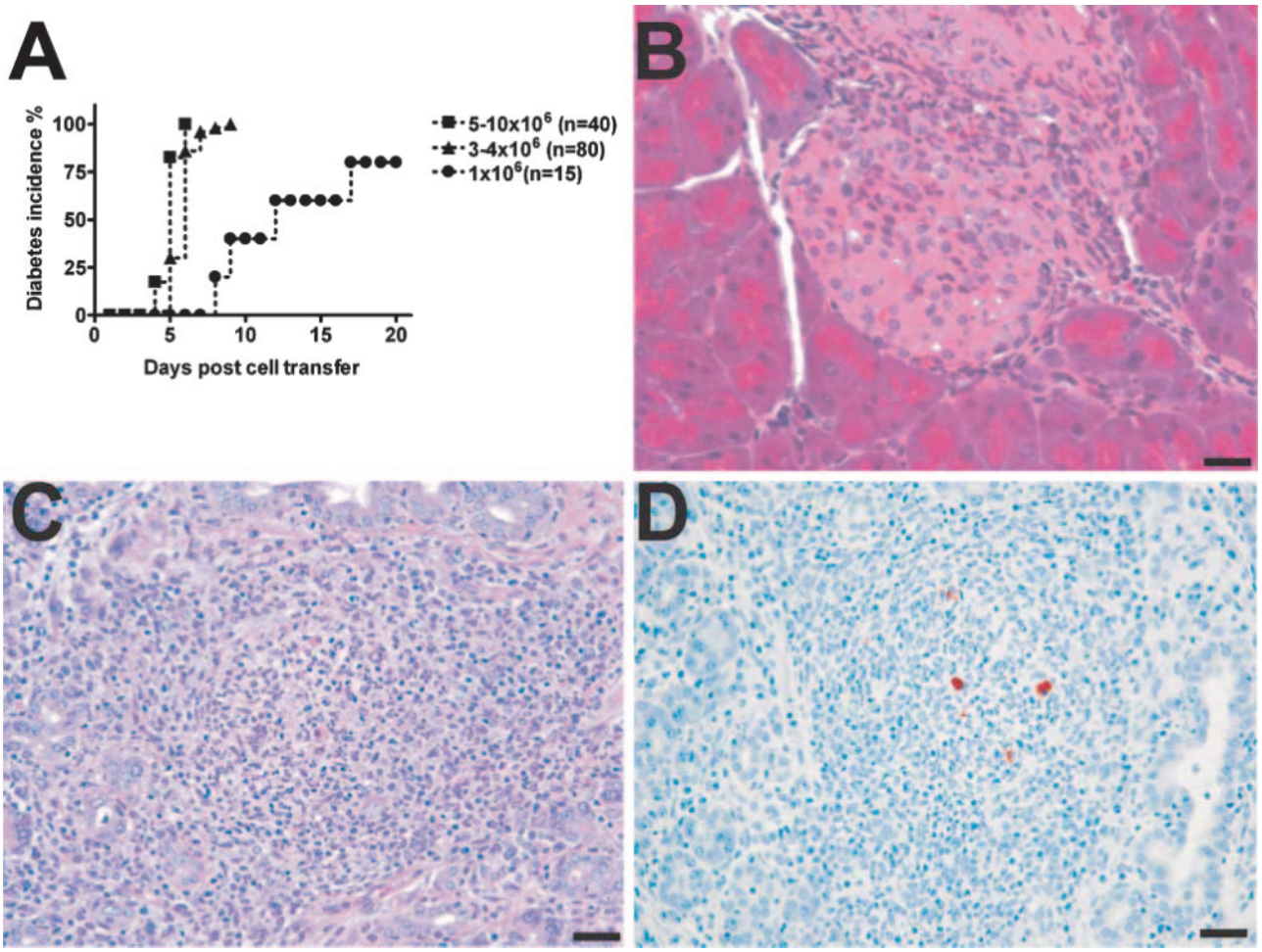


Figure 1. A: Con A-activated BDC T cells were transferred intravenously into NOD.scid recipients, which were then followed for the onset of diabetes. Depending on the numbers of injected T cells [10^6 (n = 15), 3 to 4 × 10^6 (n = 80), 5 to 10 × 10^6 (n = 40)], diabetes developed between 4 to 10 days. Mice were considered diabetic on two consecutive blood glucose measurements of ≥250 mg/dl. **B:** H&E staining of pancreas at day 3 after T-cell transfer (4 × 10^6) into NOD.scid mice. Note the early peri-insulitic lesion composed of neutrophils, macrophages, and lymphocytes. **C:** H&E staining of pancreas at day 7 after T-cell transfer (4 × 10^6). Note the extensive infiltration of islets with macrophages, neutrophils, and lymphocytes that disrupt the normal islet architecture. **D:** Same islet as in **C** stained for insulin (red). Note the decreased numbers of insulin-positive β cells that correlated with the onset of diabetes. Scale bars = 20 μm.

was extensive and few insulin-positive β cells could be found, which correlated with the onset of hyperglycemia (Figure 1, C and D).

Electron microscopy revealed the presence of cells compatible with macrophages found in close contact with β cells (Figure 2). The β cells exhibited mitochondrial swelling and extensive vacuolation with many empty vesicles; a few vesicles still contained a dense core typical of the insulin granule. Many β cells showed the condensed nuclear chromatin indicative of apoptosis (Figure 2, A–C). Images also showed the presence in macrophages of electron-dense insulin-like granules and apoptotic nuclei taken up by the macrophages (Figure 2, D–F). Neutrophils were found among the macrophages, but direct contact with β cells was not observed. The leukocytes among infiltrated islets were isolated and characterized by flow cytometry, at day 7 after transfer of BDC T cells: macrophages were the most abundant leukocytes present (46.8%) followed by NK cells (38.6%), neutrophils (8.3%), and dendritic cells (2.7%); only 3.6% of the exudate contained $CD4^+$ BDC T cells (Figure 3).

Requirement of Various Leukocytes for Development of BDC T-Cell-Induced Diabetes

Host Leukocytes

To determine whether leukocytes derived from the host were needed to initiate diabetes, the ability of BDC T cells to transfer disease was tested in the temporal absence of host leukocytes. NOD.Rag mice irradiated to eliminate bone marrow-derived leukocytes were used as recipients. As shown in Figure 4, at day 5 after transfer BDC T cells induced diabetes in 100% of the control mice while none in the irradiated mice. The irradiated recipient mice developed hyperglycemia at day 17 after transfer when hematopoiesis was restored and new host leukocytes infiltrated the pancreas (Figure 4A). Histological analysis

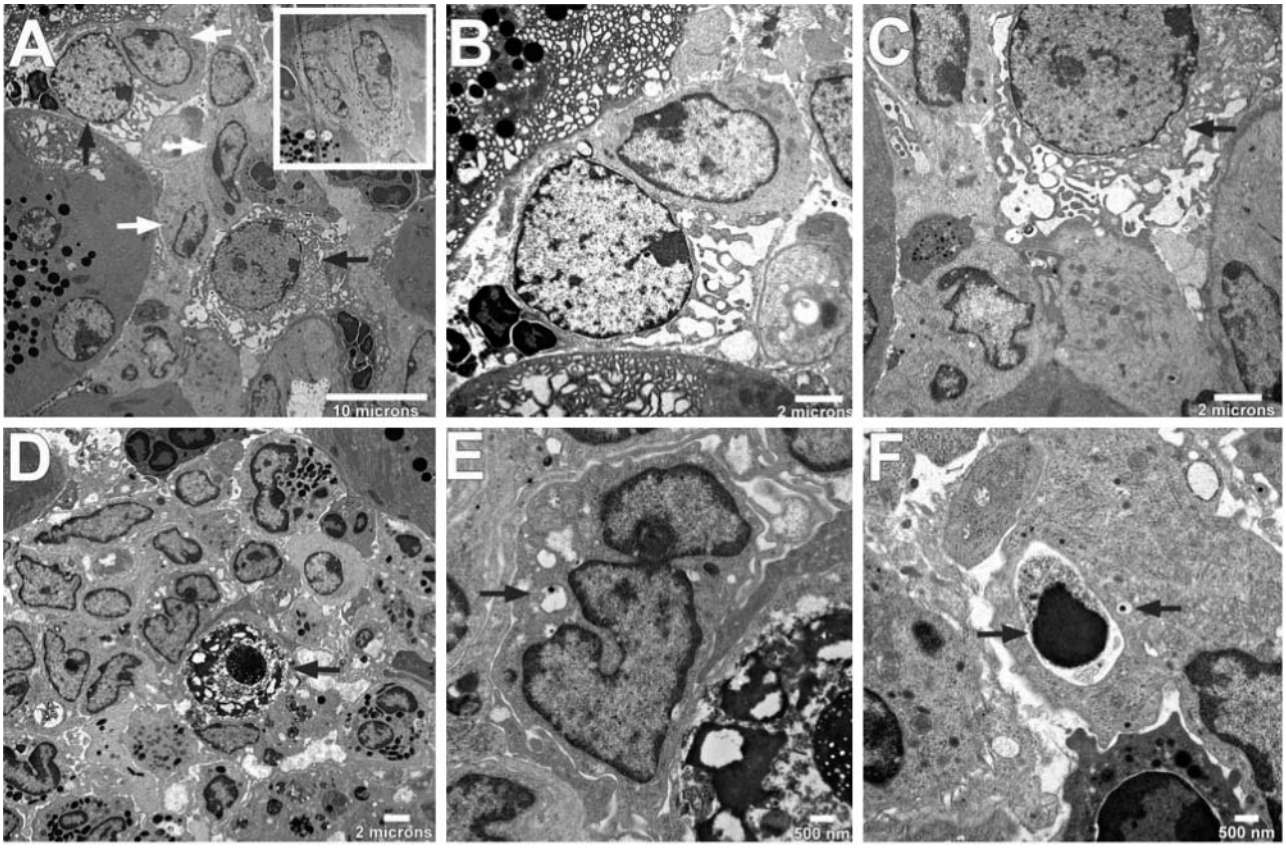


Figure 2. Electron microscopy analysis of acute diabetic mice at day 4 after BDC T-cell transfer. **A:** Infiltrated islet including two β cells (**black arrows**) showing empty vesicles and surrounded by macrophages (**white arrows**) in close contact with β cells. Small micrograph shows a normal β cell from a normal NOD.scid mouse; note the conserved cytoplasm, mitochondrias, and insulin granules. **B:** Higher magnification of a β cell in close relationship to a macrophage. **C:** Higher magnification of a β cell that exhibits mitochondrial swelling (**arrow**). **D:** Infiltrated islet showing a β cell undergoing apoptosis (**arrow**). **E:** Higher magnification of a macrophage from the previous image showing the close contact with a β cell and also what appears to be an insulin-like granule inside a vacuole (**arrow**). **F:** Apoptotic nuclei and insulin-like granule inside a vesicle of a macrophage (**arrows**).

of pancreatic islets from irradiated mice at day 5 after BDC T cell transfer (ie, the same time point at which 100% of the control unirradiated mice were diabetic) showed intact islets with the presence of few leukocytes (Figure 4B), but by day 19, the mice from the same treatment group contained islets that exhibited a disrupted architecture with an extensive leukocytic infiltrate (Figure 4C). These results demonstrated that the activated BDC T cells on their own did not cause diabetes but rather needed the presence of host leukocytes.

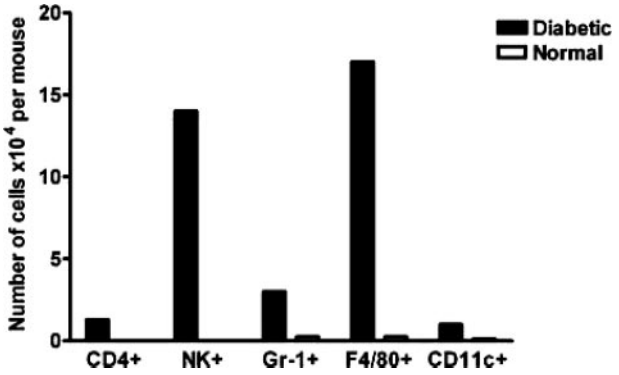


Figure 3. Distribution of islet-infiltrating leukocytes from NOD.scid mice at day 7 after BDC T-cell transfer (diabetic) and from control NOD.scid mice that did not receive any T cells (normal). Leukocytes were recovered from pancreatic islets of six mice in each group and analyzed by flow cytometry for the various leukocyte markers. The total numbers of islet-infiltrating leukocytes recovered per mouse from diabetic versus normal mice were 5.9×10^5 and 6.3×10^3, respectively. Normal NOD.scid mice contain low numbers of neutrophils, macrophages, and dendritic cells.

Neutrophils and NK Cells

For neutrophil depletion, NOD.scid recipient mice were treated with either the depleting RB6-8C5 mAb or an isotype control rat IgG and then injected with activated BDC T cells. Flow cytometry analysis of blood leukocytes indicated ~95% depletion of neutrophils, 48 hours after injection. As shown in Table 1, both sets of recipient mice developed diabetes with similar kinetics and incidence. The islet infiltrate from the neutrophil-depleting RB6-8C5 antibody-treated mice contained abundant macrophages. The reverse situation was shown in the experiment of Figure 6 reported below: selective depletion of macrophages resulted in exudates enriched in neutrophils but without damage to β cells. Moreover, twenty islets from each mouse were analyzed for the number of neutrophils present. As shown in Table 2, whereas isotype-treated mice showed a mean of 139 neutrophils per

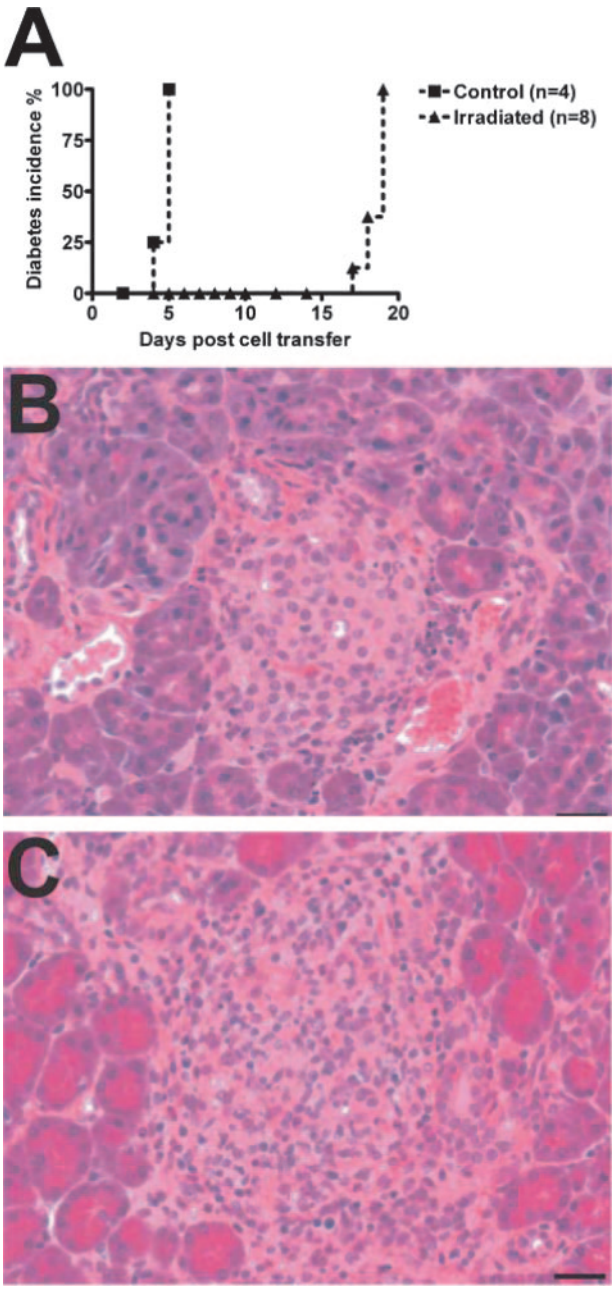


Figure 4. A: NOD.Rag1$^{-/-}$ mice irradiated with 650 rads received 10^7 activated BDC T cells and were followed for diabetes development. Although control unirradiated NOD.Rag$^{-/-}$ mice developed diabetes in 5 days (n = 4), irradiated mice showed a delayed onset of diabetes until day 19 after cell transfer (n = 8). **B:** H&E staining of an islet from an irradiated mouse at day 5 after cell transfer. Note the lack of insulitis as evidenced by few leukocytes around the islet. **C:** H&E staining of an islet from an irradiated recipient mouse that became diabetic at day 19 after BDC T-cell transfer. Islet shows severe infiltration with loss of β-cell mass. Scale bar = 20 μm.

islet, RB6-8C5 mAb-treated mice showed a mean of eight neutrophils per islet.

Two strategies that centered on *in vivo* depletion of NK cells were used to address their role: first, NOD.scid mice were treated with the anti-asialo GM1 antibody to deplete NK cells *in vivo*, and second, I-A^{g7}-expressing congenic B6 mice (B6.G7), which in contrast to NOD mice express the NK1.1 marker, were treated with the anti-NK1.1 depleting antibody. Both sets of these mice were used as recipients for activated BDC T cells. As shown in Table 1, diabetes developed with the same kinetics in both groups of experimental versus their respective isotype control antibody-treated mice. To confirm that *in vivo* depletion of NK cells was complete, we isolated peritoneal exudate cells from both sets of mice, experimental (ie, NOD.scid and B6.G7 mice treated with anti-asialo GM1 and anti-NK1.1 antibodies, respectively) or control (isotype antibody-treated mice) and tested them in a cytotoxicity assay for their ability to lyse chromium-labeled YAC-1 target cells. The peritoneal exudate cells from control mice efficiently lysed YAC-1 target cells, whereas no such activity was detected from either of the antibody-treated experimental mice (Figure 5, A and B). This result confirmed that our protocol resulted in the efficient depletion of NK cells.

Monocytes/Macrophages

In both our acute model as well as the wild-type NOD model of diabetes, leukocytes belonging to the monocyte lineage (ie, macrophage and dendritic cells) form a major component of the islet infiltrate (Figure 3).[29–33] Other studies in NOD mice and BB rat demonstrated that macrophages were essential for development of disease.[29,34–39]

To test the requirement for macrophages in BDC T-cell-induced diabetes, we used the protocol developed by N. van Rooijen and colleagues[25,26] to deplete phagocytic cells. Previous studies demonstrated the successful elimination of circulating monocytes using this protocol.[40–43] In brief, several injections of CLOD-LIP were administered to NOD.scid mice, which then received an inoculum of activated BDC T cells. Flow cytometry analysis of spleens from control or experimental mice at the 6th day after T-cell transfer showed complete depletion of phagocytic cells in the CLOD-LIP-treated mice. As shown in Figure 6, the control mice treated with PBS-LIP contained normal numbers of F4/80$^+$ and CD11b/c$^+$ cells, whereas those treated with CLOD-LIP exhibited a total absence of them (Figure 6B).

BDC T cells caused diabetes in control mice treated with PBS-LIP, whereas disease was markedly reduced in incidence, and delayed in time, in mice that received CLOD-LIP. Although 100% of the mice that received PBS-LIP were diabetic at day 6 only 33% were diabetic at day 30, 5 of 15, in the CLOD-LIP-treated group (Figure 6A). It is important to mention that the last day of treatment with CLOD-LIP or PBS-LIP was at day 4 after T-cell transfer, and the delayed onset of diabetes in experimental mice was likely attributable to the presence of new circulating monocytes that emerged after clodronate treatment. Histopathological analysis of the pancreas from control (PBS-LIP) and experimental (CLOD-LIP) mice showed inflammation; ie, there was a response to the BDC T-cell transfer (Figure 6, C–F); however, there were some unique features noted in mice treated with CLOD-LIP: 1) a high number of neutrophils were noted in the islet infiltrate; 2) macrophages were conspicuously absent; 3) abundant apoptotic bodies were found, most likely from

Table 1. Experiments of Selective Depletion of Neutrophils and NK Cells in the Acute Diabetes Model

Number of BDC T cells	Recipient strain	Treatment	Diabetes incidence
4×10^6	NOD.scid	Isotype control antibody	10 of 10 by day 6
4×10^6	NOD.scid	RB6 mAb	11 of 11 by day 6
4×10^6	NOD.scid	Anti-asialo GM1	13 of 13 by day 7
1×10^7	B6.g7*	Isotype Ab	4 of 6 by day 9
1×10^7	B6.g7*	Anti-NK1.1 mAb	4 of 4 by day 10

Male NOD.scid mice between 6 and 8 weeks of age depleted of neutrophils or NK cells received activated BDC T cells.
*These mice were irradiated with 750 rads to facilitate adoptive transfer of activated BDC T cells.

dead neutrophils; and 4) intact islet β cells that contained insulin were evident (Figure 6G).

The transfer of BDC T cells into the CLOD-LIP-treated mice resulted in severe inflammation with a paucity of activated macrophages indicating that the BDC T cells had been stimulated in the pancreas by the local antigen-presenting cells (APCs) normally residing in it. (Of note, the transfer of inflammation and diabetes required local presentation: transfer into allogeneic scid mice was completely ineffective.) Therefore, an important issue in using the clodronate-depletion protocol was to identify whether this treatment also depleted the intrapancreatic resident phagocytes. To address this, the same regimen of liposome treatment (CLOD- or PBS-LIP) was administered to normal NOD.scid mice. Although blood monocytes were entirely depleted, immunofluorescence analysis (Figure 7A) and statistical analysis (Mann-Whitney *U*-test) indicated that treatment with CLOD-LIP did not deplete intrapancreatic APCs ($F4/80^+$, $CD11b^+$, and $CD11c^+$) (Figure 7, B and C).

To demonstrate that macrophages can directly kill islet β cells, we designed an *in vitro* islet β-cell cytotoxicity assay. Primary islet β cells, isolated from NOD.scid mice,[27] were labeled with chromium and used as the targets. The phagocytes were isolated from infiltrates of NOD.scid mice 7 days after receiving activated BDC T cells. Phagocytes were enriched using CD11b/c magnetic beads, and >98% of $CD45^+$leukocytes were $CD11b/c^+$ after enrichment. Cytotoxicity of β cells was measured at various time points (0, 4, and 12 hours) at a final effector to target ratio of 50:1 (in the *in vitro* cytotoxicity assay, we detected chromium release at a ratio above 25:1). Results in Figure 8 show a time-dependent lysis of primary islet β cells by phagocytes recovered from infiltrated islets. Maximal specific lysis (~40%) was observed at 12 hours after incubation of phagocytes with islet β cells. This was a direct demonstration that macrophages recruited to the inflamed islets were capable of killing islet β cells.

Table 2. Analysis of Intra-Islet Neutrophils from RB6–8C5 mAbs and Isotype-Treated Mice at the Time of Diabetes Onset

Antibody treatment and mouse no.	Islets screened	Neutrophils counted	Neutrophil/islet mean
Isotype			
1	20	2224	
2	20	3324	
Total	40	5548	138.7
RB6–8C5			
3	20	288	
4	20	176	
5	20	140	
6	20	68	
Total	80	672	8.4

H&E-stained slides from RB6–8C5 mAb and isotype antibody-treated mice were analyzed at the time of diabetes onset.

Discussion

This study focused exclusively in an acute transfer model of T1DM in the NOD mouse in which the final effector phase of islet β-cell death could be analyzed. By selectively depleting leukocyte subsets, we could demonstrate that $CD4^+$ T cells induced diabetes via activating monocytes to differentiate into macrophage islet β-cell killers. Several observations supported this conclusion. 1) Only depletion of phagocytes, but not neutrophils or NK cells, hampered development of diabetes; neutrophils did not damage β cells even when found abundantly in the inflammatory exudate as it happened in the macrophage-depleted mice. Neither could we ascertain an involve-

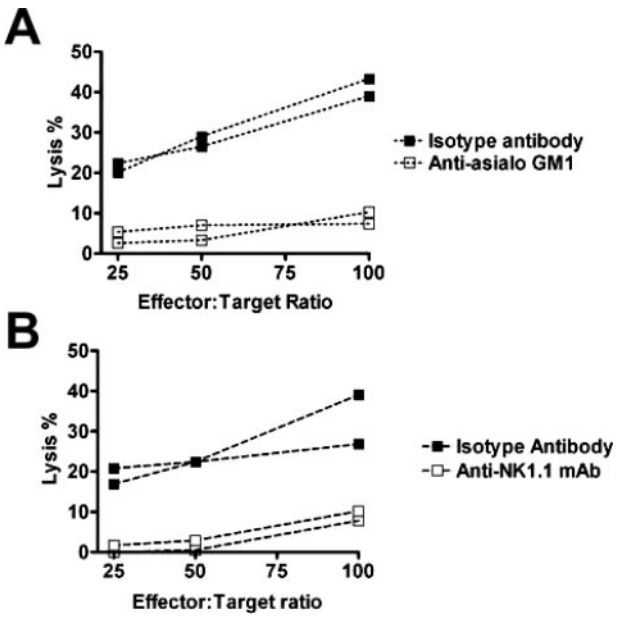
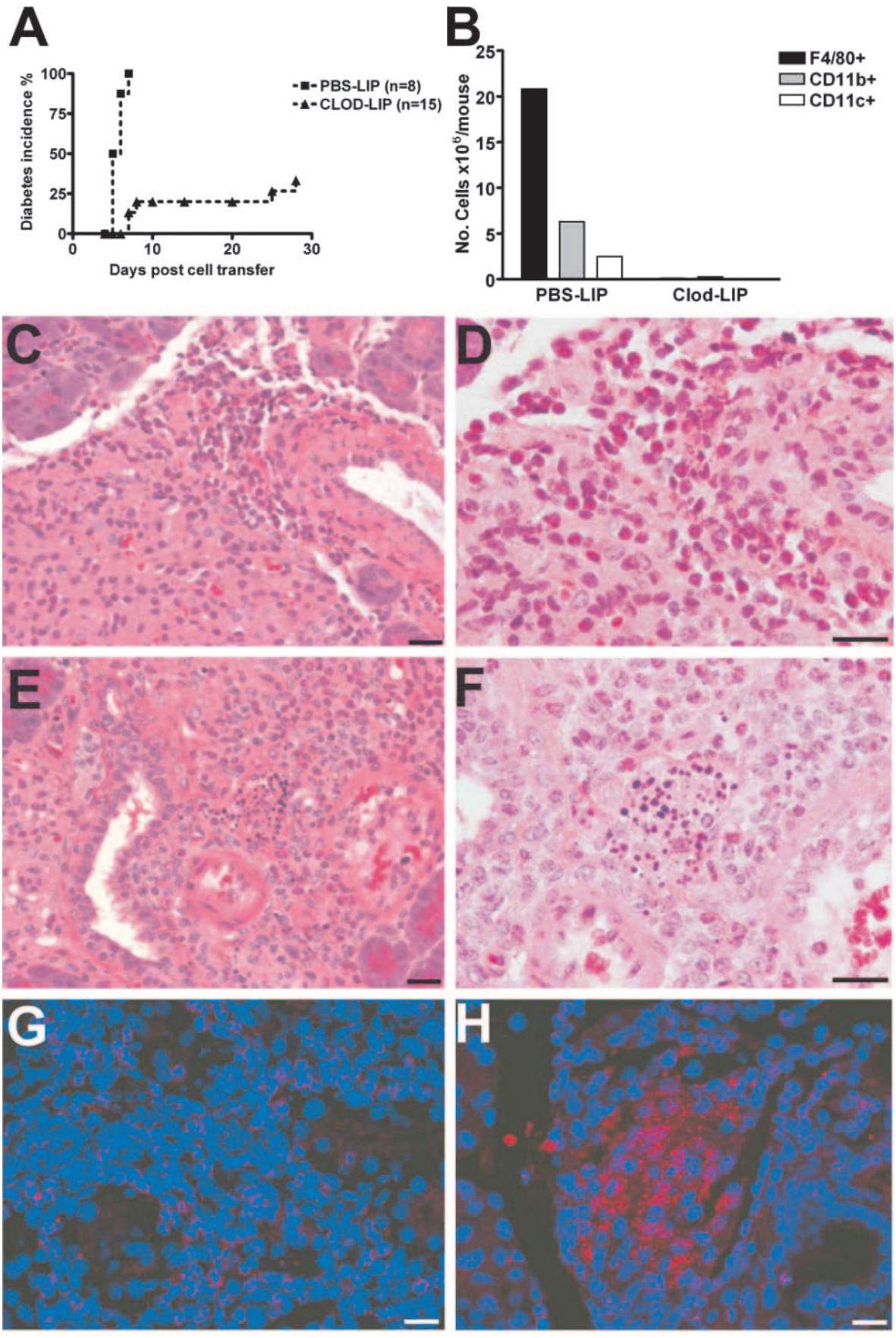


Figure 5. Cytotoxicity assay of chromium-labeled YAC-1 target cells in the presence of peritoneal exudate cells recovered at day 4 after BDC T-cell transfer. Experimental mice received either an isotype control antibody or the anti-NK cell-depleting antibody treatment. **A:** Mice that received anti-asialo GM1 antibody treatment. **B:** Mice that received anti-NK1.1 antibody treatment. Effector/target ratios of 100:1, 75:1, 50:1, and 25:1 were incubated for 4 hours. Each point represents one experimental mouse.


A
Diabetes incidence %
100
75
50
25
0
0
10
20
30
Days post cell transfer
PBS-LIP (n=8)
CLOD-LIP (n=15)
B
No. Cells x10^6/mouse
25
20
15
10
5
0
PBS-LIP
Clod-LIP
F4/80+
CD11b+
CD11c+
C
D
E
F
G
H

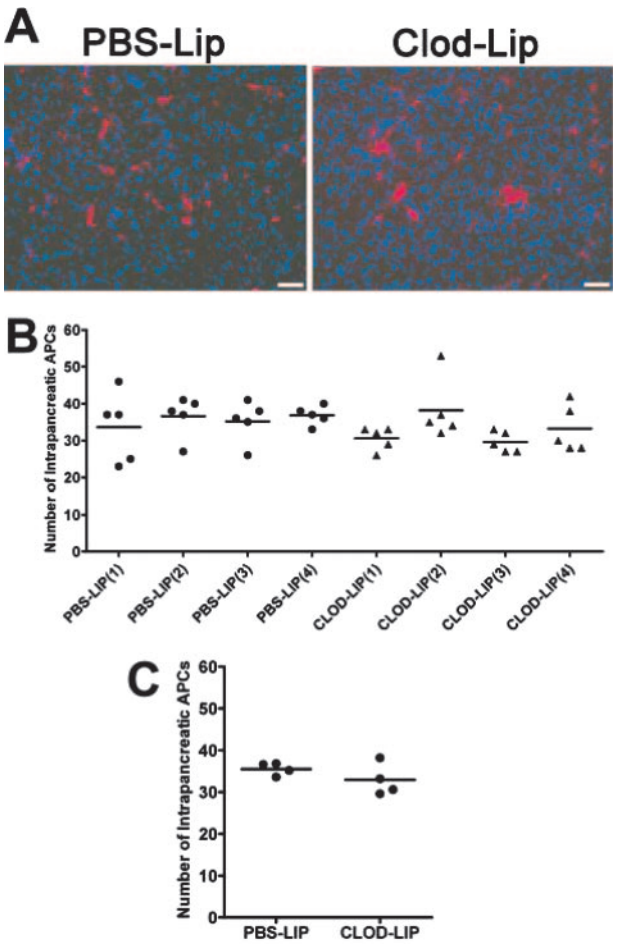


Figure 7. A: Immunofluorescence staining for F4/80$^+$, CD11b$^+$, and CD11c$^+$ leukocytes (red) in NOD.scid mice after 4 days of liposome treatment (CLOD-LIP or PBS-LIP) and analyzed at day 6. No visual differences in the numbers of APCs were observed between both groups. Nuclear stain is blue. **B:** Indicated are the data comparing the number of APCs per field between PBS-LIP- or CLOD-LIP-treated mice (n = 4 per group). Each dot represents the APCs in one field per each mouse (number in parentheses). The bar represents the mean counts. **C:** Indicated are the means of APCs per field from each mouse in each of the two groups, derived from the data of **B**. No statistical difference was observed between means of PBS-LIP and CLOD-LIP groups (Mann-Whitney U-test) (P = 0.3429). Scale bar = 40 μm.

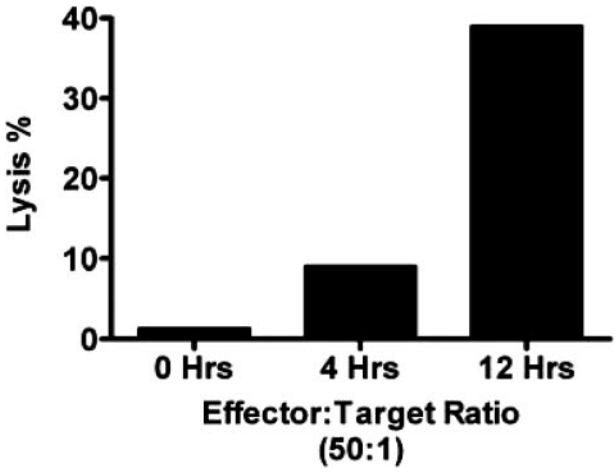


Figure 8. Cytotoxicity assay of chromium-labeled β cells in the presence of enriched phagocytes from acute diabetic pancreata. CD11b$^+$- and CD11c$^+$-enriched phagocytes were co-cultured with the chromium-labeled β cells at an effector/target ratio of 50:1 (5×10^5:1×10^4). Chromium release was measured at 0, 4, and 12 hours of incubation, and lysis percentage was calculated.

ment of NK cells. 2) Depletion of phagocytes depleted the pancreas of activated macrophages but did not affect antigen presentation of islet β-cell proteins because pancreatic-resident APCs were not affected by the CLOD-LIP treatment. 3) Histopathological analysis was compatible with an activated macrophage and β-cell interaction. 4) Macrophages isolated from islets killed β cells *in vitro*. Our data support the conclusion that macrophages are a key mediator of islet β-cell death during the later stages of disease progression.

Although past studies have noted the importance of macrophages in experimental diabetes, the precise nature of their involvement whether as direct effectors of β-cell cytotoxicity *in vivo* or as antigen-presenting cells remained unclear.[29,34,38,39] Several studies in the past suggested that inhibition of diabetes in macrophage-depleted NOD mice was a likely result of impaired priming and expansion of diabetogenic CD4$^+$ and CD8$^+$ T cells,[34–36,39] whereas other *in vitro* assays demonstrated that activated macrophages could lyse pancreatic β-tumor cells.[44–46] For example, the result that depletion of macrophages via silica particles in NOD mice or BB rats resulted in protection from diabetes was interpreted to suggest an important role for macrophages in the priming of diabetogenic T cells.[29,37,38] Another feature noted in these studies was the marked reduction in the incidence of insulitis in macrophage-depleted mice.[29,37] Other groups used clodronate-dependent elimination of macrophages in NOD mice to demonstrate protection from diabetes.[34,39] Here again, young NOD mice that were depleted of macrophages were unable to generate cytotoxic T cells, thereby implicating the macrophage as an essential accessory cell for the differentiation of effector diabetogenic T cells.[34] Moreover, as seen in the previous studies with silica administration, the clodronate-treated NOD mice also exhibited protection from insulitis,[34] which was in agreement with the lack of priming of diabetogenic T cells responsible for recruitment of the inflammatory infiltrate in the pancreas. An interesting study by Jun and colleagues[39] also followed the development

Figure 6. A: Macrophage depletion in the acute diabetes model by CLOD-LIP treatment. NOD.scid recipients were treated with CLOD-LIP or PBS-LIP on days 1, 2, 3, and 4 after transfer of 10^7 activated BDC T cells. Although all of the PBS-LIP-treated mice developed diabetes in 6 days (eight of eight), CLOD-LIP-treated mice exhibited a markedly reduced and delayed incidence of diabetes (5 of 15 by day 30). **B:** Splenocyte analysis by flow cytometry of treated mice at day 6 after cell transfer. CLOD-LIP-treated mice exhibited >99% depletion of phagocytic cells in spleen. **C** and **D:** H&E analysis showing an example of an islet with abundant neutrophils in the phagocyte-depleted mice at day 6 after T-cell transfer. **E** and **F:** Islet of a phagocyte-depleted mouse free of diabetes at day 6 showing a high number of apoptotic bodies, probably from dead neutrophils. **G:** Immunofluorescence staining for insulin (red) and nuclear staining (blue) in the pancreatic islets at day 6 after transfer of activated BDC T cells in a PBS-LIP-treated mouse. Note the lack of insulin-positive cells in the presence of leukocytic infiltrate. **H:** Nondiabetic mouse treated with CLOD-LIP. Note the presence of insulin-positive cells in the presence of leukocyte infiltration. Scale bar = 20 μm.

of diabetogenic $CD8^+$ T cells in macrophage-depleted mice. Their results clearly demonstrated that these $CD8^+$ T cells were unable to differentiate into cytotoxic T cells when the host was depleted of macrophages. Taken together, these previous reports demonstrated the important role for macrophages in many stages of diabetogenesis, including priming and differentiation of autoreactive T cells and induction of insulitis. The difference between the reported experiments and ours lies in the experimental setup: we used already activated T cells ready to induce disease, which occurred in a short period of time. Hence we bypassed the initial stages of T-cell activation to focus primarily on the final effector stages of islet β-cell killing. Thus, our studies definitively established a role of the phagocyte as an effector cell in β-cell death.

What are the molecular details of macrophage-mediated islet β-cell death? Is β-cell killing a result of direct cell-cell contact, or can soluble molecules mediate this killing? We believe that the pathway leading to islet β-cell death by macrophages is a likely consequence of cell-cell contact although we cannot discard the action of soluble mediators at this time. Our evidence presented in this report supports the action of macrophage-mediated islet β-cell death because of several reasons: electron micrographs of islet infiltrates demonstrated the presence of macrophages adjoining dying β cells, and importantly, islets from mice deficient in inducible nitric-oxide synthase were efficiently destroyed by BDC T cells.[15] Moreover, NOD mice that harbor a mutation in the inducible nitric-oxide synthase gene developed diabetes with the same kinetics and penetrance as wild-type NOD mice (personal communication with John Mudgett, Merck Inc., San Diego, CA). Further, cytotoxicity assays as shown in Figure 8 (and by others[44–46]) needed the presence of macrophages. We are currently in the process of evaluating cell-surface molecules that are expressed by activated macrophages (included among these are members of activating NK receptor family) that may interact with their cognate ligand on the surface of islet β cells. It is important to note that in our model a role for Fas/FasL in mediating β-cell death is minor because Fas-deficient islets are efficiently destroyed by activated BDC T cells.[15]

Finally, are activated macrophages also a participant in the natural disease? Certainly activated macrophages are a component of the infiltrate at the aggressive phase of the disease, that is, at the time of diabetes development.[31–33] From previous data in the wild-type NOD mice, both T-cell subsets are needed for induction of diabetes.[4–7] However, the precise role of both $CD4^+$ and $CD8^+$ T cells in inducing the death of islet β cells needs to be carefully evaluated. It is generally accepted that $CD8^+$ T-cell-mediated cytotoxicity of β cells is a result of a direct interaction involving the Fas/FasL or the perforin/granzyme pathway[8–12]; however, the observation that diabetogenic $CD8^+$ T cells in macrophage-depleted NOD mice are unable to induce diabetes suggests that the macrophage could also be operational in this situation.[39] $CD8^+$ T cells produce cytokines such as interferon-γ that activate macrophages and may also activate the phagocytic cells to differentiate into islet β-cell killers. Moreover, the nature of cooperativity that exists between $CD8^+$ and $CD4^+$ T cells in autoimmune diabetes may also involve an intermediary phagocytic cell. A previous report by Kanagawa and colleagues[47] demonstrated the need for I-A^{g7}-restricted $CD4^+$ T cells for diabetogenic $CD8^+$ T cells to cause diabetes. Deciphering the molecular details of macrophage-mediated islet β-cell death in our experimental mouse model will shed new insights into pathogenesis of T1DM and may identify new targets for modulation of autoimmune diabetes.

Acknowledgments

We thank Kathy Frederick and Patrice Bittner for their technical help with experiments involving mice; Karen Green, who was responsible for the electron microscopy examinations; the members of the Unanue Laboratory, particularly Javier A. Carrero and Matteo Levisetti for many helpful discussions; and Frances White for help with some of the ultrastructural analyses.

References

1. Tisch R, McDevitt H: Insulin-dependent diabetes mellitus. Cell 1996, 85:291–297
2. Kikutani H, Makino S: The murine autoimmune diabetes model: NOD and related strains. Adv Immunol 1992, 51:285–322
3. Nakhooda AF, Like AA, Chappel CI, Murray FT, Marliss EB: The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. Diabetes 1977, 26:100–112
4. Katz J, Benoist C, Mathis D: Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice. Eur J Immunol 1993, 23:3358–3360
5. Mora C, Wong FS, Chang CH, Flavell RA: Pancreatic infiltration but not diabetes occurs in the relative absence of MHC class II-restricted CD4 T cells: studies using NOD/CIITA-deficient mice. J Immunol 1999, 162:4576–4588
6. Matsumoto M, Yagi H, Kunimoto K, Kawaguchi J, Makino S, Harada M: Transfer of autoimmune diabetes from diabetic NOD mice to NOD athymic nude mice: the roles of T cell subsets in the pathogenesis. Cell Immunol 1993, 148:189–197
7. Yagi H, Matsumoto M, Kunimoto K, Kawaguchi J, Makino S, Harada M: Analysis of the roles of CD4+ and CD8+ T cells in autoimmune diabetes of NOD mice using transfer to NOD athymic nude mice. Eur J Immunol 1992, 22:2387–2393
8. Ogasawara K, Hamerman JA, Ehrlich LR, Bour-Jordan H, Santamaria P, Bluestone JA, Lanier LL: NKG2D blockade prevents autoimmune diabetes in NOD mice. Immunity 2004, 20:757–767
9. Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway Jr CA, Matis LA: The role of Fas in autoimmune diabetes. Cell 1997, 89:17–24
10. Kägi D, Odermatt B, Seiler P, Zinkernagel RM, Mak TW, Hengartner H: Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. J Exp Med 1997, 186:989–997
11. Thomas HE, Irawaty W, Darwiche R, Brodnicki TC, Santamaria P, Allison J, Kay TW: IL-1 receptor deficiency slows progression to diabetes in the NOD mouse. Diabetes 2004, 53:113–121
12. Suarez-Pinzon WL, Mabley JG, Strynadka K, Power RF, Szabo C, Rabinovitch A: An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in NOD mice. J Autoimmun 2001, 16:449–455
13. Katz JD, Wang B, Haskins K, Benoist C, Mathis D: Following a diabetogenic T cell from genesis through pathogenesis. Cell 1993, 74:1089–1100
14. Suri A, Katz JD: Dissecting the role of CD4+ T cells in autoimmune diabetes through the use of TCR transgenic mice. Immunol Rev 1999, 169:55–65

15. Pakala SV, Chivetta M, Kelly CB, Katz JD: In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. J Exp Med 1999, 189:1053–1062
16. Suri A, Shimizu J, Katz JD, Sakaguchi S, Unanue ER, Kanagawa O: Regulation of autoimmune diabetes by non-islet-specific T cells—a role for the glucocorticoid-induced TNF receptor. Eur J Immunol 2004, 34:447–454
17. Lacy PE: Electron microscopy of the normal islets of Langerhans; studies in the dog, rabbit, guinea pig and rat. Diabetes 1957, 6:498–507
18. Lernmark A: The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. Diabetologia 1974, 10:431–438
19. Rogers HW, Unanue ER: Neutrophils are involved in acute, nonspecific resistance to Listeria monocytogenes in mice. Infect Immun 1993, 61:5090–5096
20. Wipke BT, Allen PM: Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol 2001, 167:1601–1608
21. Habu S, Fukui H, Shimamura K, Kasai M, Nagai Y, Okumura K, Tamaoki N: In vivo effects of anti-asialo GM1. I. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. J Immunol 1981, 127:34–38
22. Kasai M, Yoneda T, Habu S, Maruyama Y, Okumura K, Tokunaga T: In vivo effect of anti-asialo GM1 antibody on natural killer activity. Nature 1981, 291:334–335
23. Mule JJ, Krosnick JA, Rosenberg SA: IL-4 regulation of murine lymphokine-activated killer activity in vitro. Effects on the IL-2-induced expansion, cytotoxicity, and phenotype of lymphokine-activated killer effectors. J Immunol 1989, 142:726–733
24. Ballas ZK, Rasmussen W: Lymphokine-activated killer (LAK) cells. IV. Characterization of murine LAK effector subpopulations. J Immunol 1990, 144:386–395
25. van Rooijen N, van Kesteren-Hendrikx E: "In vivo" depletion of macrophages by liposome-mediated "suicide." Methods Enzymol 2003, 373:3–16
26. Van Rooijen N, Sanders A: Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994, 174:83–93
27. Lacy PE, Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967, 16:35–39
28. Coligan JE, Bierer BE, Margulies DH, Shevach EM, Strober W: Current Protocols in Immunology. Edited by R Coico. Hoboken, John Wiley & Sons, Inc., 1998, pp 3.11.14–13.11.17
29. Lee KU, Amano K, Yoon JW: Evidence for initial involvement of macrophage in development of insulitis in NOD mice. Diabetes 1988, 37:989–991
30. Reddy S, Liu W, Elliott RB: Distribution of pancreatic macrophages preceding and during early insulitis in young NOD mice. Pancreas 1993, 8:602–608
31. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA: Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta-cell destruction in NOD mice. Diabetes 1994, 43:667–675
32. Reddy S, Wu D, Swinney C, Elliott RB: Immunohistochemical analyses of pancreatic macrophages and CD4 and CD8 T cell subsets prior to and following diabetes in the NOD mouse. Pancreas 1995, 11:16–25
33. Rosmalen JG, Martin T, Dobbs C, Voerman JS, Drexhage HA, Haskins K, Leenen PJ: Subsets of macrophages and dendritic cells in nonobese diabetic mouse pancreatic inflammatory infiltrates: correlation with the development of diabetes. Lab Invest 2000, 80:23–30
34. Jun HS, Yoon CS, Zbytnuik L, van Rooijen N, Yoon JW: The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Exp Med 1999, 189:347–358
35. Chung YH, Jun HS, Kang Y, Hirasawa K, Lee BR, Van Rooijen N, Yoon JW: Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes in diabetes-resistant BioBreeding rats. J Immunol 1997, 159:466–471
36. Lee KU, Pak CY, Amano K, Yoon JW: Prevention of lymphocytic thyroiditis and insulitis in diabetes-prone BB rats by the depletion of macrophages. Diabetologia 1988, 31:400–402
37. Oschilewski U, Kiesel U, Kolb H: Administration of silica prevents diabetes in BB-rats. Diabetes 1985, 34:197–199
38. Charlton B, Bacelj A, Mandel TE: Administration of silica particles or anti-Lyt2 antibody prevents beta-cell destruction in NOD mice given cyclophosphamide. Diabetes 1988, 37:930–935
39. Jun HS, Santamaria P, Lim HW, Zhang ML, Yoon JW: Absolute requirement of macrophages for the development and activation of beta-cell cytotoxic CD8+ T-cells in T-cell receptor transgenic NOD mice. Diabetes 1999, 48:34–42
40. Alves-Rosa F, Stanganelli C, Cabrera J, van Rooijen N, Palermo MS, Isturiz MA: Treatment with liposome-encapsulated clodronate as a new strategic approach in the management of immune thrombocytopenic purpura in a mouse model. Blood 2000, 96:2834–2840
41. van Lent PL, Holthuysen AE, van Rooijen N, van de Loo FA, van de Putte LB, van den Berg WB: Phagocytic synovial lining cells regulate acute and chronic joint inflammation after antigenic exacerbation of smouldering experimental murine arthritis. J Rheumatol 1998, 25:1135–1145
42. Fox A, Koulmanda M, Mandel TE, van Rooijen N, Harrison LC: Evidence that macrophages are required for T-cell infiltration and rejection of fetal pig pancreas xenografts in nonobese diabetic mice. Transplantation 1998, 66:1407–1416
43. Huitinga I, van Rooijen N, de Groot CJ, Uitdehaag BM, Dijkstra CD: Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J Exp Med 1990, 172:1025–1033
44. Schwizer RW, Leiter EH, Evans R: Macrophage-mediated cytotoxicity against cultured pancreatic islet cells. Transplantation 1984, 37:539–544
45. Appels B, Burkart V, Kantwerk-Funke G, Funda J, Kolb-Bachofen V, Kolb H: Spontaneous cytotoxicity of macrophages against pancreatic islet cells. J Immunol 1989, 142:3803–3808
46. Kolb H, Burkart V, Appels B, Hanenberg H, Kantwerk-Funke G, Kiesel U, Funda J, Schraermeyer U, Kolb-Bachofen V: Essential contribution of macrophages to islet cell destruction in vivo and in vitro. J Autoimmun 1990, 3(Suppl 1):S117–S120
47. Kanagawa O, Shimizu J, Vaupel BA: Thymic and postthymic regulation of diabetogenic CD8 T cell development in TCR transgenic nonobese diabetic (NOD) mice. J Immunol 2000, 164:5466–5473