Published in final edited form as:

Ann Surg. 2012 October ; 256(4): . doi:10.1097/SLA.0b013e31826b4ba9.

Naturally Occurring Immunoglobulin M (nIgM) Autoantibodies Prevent Autoimmune Diabetes and Mitigate Inflammation After Transplantation

Preeti Chhabra, PhD^{*}, Kailo Schlegel, MS[†], Mark D. Okusa, MD, PhD[†], Peter I. Lobo, MD, PhD[†], and Kenneth L. Brayman, MD, PhD, FACS^{*}

^{*}Department of Surgery, Division of Transplantation, University of Virginia School of Medicine, Charlottesville, VA

[†]Department of Medicine, Division of Nephrology, University of Virginia School of Medicine, Charlottesville, VA

Abstract

Objective—To investigate whether polyclonal serum naturally occurring immunoglobulin M (nIgM) therapy prevents the onset and progression of autoimmune diabetes and promotes islet allograft survival.

Background—nIgM deficiency is associated with an increased tendency toward autoimmune disease development. Elevated levels of nIgM anti-leukocyte autoantibodies are associated with fewer graft rejections.

Methods—Four- to five-week-old female nonobese diabetic (NOD) littermates received intraperitoneal nIgM or phosphate-buffered saline/bovine serum albumin/immunoglobulin G (100 μ g followed by 50–75 μ g biweekly) until 18 weeks of age. C57BL/6 recipients of 300 BALB/c or 50 C57BL/6 islet grafts received saline or nIgM.

Results—Eighty percent control mice ($n = 30$) receiving saline became diabetic by 18 to 20 weeks of age. In contrast, none of 33 of nIgM-treated mice became diabetic ($P < 0.0001$). Discontinuing therapy resulted in hyperglycemia in only 9 of 33 mice at 22 weeks postdiscontinuation, indicating development of β -cell unresponsiveness. nIgM therapy initiated at 11 weeks of age resulted in hyperglycemia in only 20% of treated animals ($n = 20$) compared with 80% of controls ($P < 0.0001$). Treatment of mildly diabetic mice with nIgM (75 μ g $3\times$ per week) restored normoglycemia ($n = 5$), whereas severely diabetic mice required minimal dose islet transplant with nIgM to restore normoglycemia ($n = 4$). The mean survival time of BALB/c islet allografts transplanted in streptozotocin-induced diabetic C57BL/6 mice was 41.2 ± 3.3 days for nIgM-treated recipients ($n = 4$, fifth recipient remains normoglycemic) versus 10.2 ± 2.6 days for controls ($n = 5$) ($P < 0.001$). Also, after syngeneic transplantation, time taken to return to normoglycemia was 15.4 ± 3.6 days for nIgM-treated recipients ($n = 5$) and more than 35 days for controls ($n = 4$).

Conclusions—nIgM therapy demonstrates potential in preventing the onset and progression of autoimmune diabetes and in promoting islet graft survival.

Copyright © 2012 by Lippincott Williams & Wilkins

Reprints: Kenneth L. Brayman, MD, PhD, FACS, Department of Surgery, Division of Transplantation, University of Virginia School of Medicine, PO Box 800709, Charlottesville, VA 22908. klb9r@hscmail.mcc.virginia.edu.

Disclosure: The research was funded by the Focus to Cure Diabetes Foundation, 200 Garrett Street, Suite O, Charlottesville, VA 22902. The funding organization had no role in the design or conduct of this research. The authors declare no conflicts of interest. Patents are pending on naturally occurring IgM anti-leukocyte autoantibodies at the University of Virginia.

Keywords

autoimmune type 1 diabetes; development of β -cell-specific unresponsiveness; islet allograft survival; naturally occurring IgM autoantibodies; prevention of onset and progression of diabetes

Autoimmune type 1 diabetes mellitus (T1D) is a multifactorial disease caused by the progressive destruction of insulin-producing pancreatic β -cells that results in an absolute loss of insulin production.^{1,2} Prevention or reversal of T1D either by preventing or arresting autoimmunity or through restoration of β -cell mass and function is the ultimate goal of clinical intervention. Diagnosis of the disease in the later stages of prehyperglycemic β -cell destruction restricts the types of immunotherapies that can be implemented. To date, pancreas and islet transplantation remain the only reliable cures of diabetes that have met with consistent success.^{3,4} However, the requisite chronic immunosuppressive therapy with its deleterious diabetogenic and nephrotoxic side effects, such as the risk associated with global deletion of T cells and the development of infections and malignancies, remains the Achilles' heel of therapeutic interventions aimed at improving islet graft survival and preventing autoimmune diabetes.^{5,6} Another major drawback of chronic immunosuppressive therapy is its ineffectiveness over the long term that results in recurrence of disease upon drug withdrawal. Therefore, the development of novel therapeutic interventions that can prevent autoimmune- and alloimmune-mediated β -cell destruction by establishing effective chronic immunological unresponsiveness in the absence of chronic immunosuppression, with minimal or no deleterious side effects, is the principal focus of ongoing multidisciplinary research. Current efforts to this end have not met with much clinical success.

Repertoires of naturally occurring autoantibodies have been intensively studied during the last 3 decades and thought to play a significant role in the immunology of health and disease. These autoantibodies are predominantly of the IgM isotype and can be found in umbilical cord blood along with the long-lived, self-renewing B-1 lymphocytes that produce them, before exposure to foreign antigens; hence, referred to as naturally occurring or natural IgM (nIgM).^{7,8} nIgMs and a subset known as nIgM anti-leukocyte autoantibodies (nIgM-ALAs) are present at low levels in normal individuals and increase during inflammatory disorders and various infections.⁹ Previous studies in our laboratory and others have demonstrated that polyclonal serum nIgM and nIgM-ALA are a heterogeneous group of antibodies that are reactive to autologous and allogeneic cells with specificities for different, largely undefined membrane receptors that include receptors with phospholipids, glycolipids, and glycoproteins, and other cells that express leukocyte receptors.⁷⁻¹⁰ Encoded by minimally or nonmutated germ line genes, nIgMs are characteristically polyreactive with low-binding affinity. Of particular importance, these autoantibodies do not mediate cytotoxicity in the presence of complement at body temperature and differ from disease-producing autoantibodies in that the latter are predominantly of the IgG isotype that bind with high affinity and specificity to autoantigens and mediate cytotoxicity at 37°C.

Elevated levels of nIgM-ALAs have been associated with lower incidence and severity of acute human heart and kidney graft rejections, thus permitting better graft survival.¹⁰⁻¹² We have previously shown that nIgM-ALA plays a regulatory role in attenuating inflammation mediated by innate and adaptive immune mechanisms, including where the inflammatory response involved Th-17 cells that were not effectively regulated by regulatory T cells.¹⁰ In addition, purified human serum nIgM immunoprecipitated human CD3 and CD4 T cells and chemokine receptors CCR5 and CXCR4 from whole-cell lysates, downregulated CD2, CD86, and CD4, inhibited leukocyte production of proinflammatory cytokines tumor necrosis factor α (TNF- α), interleukin (IL)-13, and IL-2, and inhibited T-cell activation and

proliferation as well as leukocyte chemotaxis induced by chemokines, indicating an innate mechanism that acts to downregulate T-cell-mediated inflammatory responses.⁷⁻¹⁰ Polyclonal nIgMs have also been shown to contribute to the maintenance of immune homeostasis and prevent autoimmunity.^{13,14} These findings prompted us to (a) investigate whether purified, polyclonal mouse serum nIgM autoantibody therapy could have a functional role in inhibiting the onset and progression of T1D when administered in a nonobese diabetic (NOD) mouse model of autoimmune T1D, (b) determine whether intervention at the prehyperglycemic late stage of disease development or after the establishment of overt diabetes might be feasible, and (c) investigate whether administration of nIgM could prolong graft survival after syngeneic and allogeneic islet transplantation by mitigating inflammation.

METHODS

nIgM Purification From Mouse Sera

nIgM was purified by size-exclusion column chromatography (Sephacryl S-300 HR; GE Healthcare, Piscataway, NJ) from irradiated, heat-inactivated (56°C × 1 hour) WT C57BL/6 murine sera (Innovative Research, Novi, MI), using previously described procedures⁷ and with modifications detailed later. nIgM was not isolated by dialyzing sera in water or by ammonium chloride precipitation because these techniques yield IgM with impaired functional activity. Column-purified nIgM was repassaged through Sephacryl S-300 to remove contaminating IgG and other proteins. With this approach, more than 92% of the protein fraction contained nIgM with less than 1% IgG, less than 3% albumin, and less than 1% other protein contaminants as determined by protein electrophoresis and ELISA. We did not affinity purify nIgM antibodies, as such procedures (binding of nIgM to mannan-binding protein or binding of nIgM to agarose coupled with goat anti-IgM antibodies) yield 10% to 15% of the starting IgM and have the potential to deplete certain IgM subsets. Purified nIgM was concentrated to 1.3 to 1.5 mg/mL (higher concentration led to nIgM aggregation and precipitation), dialyzed against RPMI 1640, and micro filtered using a 0.45- μ m Millipore filter, before use in cultures and for in vivo use. Purified IgM were stored at 4°C to prevent precipitation that occurs when frozen.

Mice

Female NOD mice littermates and NOD scid (severe combined immunodeficiency) mice were purchased from Jackson laboratories (Bar Harbor, ME), whereas C57BL/6 and BALB/c mice used in islet transplantation and in vitro experiments were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed in institutional vivaria. All procedures were approved by the Animal Care and Use Committee of University of Virginia and performed in accordance with National Institutes of Health and Institutional Animal Care and Use Guidelines.

nIgM Treatment Regimen in NOD Mice

NOD mice were divided into 5 groups: 2 test groups consisting of female NOD littermates that received nIgM therapy beginning at 4 to 5 weeks of age (test group 1, n = 33) or at 10 to 11 weeks of age (test group 2, n = 20) and 3 control groups that received sterile phosphate-buffered saline (PBS) (n = 30), bovine serum albumin (BSA) (n = 10), and mouse IgG (n = 10) at the same time and, in the case of BSA or IgG, in concentrations similar to nIgM administration. Briefly, mice received an initial intraperitoneal injection of 100 μ g of purified nIgM, followed by 50 to 60 μ g in 150 μ L of PBS biweekly until mice were 18 weeks old. These experiments were performed using batches of 10 littermates in each group and repeated for statistical significance. NOD mice were deemed diabetic when tail vein blood glucose (BG) level was 250 mg/dL or more (Accu-Chek Aviva glucose monitor) on 3

successive days. nIgM therapy was withdrawn after mice reached 18 weeks of age and were monitored for the appearance of T1D. In another experiment, moderately diabetic mice (BG 250–340 mg/dL) received initial intraperitoneal injection of 100 μ g of purified nIgM, followed by 75 μ g thrice weekly, whereas severely diabetic mice (BG >350 mg/dL) received this dose along with minimal dose islet transplant (75 islets).

Islet Isolation and Transplantation

Ten- to sixteen-week-old female BALB/c mice served as donors in islet allotransplantation experiments, whereas retired female C57BL/6 mice were used as donors in syngeneic islet transplantation experiments. In some experiments, diabetic NOD mice recipients were transplanted with marginal dose NOD scid islets (75 islets). Briefly, islets were isolated by collagenase P digestion (Roche Molecular Biochemicals, Indianapolis, IN) and purified by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St Louis, MO) according to a previously described procedure.¹⁵ After overnight culture (37°C and 5% CO₂), 300 BALB/c or 50 C57BL/6 islets were transplanted under the kidney capsule of 10- to 12-week-old female C57BL/6 recipients that were rendered diabetic by a single intraperitoneal injection of streptozotocin (250 mg/kg; Sigma) as described elsewhere.¹⁵ Postoperatively, mice were administered analgesia and maintained at 35 to 37°C before returning to the vivarium. Test group islet recipients received 100 μ g of nIgM predialyzed in RPMI 1640 (BioWhittaker, Walkersville, MD) intraperitoneally, 24 hours before transplantation and 75 μ g thrice weekly every 48 hours; whereas control group islet recipients received PBS/BSA. A third group did not receive islet transplant but received nIgM therapy alone. Tail vein BG 300 mg/dL or more on 3 successive days was indicative of graft rejection. In syngeneic islet transplantation, cure was defined as a return to normoglycemic state.

Adoptive Transfer of T1D

Five- to six-week-old immunodeficient NOD scid mice served as adoptive transfer recipients and were divided into 2 groups: nIgM therapy–receiving test group and BSA-injected control group. Recipients received 75 μ g of nIgM or BSA biweekly. A total of 2×10^7 splenocytes from diabetic NOD mice in 200 μ L of sterile PBS were injected intraperitoneally into both groups, and mice were monitored for hyperglycemia. Alternatively, CD3 T-cell subsets were prepared by negative selection with magnetic beads, using the MagCelect Mouse CD3⁺ T-Cell isolation kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Approximately 97.5% purity was confirmed by flow cytometric analysis. A total of 7.5×10^6 CD3 T cells were transferred intraperitoneally into NOD scid recipients. In addition, a third group received either splenocytes or CD3 T cells from 2- to 3-week-old prediabetic NOD mice, followed with BSA administration.

Histology

Pancreases from NOD mice were excised, fixed (10% neutral buffered formalin), and prepared for routine paraffin histology. Five-micrometer sections obtained at multiple levels were stained by hematoxylin and eosin (H&E) and observed for peri-insulitis, insulitis, and perivascular and periductular inflammation and β -cell regeneration.

Anti-CD3 and Lipopolysaccharide Activation of Splenocytes

Single-cell splenocyte suspensions were obtained from prediabetic 2- to 5-week-old NOD mice (n = 4). Erythrocytes were removed using RBC lysing buffer (eBioscience, San Diego, CA). The cells were washed thrice with RPMI 1640–supplemented 2% fetal bovine serum (Fisher Scientific, Pittsburgh, PA). Cell concentration was adjusted to 1×10^6 cells/0.5 mL in RPMI 1640 containing 4.5 g/L of D-glucose, 2.383 g/L of HEPES, L-glutamine, 110 mg/

L of sodium pyruvate, and 1.5 g/L of sodium bicarbonate and supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and β -mercaptoethanol (1:1000) (Invitrogen, New York, NY). The cells were initially activated with anti-mouse CD3e antibody (1 μ g/mL; eBioscience 145-2C11) and lipopolysaccharide (LPS) (0.35 μ g/mL; Sigma-Aldrich) and cultured for 4 days at 37°C in 5% CO₂ in triplicate with or without IgM (~3–10 μ g). TNF- α , IFN- γ , and IL-17A in culture supernatants were quantitated using ELISA kits.

Statistical Analyses

Percent diabetes incidence data were analyzed using Kaplan-Meier statistics, with significance determined by the log-rank test was used to analyze the diabetes incidence data. Differences were considered statistically significant at $P < 0.001$.

RESULTS

nIgM Therapy Inhibits Onset and Progression of Autoimmune Diabetes When Initiated in the Early-and Late-Onset Stage of Prehyperglycemic β -Cell Destruction

In our studies, 80% of the control mice ($n = 30$) receiving saline became diabetic by 18 to 20 weeks of age and 90% by 25 weeks (Fig. 1A). BSA administration resulted in 70% of diabetes ($n = 10$), whereas IgG administration resulted in 50% of diabetes ($n = 10$) at 25 weeks of age (Fig. 1B). In contrast, none of nIgM-treated mice (0/30) that received therapy beginning at 4 to 5 weeks of age became diabetic ($P < 0.0001$). Comparison of percent diabetes incidence curves showed statistically significant difference between nIgM-treated group compared with PBS, BSA, and IgG administration ($P < 0.0001$) (Figs. 1A, B). These results indicate that nIgM therapy initiated in the early-onset, prehyperglycemic phase of diabetes is capable of completely preventing the onset of autoimmune T1D development.

nIgM therapy initiated at 11 weeks of age in the late-onset, prehyperglycemic phase of disease development resulted in hyperglycemia in only 20% of nIgM-treated animals ($n = 20$) at 25 weeks compared with 90% of controls ($P < 0.0001$) (Fig. 1A). This is a clinically significant result because in the majority of studies, treatment begun after 9 weeks of age has proven ineffectual in protecting from β -cell destruction.^{16,17} Thus, our results indicate that increasing serum levels of nIgM prevents the onset and progression of autoimmune diabetes.

nIgM Therapy May Result in Permanent Protection From Autoimmune β -Cell Destruction

In mice that received nIgM therapy beginning at 4 to 5 weeks of age, discontinuation of therapy at 18 weeks of age resulted in hyperglycemia in only 9 of 33 mice (~27%) at 22 to 28 weeks post-discontinuation, indicating that nIgM therapy acts in an immunomodulatory capacity that results in possible permanent protection from β -cell destruction in the remaining 73% mice.

nIgM Therapy Inhibits Insulitis and Promotes β -Cell Neogenesis

Examination of H&E-stained pancreas sections indicated the presence of severe insulitis as well as severe periductular and perivascular inflammation in the control diabetic mice group (Figs. 2B, C). In contrast, in pancreas sections obtained from the nIgM-treated, non-diabetic mice group at 18 to 25 weeks of age, normal appearance or mild insulitis/peri-insulitis was observed in the nIgM-treated islets, accompanied by some periductular and perivascular inflammation (Fig. 2D). In addition, numerous big islets near ducts, a process classically observed in β -cell neogenesis, were also observed (Fig. 2E). These results indicate that nIgM therapy promotes β -cell regeneration while inhibits β -cell destruction.

nIgM Therapy With Islet Transplantation Restores Normoglycemia in Overtly Diabetic NOD Mice

In our studies, injecting control diabetic NOD mice that were mildly diabetic (BG = 250–340 mg/dL) with 75 μ g of nIgM thrice weekly restored normoglycemia ($n = 5$). This dose did not restore normoglycemia in severely diabetic mice (BG >350 mg/dL) ($n = 4$). However, combining nIgM therapy with minimal dose islet transplant (75 islets) could permanently restore normoglycemia ($n = 4$) in severely diabetic mice. This result is clinically significant because it demonstrates the therapeutic effect of elevated serum nIgM levels even after the initial appearance of mild hyperglycemia and indicates its clinical potential in islet transplantation.

nIgM Therapy Promotes Syngeneic and Allogeneic Graft Survival

In the syngeneic islet transplantation studies using minimal dose islet transplant (50 islets), diabetic mice receiving 75 μ g of nIgM thrice weekly returned to normoglycemia within 15.4 ± 3.6 days ($n = 5$). Of 4 diabetic recipient mice that received syngeneic islet transplants with PBS/BSA administration instead of nIgM therapy, 2 mice returned to normoglycemia in 37 ± 2.8 days whereas 2 did not achieve normoglycemia and remained hyperglycemic (Fig. 3, $P < 0.001$).

Similarly, in islet allotransplantation studies, of 5 diabetic C57BL/6 recipient mice that received 300 BALB/c islet transplants, the mean survival time was 41.2 ± 3.3 days for 4 of the nIgM-treated recipients whereas the fifth recipient remained permanently normoglycemic. In the control group that received PBS/BSA instead of nIgM therapy, the mean survival time of BALB/c islet allografts was 10.2 ± 2.6 days for controls ($n = 5$) (Fig. 4, $P < 0.001$). These results demonstrate that nIgM therapy promotes longitudinal islet allograft survival and may prove to be an important adjunctive therapy to advance successful islet engraftment and transplant outcomes.

nIgM Therapy Delays the Incidence of Diabetes (Appearance of Hyperglycemia) in Adoptive Transfer Model

T1D can be adoptively transferred to immunodeficient NOD scid mice by splenocytes or CD3 T cells from diabetic NOD mice.¹⁸ In our studies involving intraperitoneal adoptive transfer of splenocytes from diabetic NOD mice to 5- to 6-week-old NOD scid recipient mice, the appearance of diabetes (hyperglycemia) was significantly delayed in recipient mice receiving nIgM therapy (49.4 ± 14.7 days; $n = 5$) when compared with the control group receiving PBS/BSA (30.4 ± 6.5 days; $n = 5$) ($P < 0.001$) (Fig. 5A). A third group of recipient NOD scid mice that received splenocytes from 2- to 3-week-old nondiabetic NOD mice ($n = 5$) with PBS/BSA administration did not become diabetic (Fig. 5A).

Similar beneficial results were observed with nIgM therapy when CD3 T cells from diabetic NOD mice were transferred intraperitoneally to 5- to 6-week-old NOD scid recipients. The appearance of diabetes was significantly delayed in NOD scid mice receiving nIgM therapy (53.0 ± 19.1 days; $n = 7$) when compared with the control group (24.0 ± 1.0 days; $n = 7$) that received PBS/BSA ($P < 0.001$) (Fig. 5B). Recipient NOD scid mice that received CD3 T cells from 2 to 3 weeks old nondiabetic NOD mice ($n = 3$) with PBS/BSA administration did not become diabetic (Fig. 5B). These results indicate that elevation of serum nIgM levels delays the incidence of diabetes through immunomodulation of T cells, suggesting an immunoregulatory role in the possible development of tolerance.

nIgM Inhibits Proinflammatory Cytokine Production From Activated Splenocytes From Prediabetic NOD Mice In Vitro

T1D is thought to be a Th-1–dominant disease with type 1 cytokines such as IFN- γ , TNF- α , IL-2, IL-12, and IL-18 actively involved in β -cell destruction.⁴ Of interest is the recent discovery that Th-17 cells are potent inducers of tissue inflammation and autoimmunity and may have a role in T1D.^{19–21} Therefore, we decided to study the effect of nIgM on in vitro production of proinflammatory cytokine from activated 5×10^6 cells freshly isolated splenocytes obtained from 3- to 5-week-old NOD mice ($n = 4$) and activated with LPS, and soluble anti-CD3 were cultured for 4 days at 37°C, 5% CO₂, without or with purified nIgM. Controls received BSA instead of nIgM. TNF- α , IFN- γ , and IL-17A in supernatants were quantitated using ELISA kits. We found that nIgM significantly inhibited TNF- α secretion (activated splenocytes cultured with BSA vs with nIgM; 4443.1 ± 210.5 vs 101.1 ± 52.5 pg/mL; $P < 0.001$); IFN- γ (3244.2 ± 160.6 vs 0 pg/mL; $P < 0.0001$) and IL-17A secretion (732.2 ± 66.4 vs 5.3 ± 9.2 pg/mL, $P < 0.0001$) from LPS- and anti-CD3–activated splenocytes in vitro when compared with controls (Fig. 6). These results indicate that nIgM acts by inhibiting the nonspecific, proinflammatory immune response, including where the inflammatory response involves Th-17 cells.

nIgM Therapy Does Not Affect Islet Function

Incubation of mouse or human islets in vitro with 2 and 8 μ g of nIgM did not affect insulin secretion in response to glucose challenge compared with controls in glucose-stimulated insulin secretion assay. The stimulation index, for 2 and 8 μ g of nIgM versus BSA is 1.9 and 2.0 versus 1.9 in mice and 2.2 and 1.9 versus 1.8 in humans. These results indicate that islets remain viable and functional in the presence of nIgM.

DISCUSSION

Immunomodulatory strategies to prevent or arrest autoimmune T1D are aimed at reversing immune autoreactivity and restoring β -cell mass.²² The former has mainly focused on targeting T cells and includes the use of, among others, monoclonal antibodies specific to membrane-expressed receptors such as CD3, CD4, CD40L, and B7—2,^{16,17,23,24} antibodies that interfere with antigen recognition (anti-class II, anti-TCR^{25,26}), cellular activation (anti-B7),¹⁷ and homing to the pancreas (anti-L-selectin and anti-VLA-4),²⁷ and antibodies that target proinflammatory cytokines related to Th-1 activity (anti-IFN- γ , anti-TNF- α , and anti-IL-12).²⁸ These strategies have met with limited success, providing transient protection and effective mostly when initiated in the early-onset stage of disease development. Clinical islet transplantation represents a definitive intervention in patients diagnosed with T1D.^{2–4} However, the requisite long-term use of chemical immunosuppressants, corticosteroids, and anti-inflammatory agents that are associated with deleterious side effects linked to drug toxicity and increased risk of infections and tumors, as well as the transient nature of protection, are major hindrances for its widespread applicability. Our study using polyclonal serum nIgM was aimed at harnessing the power of innate immunity by using a benign, naturally occurring immunoregulatory mechanism to reprogram the immune system to induce the development of β -cell protection and mitigate inflammation in the absence of chronic immunosuppression.

The NOD mouse has been used extensively to study the many features of the disease shared with human T1D.²⁹ The disease begins 4 to 5 weeks after birth, and the initial phase is characterized by a silent and nondestructive infiltration of the perivascular and periductular regions in the pancreas as well as the peripheral islet regions by a heterogeneous mixture of CD4 and CD8 T cells, B cells, macrophages, and dendritic cells (peri-insulinitis).^{4,30} In the invasive phase that begins at 8 to 12 weeks of age, the immune infiltrate enters the islet

(insulinitis) inducing apoptotic β -cell destruction. Significant destruction first becomes evident around 12 to 13 weeks of age, with mice exhibiting overt diabetes.^{30,31}

We have demonstrated that nIgM therapy intervention initiated in the early-onset stage of disease development prevented disease. On the basis of our previously published results,^{7–10} we postulate that this abrogation of autoimmunity might be attributable to nIgM-ALA–induced interruption of costimulatory CD28/CD86 signaling, inhibition of proinflammatory cytokine production and chemokine binding, as well as inhibition of autoreactive T-cell activation/proliferation. This is supported by our present data wherein nIgM (*a*) significantly delayed the progression of diabetes development in NOD scid recipients of adoptively transferred CD3 T cells obtained from overtly diabetic NOD mice, indicating that nIgM may act by binding to membrane receptors on autoreactive T cells and (*b*) inhibited the production of proinflammatory cytokines from NOD splenocytes *in vitro*. It is conceivable that nIgM-mediated inhibition of autoimmune effector cells allows a slow but continuous increase in β -cell mass by means of β -cell neogenesis in the absence of β -cell apoptosis, a view supported by our histological studies that demonstrated a lack of insulinitis, some peri-insulinitis, and numerous big islets near ducts, a process classically observed in β -cell neogenesis, in pancreatic sections obtained from nIgM-treated nondiabetic mice. We have also demonstrated that approximately 73% of mice remained normoglycemic at 22 to 28 weeks postdiscontinuation of nIgM therapy, indicating nIgM mediated immunomodulation resulting in the induction of β -cell–directed immune ignorance, a significant result with attractive therapeutic potential. Our preliminary flow cytometric data (unpublished) indicated higher CD4⁺CD25⁺Foxp3⁺ expression levels in splenocytes obtained from nIgM-treated nondiabetic NOD mice than controls. This observation gains significance when compared with other studies where new-onset T1D or cyclophosphamide-induced T1D was associated with a reduction in CD4⁺CD25⁺Foxp3⁺ regulatory T cells^{32,33} and where diabetes could be prevented by the transfer of CD4⁺CD25⁺ regulatory T cells.³³ Detailed flow cytometric analyses of immune cells in nIgM-treated mice that have remained long-term normoglycemic after discontinuation of therapy as well as *in vivo* experiments involving adoptive transfer of tolerance from these mice into NOD scid mice are underway.

Interestingly, most existing immunosuppressive strategies are unable to prevent diabetic disease progression when administered at more than 10 weeks of age or are unable to reverse disease after establishment of hyperglycemia.^{16,17} Limited data are available on treatments administered in the late-onset, prehyperglycemic phase of disease development (ie, replicating the clinical scenario where euglycemic patients demonstrate elevated T1D-related autoantibody levels) aimed at suppressing progressive β -cell destruction. Our studies found a significant reduction in the incidence of diabetes (20%) when nIgM therapy was initiated at 10 to 11 weeks of age, a point in time in the later stages of β -cell destruction. It is possible that in the 20% of mice that did become diabetic, nIgM administration at the low dosage used (50 μ g of nIgM biweekly) may not have succeeded in completely inhibiting autoreactive T cells in the presence of advanced insulinitis. This perspective is supported by the fact that treatment with a higher dose (75 μ g of nIgM administered thrice weekly) could completely restore normoglycemia in mildly diabetic NOD mice with BG in the range of 250 to 340 mg/dL. nIgM therapy might also induce a new population of regulatory T cells that can lead to a reversal of overt diabetes. Because autoimmune T1D is a disease that is associated with a loss of T-cell tolerance leading to the activation, differentiation, and pathogenicity of autoreactive T cells and that nIgM therapy inhibits disease when applied at the later stages of disease development and after establishment of disease (albeit only in the mildly diabetic NOD mice when administered alone), the absence of chronic immunosuppression is significant.

Optimal T-cell response initiation requires costimulatory molecule signaling, in the absence of which T cells perceiving antigen become anergic.³⁴ It is now known that OX40/OX40L costimulatory molecule expression on T cells in inflamed tissue and on activated antigen-presenting cells (eg, CD11c⁺CD11b⁺ dendritic cells) coincides late in the life span of NOD mice (at 11 weeks of age), thereby representing a late checkpoint in T1D development.³¹ OX40 signaling results in T-cell proliferation, proinflammatory cytokine production, and augmentation of Th-17 cell function.^{35,36} OX40 is also upregulated in the majority of primed “memory” T cells after antigen reencounter. Targeting OX40/OX40L interactions at late onset significantly reduces the incidence of T1D in NOD mice.³¹ It is possible that nIgM acts by downregulating expression of these costimulatory molecules on immune cells similar to its downregulation of CD86, CD4, and CD2, thereby interrupting signaling and inhibiting autoreactive T-cell activation and proliferation at this later checkpoint in the progression of T1D.

Several groups have associated elevated Th-17 cells and IL-17 expression with spontaneous T1D in NOD mice.^{19,20} In fact, neutralization of IL-17 postinitiation of insulinitis was shown to prevent the development of diabetes, suggesting interference at the effector phase of T1D.²¹ Our in vitro results also indicate inhibition of proinflammatory TNF- α , IFN- γ , and IL-17A secretion from NOD mouse splenocytes. We have previously shown that nIgM decreases the frequency of Th-17 and CD4 T cells in inflamed tissue and inhibits proinflammatory cells from proliferating and producing IFN- γ and IL-17 in response to alloantigens, anti-CD3, and α -galactosyl ceramide.^{7–10} Regulatory T cells could not inhibit both Th-17 cell activation and IFN- γ /IL-17 production induced by LPS or in a mixed lymphocyte reaction assay indicating that inhibition was nIgM specific. Taken together, these data highlight the importance of nIgMs and nIgM-ALAs in providing another mechanism to regulate excess inflammation mediated by innate and adaptive immune mechanisms, including IL-17-producing cells.

Preclinical and clinical studies indicate that innate host inflammatory responses occurring in the microenvironment of the graft mediate the early loss of approximately half of the transplanted islet mass in the peritransplant period.^{15,37} These innate inflammatory responses likely promote alloantigen presentation, thereby strengthening and accelerating subsequent cell-mediated allogeneic immune responsiveness. Th-1 and Th-17 subsets produce proinflammatory cytokines, which activate infiltrating leukocytes to mediate the allograft rejection. Our studies have demonstrated that low-dose nIgM therapy could promote graft survival in both syngeneic and allogeneic islet transplantation. We are currently investigating whether combining low-dose nIgM therapy with transient immunosuppression or with agents that promote β -cell regeneration or by increasing the dosage of nIgM to 175 μ g of nIgM biweekly (which would elevate nIgM serum levels by 150–200 μ g/mL) would result in permanent islet allograft survival. In our previous cardiac allotransplantation experiments, high-dose nIgM therapy could inhibit severe and rapid BALB/c cardiac allograft rejection in wild-type B6 recipients.¹⁰ Control C57BL/6 recipients rejected cardiac allografts by 5 to 7 days, with histology demonstrating abundant Th-17 cells, while recipients receiving nIgM had none/minimal rejection. Furthermore, a significant lack of infiltrating leukocytes in the cardiac parenchyma of nIgM-treated recipients, decreased CXCL1 production, and none or minimal fragmentation of capillaries suggested that nIgM mediated its protective effect by inhibiting processes involved in leukocyte activation and migration. Replenishing IgM in IgM knockout mice with intact B cells and regulatory T cells significantly attenuated inflammation associated with ischemic reperfusion injury and inhibited cardiac allograft rejection, processes involving IFN- γ and IL-17. We have also shown that nIgM can directly inhibit Th-17 differentiation and prevent Foxp3⁺ regulatory T cells from becoming proinflammatory under Th-17 inflammatory conditions.¹⁰ Taken together, elevated nIgM-ALA levels could, alone or in the presence of

regulatory T cells, attenuate severe inflammatory processes by inhibiting Th-1 and Th-17 proliferation and differentiation and promote allograft survival.

To conclude, our studies indicate that purified polyclonal serum nIgM therapy may represent an innate, effective, safe, and benign therapeutic interventional agent that, in the future, may be effectively used to preemptively treat high-risk patients who might develop T1D and those with elevated T1D related autoantibodies or in conjunction with islet transplantation. Our observations also highlight the clinical potential of nIgM therapy in promoting islet graft survival after allotransplantation by regulating excess inflammation mediated by both innate and adaptive immune mechanisms and where the inflammatory responses involves Th-17 cells that are not effectively regulated by regulatory T cells. In addition, through possible induction of β -cell-specific unresponsiveness, nIgM therapy may also prevent recurrence of the disease after islet transplantation. As no generalized immunosuppression was observed with nIgM therapy, it seems to be a safe, naturally occurring, interventional agent for long-term continuous administration without complications usually associated with chronic use of immunosuppressive drugs to prevent T1D.

References

1. Yoon JW, Jun HS. Autoimmune destruction of pancreatic beta cells. *Am J Ther.* 2005; 12:580–591. [PubMed: 16280652]
2. Huang X, Moore DJ, Ketchum RJ, et al. Resolving the conundrum of islet transplantation by linking metabolic dysregulation, inflammation, and immune regulation. *Endocr Rev.* 2008; 29:603–630. [PubMed: 18664617]
3. Jahansouza C, Kumer SC, Ellenbogen M, et al. Evolution of β -cell replacement therapy in diabetes mellitus: pancreas transplantation. *Diabetes Technol Ther.* 2011; 13:395–418. [PubMed: 21299398]
4. Chhabra P, Brayman KL. Current status of immunomodulatory and cellular therapies in preclinical and clinical islet transplantation. *J Transplant.* 2011; 2011:637–692.
5. Mineo D, Sageshima J, Burke GW, et al. Minimization and withdrawal of steroids in pancreas and islet transplantation. *Transplant Int.* 2009; 22:20–37.
6. Rother KI, Harlan DM. Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus. *J Clin Invest.* 2004; 114:877–883. [PubMed: 15467822]
7. Lobo PI, Schlegel KH, Spencer CE, et al. Naturally occurring IgM anti-leukocyte autoantibodies (IgM-ALA) inhibit T cell activation and chemotaxis. *J Immunol.* 2008; 180:1780–1791. [PubMed: 18209075]
8. Lobo PI, Schlegel KH, Vengal J, et al. Naturally occurring IgM anti-leukocyte autoantibodies inhibit T-cell activation and chemotaxis. *J Clin Immunol.* 2010; 30(suppl 1):S31–S36. [PubMed: 20401525]
9. Lobo PI, Schlegel KH, Yuan W, et al. Inhibition of HIV-1 infectivity through an innate mechanism involving naturally occurring IgM anti-leukocyte autoantibodies. *J Immunol.* 2008; 180:1769–1779. [PubMed: 18209074]
10. Lobo PI, Bajwa A, Schlegel KH, et al. Natural IgM anti-leukocyte autoantibodies attenuate excess inflammation mediated by innate and adaptive immune mechanisms involving Th-17. *J Immunol.* 2012; 188:1675–1785. [PubMed: 22262657]
11. Kerman RH, Susskind B, Buyse I, et al. Flow cytometry-detected IgG is not a contraindication to renal transplantation: IgM may be beneficial to outcome. *Transplantation.* 1999; 68:1855–1858. [PubMed: 10628764]
12. Przybylowski P, Balogna M, Radovancevic B, et al. The role of flow cytometry-detected IgG and IgM anti-donor antibodies in cardiac allograft recipients. *Transplantation.* 1999; 67:258–262. [PubMed: 10075590]
13. Boes M. Role of natural and immune IgM antibodies in immune responses. *Mol Immunol.* 2000; 37:1141–1149. [PubMed: 11451419]

14. Manson JJ, Mauri C, Ehrenstein MR. Natural serum IgM maintains immunological homeostasis and prevents autoimmunity. *Springer Semin Immunopathol.* 2005; 26:425–432. [PubMed: 15611856]
15. Chhabra P, Wang K, Zeng Q, et al. Adenosine A(2A) agonist administration improves islet transplant outcome: evidence for the role of innate immunity in islet graft rejection. *Cell Transplant.* 2010; 19:597–612. [PubMed: 20350347]
16. Balasa B, Krah T, Patstone G, et al. CD40 ligand-CD40 interactions are necessary for the initiation of insulinitis and diabetes in nonobese diabetic mice. *J Immunol.* 1997; 159:4620–4627. [PubMed: 9379064]
17. Lenschow DJ, Ho SC, Sattar H, et al. Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. *J Exp Med.* 1995; 181:1145–1155. [PubMed: 7532678]
18. Christianson SW, Shultz LD, Leiter EH. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4⁺ and CD8⁺ T cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. *Diabetes.* 1993; 42:44–55. [PubMed: 8093606]
19. Emamaullee JA, Davis J, Merani S, et al. Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice. *Diabetes.* 2009; 58:1302–1311. [PubMed: 19289457]
20. Honkanen J, Nieminen JK, Gao R, et al. IL-17 immunity in human type 1 diabetes. *J Immunol.* 2010; 185:1959–1967. [PubMed: 20592279]
21. Jain R, Tartar DM, Gregg RK, et al. Innocuous IFN γ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. *J Exp Med.* 2008; 205:207–218. [PubMed: 18195074]
22. Boettler T, von Herrath M. Immunotherapy of type 1 diabetes—how to rationally prioritize combination therapies in T1D. *Int Immunopharmacol.* 2010; 10:1491–1495. [PubMed: 20667487]
23. Chatenoud L, Thervet E, Primo J, et al. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci U S A.* 1993; 91:123–127. [PubMed: 8278351]
24. Shizuru JA, Taylor-Edwards C, Banks BA, et al. Immunotherapy of the nonobese diabetic mouse; treatment with an antibody to T helper lymphocytes. *Science.* 1988; 240:659–662. [PubMed: 2966437]
25. Boitard C, Bendelac A, Richard MF, et al. Prevention of diabetes in nonobese diabetic mice by anti-I-A monoclonal antibodies: transfer of protection by splenic T cells. *Proc Natl Acad Sci U S A.* 1988; 85:9719–9723. [PubMed: 3264405]
26. Sempe P, Bedossa P, Richard MF, et al. Anti-a/b T cell receptor monoclonal antibody provides an efficient therapy for autoimmune diabetes in nonobese diabetic (NOD) mice. *Eur J Immunol.* 1991; 21:1163–1169. [PubMed: 1828030]
27. Yang XD, Karin N, Tisch R, et al. Inhibition of insulinitis and prevention of diabetes in nonobese diabetic mice by blocking L-selectin and very late antigen 4 adhesion receptors. *Proc Natl Acad Sci U S A.* 1993; 90:10494–10498. [PubMed: 7504266]
28. Rabinovitch A. Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation. *Diabetes.* 1994; 43:613–621. [PubMed: 8168635]
29. Chaparro RJ, Diloranzo TP. An update on the use of NOD mice to study autoimmune (Type 1) diabetes. *Expert Rev Clin Immunol.* 2010; 6:939–955. [PubMed: 20979558]
30. Tisch R, McDevitt H. Insulin-dependent diabetes mellitus. *Cell.* 1996; 85:291–297. [PubMed: 8616883]
31. Pakala SV, Bansal-Pakala P, Halteman BS, et al. Prevention of diabetes in NOD mice at a late stage by targeting OX40/OX40 ligand interactions. *Eur J Immunol.* 2004; 34:3039–3046. [PubMed: 15368274]
32. Takiishi T, Korf H, Van Belle TL, et al. Reversal of autoimmune diabetes by restoration of antigen-specific tolerance using genetically modified *Lactococcus lactis* in mice [published online ahead of print April 9, 2012]. *J Clin Invest.* 2012; 122:1717–1725. <http://www.jci.org/articles/view/60530#B22>. 10.1172/JCI60530 [PubMed: 22484814]

33. Brode S, Raine T, Zacccone P, et al. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *J Immunol.* 2006; 177:6603–6612. [PubMed: 17082572]
34. Snanoudj R, de Préneuf H, Créput C, et al. Costimulation blockade and its possible future use in clinical transplantation. *Transplant Int.* 2006; 19:693–704.
35. Ishii N, Takahashi T, Soroosh P, et al. OX40-OX40 ligand interaction in T-cell-mediated immunity and immunopathology. *Adv Immunol.* 2010; 105:63–98. [PubMed: 20510730]
36. Zhang Z, Zhong W, Hinrichs D, et al. Activation of OX40 augments Th17 cytokine expression and antigen-specific uveitis. *Am J Pathol.* 2010; 177:2912–2920. [PubMed: 20952591]
37. Jahansouz C, Jahansouz C, Kumer SC, et al. Evolution of β -cell replacement therapy in diabetes mellitus: islet cell transplantation. *J Transplant.* 2011; 2011:247959. [PubMed: 22013505]

FIGURE 1.

nIgM therapy inhibits onset and progression of autoimmune diabetes. Groups of NOD mice were injected biweekly, beginning at 4 to 5 or 10 to 11 weeks of age with nIgM or PBS (A) or BSA or mouse IgG up to 18 weeks of age (B). Diabetes incidence was monitored by measuring tail vein BG levels and hyperglycemia defined as BG 250 mg/dL or more on 3 successive days.

FIGURE 2.

nIgM therapy inhibits insulinitis and promotes β -cell neogenesis. H&E-stained pancreas sections were examined histologically. Representative sections are shown. A, Islet from nondiabetic 4- to 5-week-old NOD mouse; B, Islet from 18-week-old diabetic NOD mouse showing insulinitis; and C, perivascular and periductular inflammation. D, Islet from 18-week-old nIgM-treated, nondiabetic NOD mouse showing no insulinitis and mild perivascular and periductular inflammation; E, Islet from 18-week-old, nIgM-treated, nondiabetic NOD mouse showing mild insulinitis/peri-insulinitis and β -cell neogenesis. Thick arrow indicates islets; thin arrow, insulinitis, peri-insulinitis, or periductular/perivascular inflammation.

FIGURE 3.

nIgM therapy promotes syngeneic graft survival. 50 C57BL/6 syngeneic islets were transplanted under the renal capsule of streptozotocin-induced diabetic C57BL/6 mice receiving nIgM or PBS/BSA (75 μ g thrice weekly). Mice with BG levels 300 mg/dL or more were considered diabetic. nIgM therapy reduced the time taken to return to normoglycemia, significantly improving islet graft survival compared with the control group ($P < 0.001$).

FIGURE 4.

nIgM therapy promotes allogeneic graft survival. A total of 300 allogeneic BALB/c islets were transplanted under the renal capsule of streptozotocin-induced, diabetic C57BL/6 mice receiving nIgM or PBS/BSA (75 μ g thrice weekly). A third group of control diabetic mice received nIgM therapy alone without islet transplants. BG levels of 300 mg/dL or more were an indication of graft rejection. nIgM therapy significantly increased the mean survival time of islet allografts compared with controls ($P < 0.001$).

FIGURE 5.

nIgM delays the incidence of diabetes (appearance of hyperglycemia) in adoptive transfer model. nIgM therapy significantly delays the incidence of diabetes (appearance of hyperglycemia) after adoptive transfer of diabetic NOD splenocytes (A) or CD3 T cells (B) into NOD scid recipient mice when compared with control mice receiving BSA ($P < 0.001$). Transfer of splenocytes or CD3 T cells from nondiabetic 2- to 3-week-old NOD mice did not transfer disease.

FIGURE 6.

nIgM inhibits proinflammatory cytokine production from activated splenocytes in vitro. nIgM significantly inhibits proinflammatory cytokine production in vitro from splenocytes obtained from 3- to 6-week-old NOD mice ($n = 4$) that were activated with LPS and soluble anti-CD3 and cultured for 4 days at 37°C, 5% CO₂, with BSA or purified nIgM.