Healthy Donor Polyclonal IgMs Diminish B-Lymphocyte Autoreactivity, Enhance Regulatory T-Cell Generation, and Reverse Type 1 Diabetes in NOD Mice

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Diabetes 2018;67:2349–2360 | https://doi.org/10.2337/db18-0456

Autoimmune diseases such as type 1 diabetes (T1D) arise from unrestrained activation of effector lymphocytes that destroy target tissues. Many efforts have been made to eliminate these effector lymphocytes, but none has produced a long-term cure. An alternative to depletion therapy is to enhance endogenous immune regulation. Among these endogenous alternatives, naturally occurring Igs have been applied for inflammatory disorders but have lacked potency in antigen-specific autoimmunity. We hypothesized that naturally occurring polyclonal IgMs, which represent the majority of circulating, noninduced antibodies but are present only in low levels in therapeutic Ig preparations, possess the most potent capacity to restore immune homeostasis. Treatment of diabetes-prone NOD mice with purified IgM isolated from Swiss Webster (SW) mice ($nIgM_{SW}$) reversed new-onset diabetes, eliminated autoreactive B lymphocytes, and enhanced regulatory T-cell (Treg) numbers both centrally and peripherally. Conversely, IgM from prediabetic NOD mice could not restore this endogenous regulation, which represents an unrecognized component of T1D pathogenesis. Of note, IgM derived from healthy human donors was similarly able to expand human CD4 Tregs in humanized mice and produced permanent diabetes protection in treated NOD mice. Overall, these studies demonstrate that a potent, endogenous regulatory mechanism, nIgM, is a promising option for reversing autoimmune T1D in humans.

Type 1 diabetes (T1D) remains a devastating, chronic immune disorder for which incidence continues to rise (1). The personal and economic burden of this disease is monumental despite improvements in insulin therapy. Attempts to target the immune system to dissuade it from attacking and destroying insulin-producing β-cells have focused largely on depleting immune cells. These therapies have been mostly unsuccessful because of reemergence of autoreactive cells after therapy is discontinued, pointing to ongoing deficiencies in immune tolerance despite therapy.

Nonetheless, an immunologic approach to the resolution of T1D seems attainable. Important insights have been gleaned from human blood and pancreas samples about the pathogenesis of T1D, including the roles of specific cell types that target antigen (2–4). These studies continue to point to the important role of autoreactive T cells and their collusion with islet-reactive B lymphocytes. The essential contribution of this interaction has been born out repeatedly in animal studies (5–8). More importantly, the presence of two anti-islet antibodies, which are produced by islet-reactive B lymphocytes, now is defined as a diagnosis of stage 1 T1D (9). Individuals with T1D defined by this biomarker will develop new insulin requirements at a rate of 11% per year. Nearly every child facing this circumstance will progress to β-cell failure in his or her lifetime, making the understanding and inhibition of this pathologic process paramount.

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Received 20 April 2018 and accepted 12 August 2018.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db18-0456/-/DC1.

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Although the central role of autoreactive B lymphocytes is well documented, B lymphocytes have been the target of only one clinical intervention trial, which involved the treatment of patients with new-onset T1D with Rituxan (anti-CD20). This trial demonstrated a transient improvement in β-cell function, as defined by C-peptide secretion, followed by a resumption of β-cell functional decline (10–12). Analysis of blood samples in treated patients demonstrated initial depletion of autoreactive B lymphocytes followed by the generation of new, equally reactive cells after the therapeutic effects waned (13). In addition, the therapy seemed unable to target highly activated B lymphocytes that downregulated the CD20 molecule and may be important in sustaining destructive autoimmunity (8). Despite this initial success, no additional B-lymphocyte–targeted therapies have been investigated.

In contrast to depleting pharmacotherapies, the healthy human immune system possesses endogenous regulatory mechanisms that prevent self-injury by restraining inappropriate immune activation. These endogenous processes often are long-lived, self-renewing, and nontoxic. Harnessing these endogenous regulatory mechanisms represents a paradigm shift away from immunosuppressive strategies to treat T1D. Initial studies in this area have focused on isolation and expansion of regulatory T cells (Tregs), which are critically important for restraining tissue-injurious immunity. Although $CD4^+$ Tregs are a well-known form of immune regulation in T1D, B lymphocytes now are known to secrete IgM with immunoregulatory properties, but their role in T1D has not been explored (14–19).

Present at low levels in healthy individuals, IgMs increase during inflammatory disorders and various infections (20). Studies in animal models have indicated that these natural IgMs are an important part of the normal homeostatic mechanisms of the immune system because they limit inflammatory responses (21–23). Secreted IgM also is an important endogenous regulator of B-lymphocyte development. Mice that lack the ability to secrete IgM ($\mu s^{-/-}$) or the ability to detect IgM through the TOSO receptor ($Fc\mu R^{-/-}$), demonstrate perturbations in B-lymphocyte development that are similar to B-lymphocyte development in NOD mice and that permit the maturation of autoreactive B lymphocytes (17,19,24,25). Treatment with therapeutic IgM, therefore, has the potential to alter the development of autoreactive lymphocytes and prevent or reverse T1D.

In this study, we demonstrate the potent immunoregulatory capacity of murine and human IgM. We found that polyclonal IgM is a natural regulator of B-lymphocyte development that selectively diminishes autoreactive B-lymphocyte numbers and function. IgM also acts on B lymphocytes within the thymus to promote the thymic development of potent and long-lasting Tregs. Unexpectedly, IgMs derived from prediabetic NOD mice, the primary preclinical model of T1D, are unable to treat disease or expand regulatory cells, revealing a new mechanism that may permit T1D pathogenesis. Critically, we confirm that IgM derived from healthy human donors prevents diabetes onset in the NOD model and causes a significant expansion of human Tregs in a humanized mouse model, indicating a potential for future clinical application.

RESEARCH DESIGN AND METHODS

Animals

C57BL/6J (B6), NOD/ShiLtJ (NOD), and immunodeficient NSG mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and Swiss Webster (SW) mice were purchased from Charles River Laboratories (Wilmington, MA). V_H125^{SD}.B6 and V_H125^{SD}.NOD (NOD.129P2(*Cg*)-*Igh*$^{tm1.1Jwt}$/J) were constructed by our collaborator J.W. Thomas (Vanderbilt University). Mice were housed in a specific-pathogen–free facility at Vanderbilt University. B-cell–deficient NOD.μMT mice were a gift from D. Serreze (The Jackson Laboratory). BLT mice were created as previously described (26,27).

Purification of IgM

IgM was purified by size-exclusion column chromatography (Sephacryl S-300 HR; GE Healthcare, Piscataway, NJ) from irradiated, heat-inactivated (56°C × 1 h) SW or NOD murine sera using previously described procedures (28) and with modifications as detailed herein. Mouse sera were obtained from mice housed locally at the University of Virginia. 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, >92% of the protein fraction contained nIgM with <1% IgG, <3% albumin, and <1% other protein contaminants as determined by protein electrophoresis and ELISA. We did not affinity purify nIgM antibodies because such procedures (binding of nIgM to mannan-binding protein or binding of nIgM to agarose coupled with goat anti-IgM antibodies) yield 10–15% of the starting IgM and have the potential to deplete certain IgM fractions. Purified nIgM was concentrated to 1.3–1.5 mg/mL (the higher concentration led to nIgM aggregation and precipitation), dialyzed against RPMI medium, and microfiltered using a 0.45-μm Millipore filter before use in cultures or in vivo. Purified IgMs were stored at 4°C to prevent the precipitation that occurs when frozen. All preparations had undetectable endotoxin activity.

Dosage of nIgM, hIgM, Anti-CD25, and Anti–B-Cell Activating Factor

For diabetes reversal, NOD mice were treated by i.p. injection with two doses (100 μg) of NOD or $nIgM_{SW}$ on days 1 and 4 after diabetes onset. For cellular analysis, NOD and B6 mice were treated by i.p. injection with an initial dose of 100 μg SW nIgM on day 1 followed by 50-μg doses on days 3, 5, 7, and 10 and sacrificed on day 13. For depletion of Tregs, IgM-treated, reversed NOD mice were injected with anti-CD25 (2 mg per mouse) (PC61; Bio X

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Cell) on day 1 and then again on day 7. For human IgM testing, NOD WT mice or humanized BLT mice were treated by i.p. injection of 100 μg human IgM on day 1 followed by 70 μg on days 5 and 10 with analysis on day 13. In experiments where anti–B-cell activating factor (BAFF) (Sandy-2; Adipogen Life Sciences) was used, 100 μg was injected into NOD mice on day −2 concomitantly with 100 μg $nIgM_{SW}$ on day 1 and another dose of anti-BAFF and nIgM on day 3 before sacrifice and analysis on day 4.

Measurement of Insulin Autoantibodies

Longitudinal evaluation of insulin autoantibody (IAA) levels was conducted using plasma samples obtained from female NOD/ShiLtJ mice injected with saline or IgM. Plasma IAA was measured by micro-IAA radioimmunoassay at the Barbara Davis Center for Childhood Diabetes (Aurora, CO).

Thymus Histology and Analysis

Thymuses were harvested into 10% formalin. Slides were then embedded in paraffin and sectioned in 5-μm sections. The slides were stained for hematoxylin-eosin (H-E) and double stained for B220 and Foxp3. Thymus sections then were imaged on a brightfield Aperio ScanScope and acquired at 20× using Aperio ImageScope. Sections were analyzed for colocalization using HALO software (Indica Labs). Medullary spaces were defined as areas of light H-E staining.

Flow Cytometry and Antibodies

Spleen and thymus were rendered to single-cell suspensions by crushing through a 70-μm filter. Splenocytes or thymocytes were stained with fluorophore-conjugated antibodies purchased from either BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), Cell Signaling Technologies (Danvers, MA), or MBL International (Woburn, MA). A complete antibody list for time-of-flight mass cytometry is provided in Supplementary Table 1.

Statistics

Statistical analysis was performed with Prism 5 software (GraphPad, La Jolla, CA) using the Mann-Whitney *U* test. One- or two-way ANOVA followed by Bonferroni posttest was used to compare multiple groups. Statistical comparisons with $P \leq 0.05$ were deemed significant.

Study Approval

The institutional animal care and use committee or institutional review boards at Vanderbilt University and University of Virginia approved all procedures carried out during this study. Written informed consent was obtained from all human subjects before participation.

RESULTS

$nIgM_{SW}$ Reverses Diabetes and Modulates Immune Cell Subsets in NOD Mice

Because humans who will progress to T1D rarely are identified before experiencing β-cell loss, developing therapies that facilitate β-cell recovery after patients present with hyperglycemia remains important. To this end, we tested the ability of IgM derived from SW donor mice ($nIgM_{SW}$) to reverse hyperglycemia in newly diabetic NOD mice (blood glucose 200–300 mg/dL on two consecutive measurements). Mice were administered two doses of $nIgM_{SW}$ (100 μg) by i.p. injection on days 1 and 4 after onset (n = 11) or were left untreated (n = 15). By using this strategy, we determined that $nIgM_{SW}$ normalized hyperglycemia and maintained blood glucose ≤200 mg/dL in 63% of treated mice (Fig. 1*A* and *B*). To determine whether $nIgM_{SW}$ mediated this effect by targeting immune cell subsets, we analyzed spleens of treated and untreated prediabetic NOD mice. We noted that splenic size and cellularity were increased compared with control, IgG, or monoclonal IgM-injected mice (data not shown). To determine what immune subsets were modulated by $nIgM_{SW}$, we used time-of-flight mass cytometry to analyze 24 markers of multiple cell subsets of the immune system simultaneously; antibodies and metal conjugates are listed in Supplementary Table 1. We then used Spanning Tree Progression Analysis of Density Normalized Events to visualize changes in immune cell subsets in $nIgM_{SW}$-treated NOD mice compared with control mice in an unbiased fashion (29). We observed a massive expansion of the Gr-1^+CD$11b^+$ subset with a myeloid-derived suppressor cell (MDSC) phenotype and modulation of B-lymphocyte subsets in the spleen (Supplementary Fig. 1*A* and *B*). To determine whether the MDSC-like population accounted for diabetes protection, we transferred purified Gr-1^+CD$11b^+$ cells from $nIgM_{SW}$-treated donors into NOD.RAG mice with splenocytes from a donor with diabetes. These MDSC-phenotype cells did not delay or prevent T1D onset compared with control mice that received only diabetic splenocytes despite being transferred at a high ratio, indicating that other immunologic changes mediated the protection from diabetes (Supplementary Fig. 1*C*). Analysis of splenic CD4, CD8, and B lymphocytes demonstrated that B lymphocytes underwent expansion in total cell numbers up to the levels observed in healthy B6 animals (Fig. 1*C*). Unlike the correction of perturbed homeostasis seen with therapy in NOD, cell distribution in healthy B6 animals was unchanged by treatment with $nIgM_{SW}$.

Therapy With $nIgM_{SW}$ Restores B-Lymphocyte Homeostasis and Eliminates Autoreactive B Lymphocytes

Therapy with $nIgM_{SW}$ normalized the abnormal B-lymphocyte subset distribution of transitional and marginal zone B lymphocytes in NOD (Fig. 2*A* and *B*). We hypothesized that this correction of B-lymphocyte developmental defects in NOD mice would foster the elimination of autoreactive B lymphocytes and thus eliminate the B lymphocytes important for initiation and progression of disease. To test this hypothesis, we used transgenic models with increased frequency of anti-insulin B lymphocytes. The V_H125^{SD}.NOD and V_H125^{SD}.B6 mice have heavy

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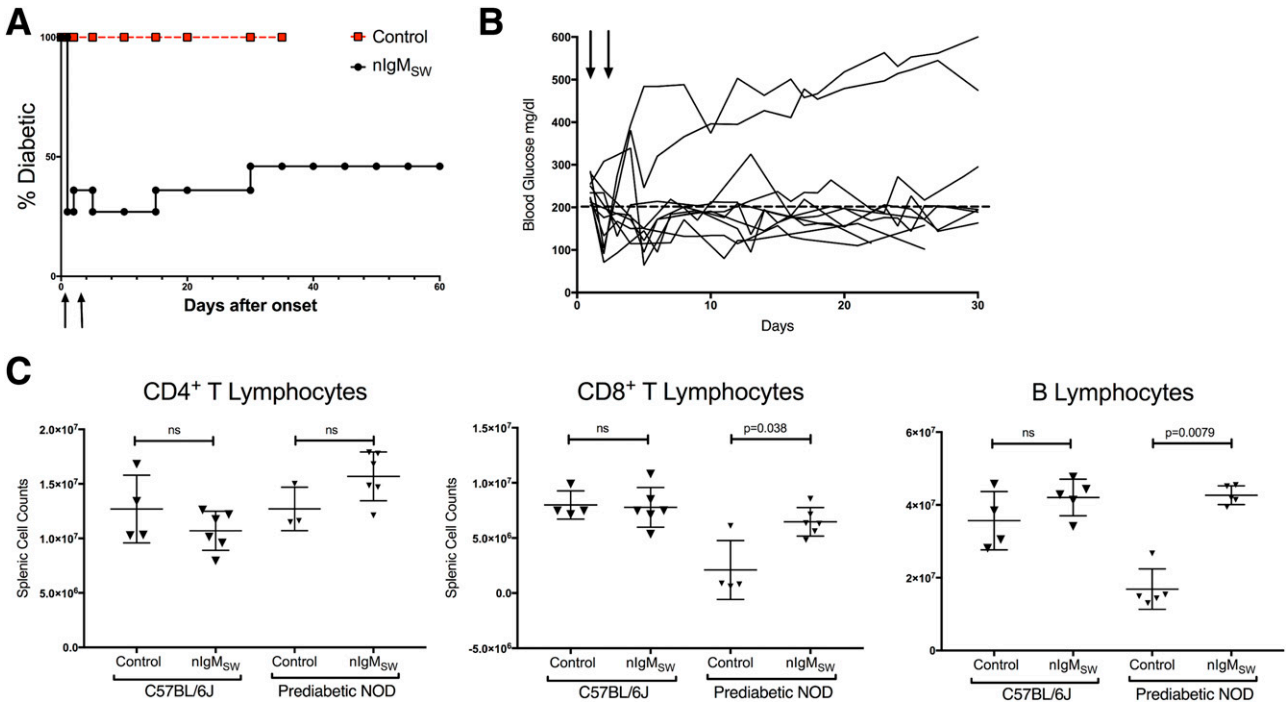

Figure 1—nIgM$_{SW}$ reverses T1D and modifies immune subsets in NOD mice. *A* and *B*: NOD mice were allowed to become diabetic as defined by two consecutive blood glucose readings between 200 and 300 mg/dL. These mice were then treated with two injections of 100 μg nIgM isolated from SW mice (nIgM$_{SW}$) on days 1 and 4 postdiagnosis. Blood glucose was monitored serially, and ~63% of mice reversed and maintained blood glucoses <200 mg/dL for the duration of the time they were monitored (n = 11 mice and n = 15 controls). *C*: Flow cytometric analysis revealed no significant changes in CD4 and a small increase in CD8 T lymphocytes in nIgM$_{SW}$-treated mice (representative data of at least seven experimental repeats are shown). NOD mice demonstrated an increase in B lymphocytes after nIgM$_{SW}$ injection. ns, not significant.

chains specific for insulin knocked into the endogenous heavy chain locus, which combines with endogenous light chains to produce a functional B-cell receptor. On both the NOD and B6 backgrounds, these mice develop a small, but identifiable population of insulin-binding B lymphocytes; in the NOD background, these B lymphocytes drive accelerated diabetes onset (30). NOD or B6 mice on the V_H125^{SD} background were treated with nIgM$_{SW}$ by i.p. injection, and insulin-reactive B lymphocytes were analyzed through flow cytometry using a biotin-conjugated human insulin followed by a streptavidin-conjugated fluorophore. V_H125^{SD}.NOD mice treated with nIgM$_{SW}$ showed a complete loss of detectable insulin-reactive lymphocytes in the spleen (Fig. 2*C* and *D*). Similarly, we identified a complete abrogation of IAA production in NOD mice treated with nIgM$_{SW}$ as detected by radioimmunoassay (Fig. 2*E*). Taken together, the data demonstrate that nIgM$_{SW}$ therapy corrects defects in B-lymphocyte selection characteristic of autoimmune diabetes in NOD mice and that predict disease in humans, thus demonstrating that nIgM interferes with the pathologic process in T1D.

Tregs Expand and Restrain Diabetes in nIgM$_{SW}$-Treated NOD Mice

In addition to the profound effects on B-lymphocyte development, we observed a trend toward increased CD4$^+$ T cells in the spleen (compare with Fig. 1*C*). Although not statistically different, this suggests that certain CD4 T-cell subsets could be expanded. In particular, Foxp3$^+$ CD4 Tregs are instrumental in restraining deleterious interactions between B and T lymphocytes that incite autoimmunity and have been demonstrated repeatedly as capable of reversing T1D. Direct analysis of CD4$^+$ Tregs in the spleen revealed that nIgM$_{SW}$ expands Tregs, suggesting that CD4$^+$ Tregs promote reversal of T1D by nIgM$_{SW}$ (Fig. 3*A* and *B*). To determine whether CD4$^+$ Tregs were requisite for durable disease reversal after nIgM$_{SW}$ therapy, we depleted Tregs from NOD mice that had their disease reversed with nIgM$_{SW}$ for >30 days by administering two injections of anti-CD25 (PC61), an approach that consistently breaks Treg-dependent tolerance. These mice developed hyperglycemia in ~21 days after the initial administration of anti-CD25 compared with control mice that received no anti-CD25 and remained euglycemic (n = 3 in each group) (Fig. 3*C*). Transfer of Tregs into NOD/SCID mice with diabetic splenocytes also demonstrated the ability of these cells to restrain disease (data not shown).

Thymic Tregs Expand in a B-Lymphocyte–Dependent Manner in nIgM$_{SW}$-Treated Mice

Although expansion of Tregs was apparent in nIgM$_{SW}$-treated NOD mice, this expansion may occur from peripheral

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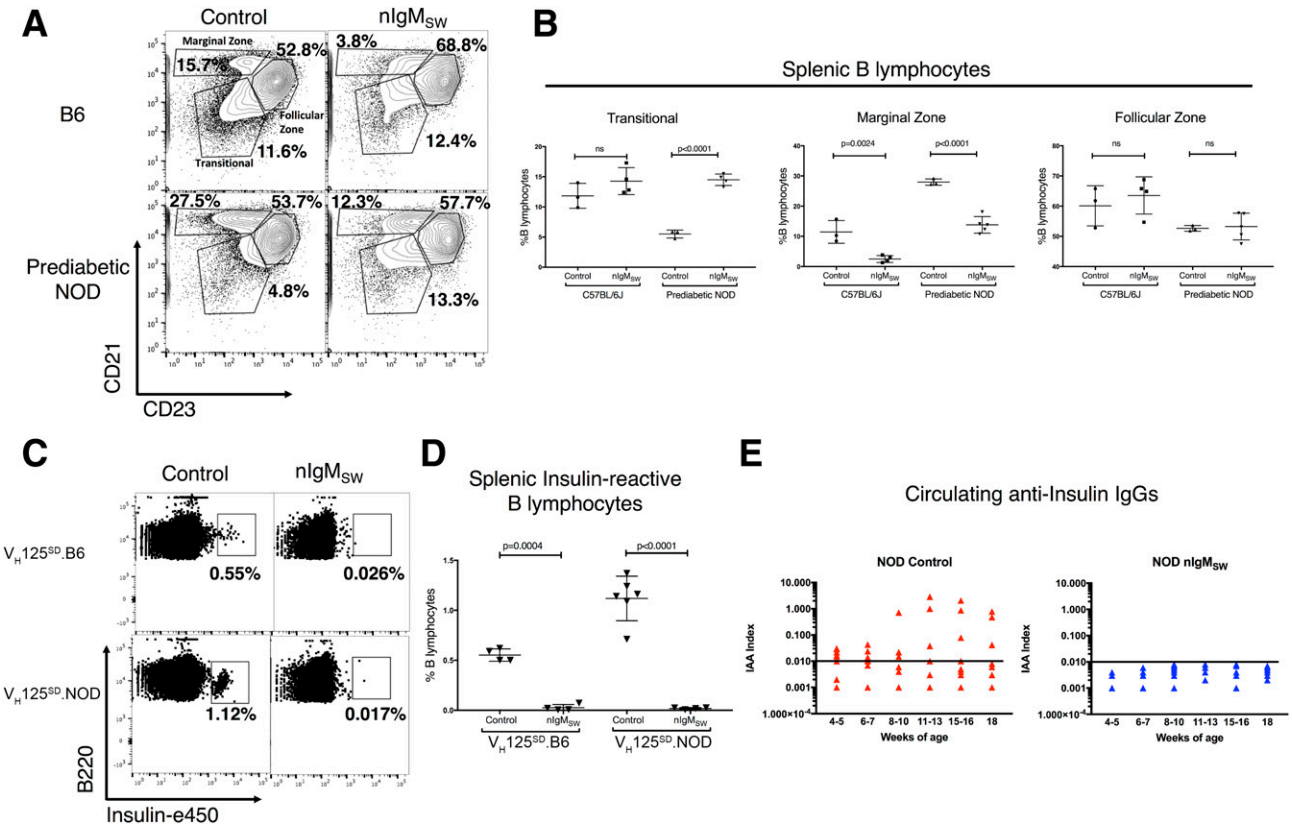

Figure 2—nIgM$_{SW}$ corrects B-lymphocyte homeostatic defects and eliminates autoreactive B-lymphocyte clones. *A*: Flow cytometric analysis of B220$^+$ cells revealed normalization of marginal zone and transitional B-lymphocyte subsets in NOD mice after nIgM$_{SW}$ treatment. Marginal zone expansion and loss of the transitional compartment are hallmark defects of B-lymphocyte development in NOD mice that were corrected by nIgM$_{SW}$ therapy. *B*: Quantification of subsets (representative data of at least seven experimental repeats). *C* and *D*: V$_H$125^{SD}.B6 and V$_H$125^{SD}.NOD possess a heavy chain specific for human insulin knocked into the endogenous IgM locus. This transgenic heavy chain combines with endogenous light chains to produce a population of insulin-reactive B lymphocytes in NOD mice. These mice were treated with nIgM$_{SW}$, and insulin-reactive B lymphocytes were identified by staining with biotinylated human insulin followed by streptavidin e450. We were unable to detect insulin-reactive B lymphocytes in NOD mice after treatment, which is quantified in *D*. *E*: A longer course of treatment was undertaken to determine how IgM$_{SW}$ affected production of anti-insulin Igs. A radioimmunoassay of circulating IgGs reactive to insulin revealed loss of anti-insulin antibodies in nIgM$_{SW}$-treated mice, whereas control NOD mice possessed insulin-reactive IgGs as expected. ns, not significant.

precursors or from contributions from thymic development, where newly generated clones may be important for long-term tolerance (30). We assessed the production of CD4$^+$ Foxp3$^+$ Tregs in the thymuses of B6 and NOD nIgM$_{SW}$-treated and untreated mice. After nIgM$_{SW}$ treatment, NOD mice showed a twofold expansion in Tregs in the thymus (Fig. 4*A*). Of note, we also noticed a robust expansion of B lymphocytes in thymuses of nIgM$_{SW}$-treated NOD mice (Fig. 4*B* and *C*). To test the hypothesis that these interactions between thymic B cells and developing T cells are required for Treg generation, we injected nIgM$_{SW}$ into NOD.μMT mice that lacked B lymphocytes. With treatment, thymic Tregs did not expand in B-cell–deficient NOD mice (Fig. 4*D*). Histologic analysis of treated thymuses revealed considerable infiltration of B lymphocytes into the medullary spaces of the thymus, an area essential for Treg development (31), in treated NOD mice (Fig. 4*E*). Colocalization analysis revealed that the B220$^+$ and Foxp3$^+$ cells were localized more closely in the medulla of treated NOD mice than in untreated control groups (Fig. 4*F*). These data demonstrate a unique role for B-lymphocyte location in fostering Treg development, which is abnormal in untreated NOD mice and enhanced by treatment with nIgM$_{SW}$.

BAFF Is Required for Expansion of Thymic B Lymphocytes and Thymic Tregs

Overexpression of BAFF in mice on a B6 background previously demonstrated expansion of thymic B lymphocytes, colocalization of B lymphocytes with Tregs, and an increase in thymic Treg output. Of note, the action of BAFF depended on B lymphocytes, as BAFF-overexpressing mice did not show Treg expansion when placed on a B-cell–deficient background (32). To date, the role of BAFF in autoimmune disease has been believed to be deleterious, especially in the progression of T1D (33). Of note, nIgM$_{SW}$

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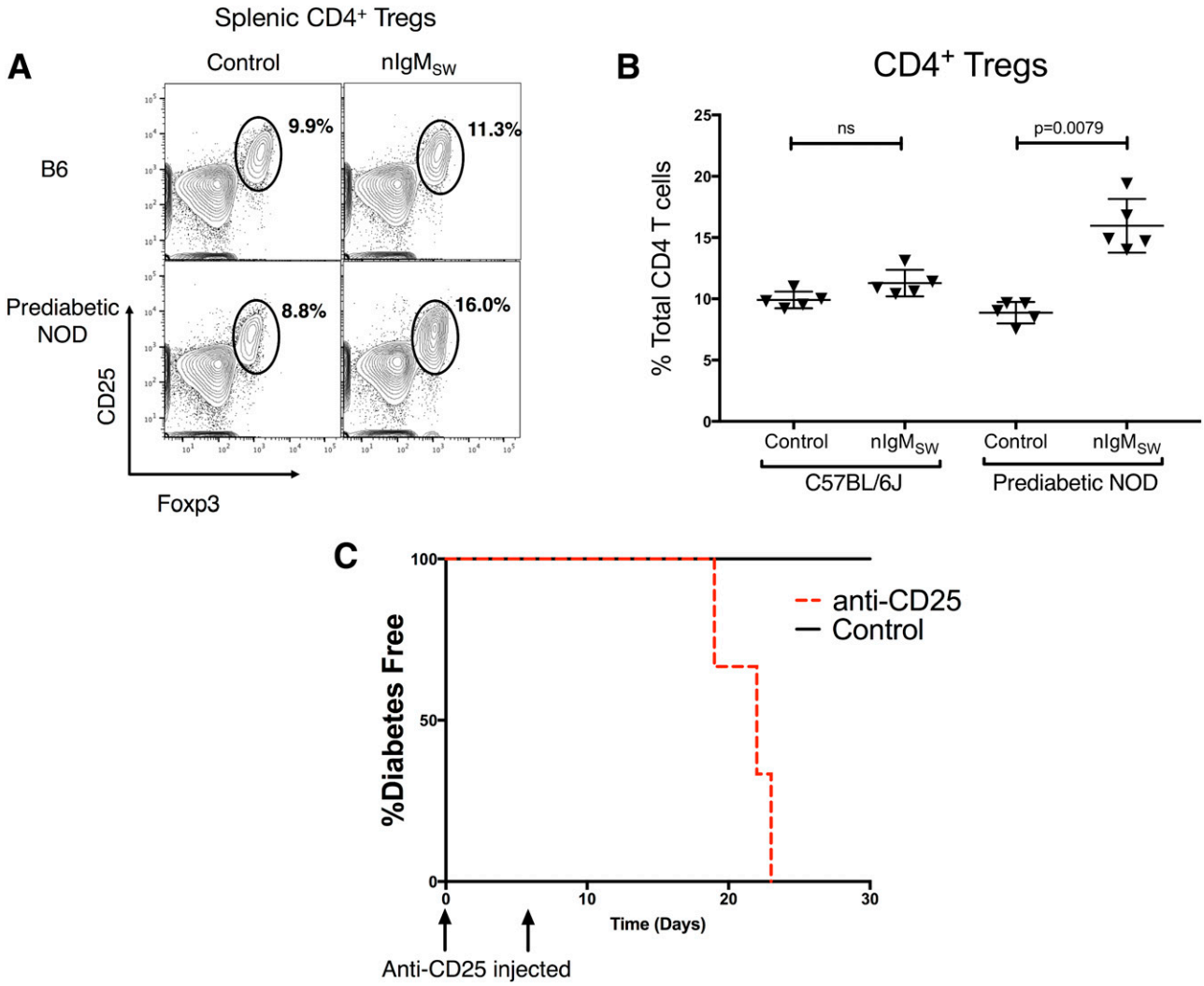

Figure 3—nIgM$_{SW}$ expands Tregs, and these cells are essential for diabetes reversal. *A*: B6 and NOD mice were treated with nIgM$_{SW}$, and spleens were analyzed for CD25$^+$ FoxP3$^+$ CD4 T cells. *B*: Treated NOD mice had an almost threefold increase in peripheral Tregs after the therapy. B6 mice showed only a modest increase in Tregs after therapy. (Representative data of at least seven experimental repeats are shown.) *C*: To determine whether Tregs were responsible for stably preserving β-cell mass and preventing hyperglycemia, mice that had remained stably reversed with nIgM$_{SW}$ for 30 days were then treated with 2 mg/kg T-cell–depleting anti-CD25 antibody (PC61) on days 1 and 7 (n = 3). PC61-treated mice became hyperglycemic ∼3 weeks after the first injection of anti-CD25 and were sacrificed according to animal protocols. Control mice (n = 3) remained euglycemic even out to 60 days after initial nIgM$_{SW}$ therapy.

therapy increased circulating BAFF almost eightfold in NOD mice (Fig. 5*A*). To determine the role of BAFF in thymic Treg expansion, we blocked circulating BAFF with anti-BAFF (Sandy-2) and treated NOD mice with nIgM$_{SW}$. Blockade of BAFF led to a decrease in both thymic B lymphocytes and thymic Tregs (Fig. 5*B* and *C*), indicating an essential role for BAFF in the generation of thymic Tregs in NOD mice.

NOD nIgM (IgM$_{NOD}$) Does Not Possess the Immunoregulatory Properties of nIgM$_{SW}$

Because nIgM isolated from SW donors reversed disease and modulated the immune compartment of NOD mice, we hypothesized that NOD-derived nIgM may lack the capacity to reverse disease and restore immune homeostasis. IgMs were prepared from prediabetic NOD donors at age 8–12 weeks. Diabetic NOD mice were treated with two doses of 100 μg nIgM$_{NOD}$, and blood glucose was monitored. Treated NOD mice experienced an early reprieve from high blood glucose at the beginning of the treatment course but returned to hyperglycemia shortly thereafter (n = 4) (Fig. 6*A*). To determine the immune changes induced by nIgM$_{NOD}$, we treated prediabetic NOD mice with nIgM$_{NOD}$ in the same fashion as nIgM$_{SW}$. We then assessed B-lymphocyte numbers and subset distribution through flow cytometry. We did not see an increase in total B-lymphocyte numbers (Fig. 6*B*), but we did note a decrease in marginal zone B lymphocytes and a slight

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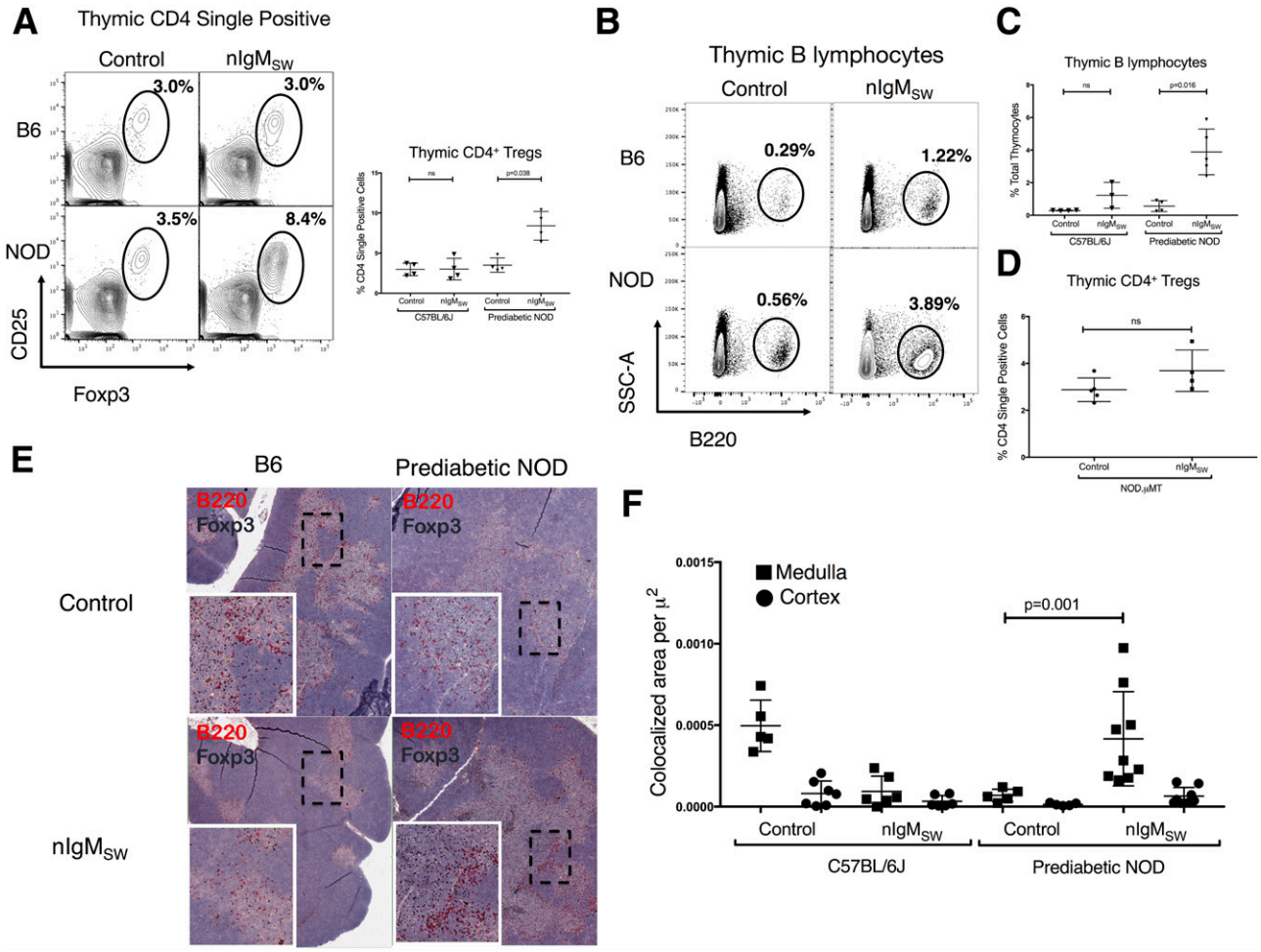

Figure 4—nIgM$_{SW}$ expands thymic Tregs in a B-lymphocyte–dependent manner. *A*: B6 and NOD mice were treated with nIgM$_{SW}$, and thymuses were analyzed for CD25$^+$ FoxP3$^+$ CD4 T cells. Treated NOD mice had a twofold increase in thymic Tregs after the therapy. B6 mice had no increase in Tregs. Representative flow data are shown. *B* and *C*: Thymic B lymphocytes also increased as shown by measurement of B220$^+$ cells. Additional staining demonstrated that these cells are also CD19$^+$ and IgM$^+$. This is quantified in *C*. *D*: To determine whether Treg expansion relied on B cells, NOD.μMT mice that are genetically deficient of B cells were treated with nIgM$_{SW}$. These mice demonstrated no increase in thymic Tregs, indicating a role for B lymphocytes in thymic Treg induction. *E*: We assessed whether B lymphocytes and Tregs were located in proximity to each other in the thymus by staining B220 (red) and Foxp3 (blue). We observed clusters of B lymphocytes near the thymic medulla (indicated by the fainter H-E staining) in B6 control and treated groups. In NOD control mice, we observed B lymphocytes in the medulla but at a much lower frequency than B6. After nIgM$_{SW}$ treatment, the number of B lymphocytes near the thymic medulla greatly expanded in NOD thymuses (original magnification ×4, inset at ×20). *F*: Colocalization analysis revealed that the medullary spaces of the thymus have the most Treg and B-lymphocyte interactions in B6. In NOD mice, this interaction occurs at a much lower frequency. Treatment of NOD mice increased the B-Treg interaction in the thymic medullary spaces. ns, not significant; SSC-A, side scatter area.

increase in transitional B lymphocytes, although not to the levels of nIgM$_{SW}$ treatment (Fig. 6*C* and *D*). Peripheral Tregs also expanded with nIgM$_{NOD}$ (Fig. 6*E*), but when we assessed the thymus, we determined that there was no significant expansion of thymic B lymphocytes or Tregs in the thymus of treated mice (Fig. 6*F–H*). Because immunostimulation has been demonstrated to prevent diabetes, we measured nuclear factor-κB activity in B lymphocytes as a marker of potential immune activation after treatment. We noted that nIgM$_{SW}$ reduced the abnormal phosphorylation of p65 in NOD B lymphocytes, indicating that the remaining B lymphocytes were not activated by therapy (Supplementary Fig. 2*A* and *B*). In further analysis of the SW-derived IgM, we determined that outbred SW mice have the capacity to express both IgMa and IgMb, whereas NOD mice express only IgMb (Supplementary Fig. 3*A*). Although we did not observe immunostimulation by nIgM$_{SW}$, we investigated whether alloreactivity could drive any of the immune changes seen in NOD mice treated with nIgM$_{SW}$. Treatment of NOD mice with a monoclonal antibody of IgMa allotype demonstrated that IgMa did not possess the capacity to induce immune changes like nIgM$_{SW}$ (Supplementary Fig. 3*B–H*). Taken together, these data demonstrate that IgM isolated from NOD mice is deficient in components important for long-term reversal of diabetes, including thymic Treg expansion.

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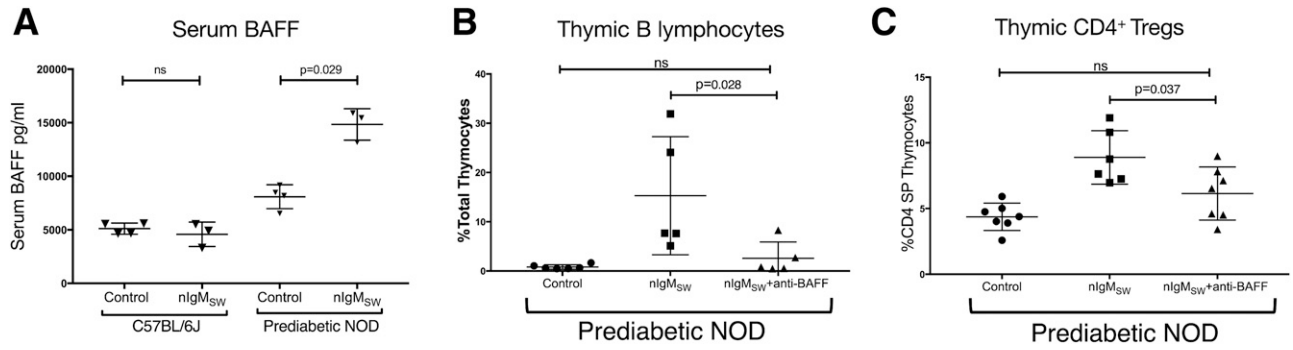

Figure 5— BAFF is essential for thymic Treg and B-lymphocyte expansion in nIgM$_{SW}$-treated mice. *A*: Serum BAFF levels were demonstrated to be increased almost eightfold in NOD mice treated with nIgM$_{SW}$. *B* and *C*: Blockade of BAFF with anti-BAFF (Sandy-2) led to a reduction in the ability of nIgM$_{SW}$ to expand B cells and Tregs in the thymuses of NOD mice (shown in *C*). ns, not significant; SP, single positive.

Human IgM Expands Tregs to Prevent Diabetes in NOD Mice and Expands Human Tregs

To assess the translational potential of this therapy, we obtained human IgM from a healthy donor, which we injected i.p. at the same dosage as nIgM$_{SW}$. We noted moderate normalization of B-lymphocyte subsets (Supplementary Fig. 4*A* and *B*) and the expansion of Tregs in these mice (Fig. 7*A*). We determined that human IgM was strikingly effective at preventing diabetes, with complete protection of treated NOD mice lasting for >12 weeks after therapy was discontinued (Fig. 7*B*). Having determined that IgM immunotherapy expands thymic Tregs to promote long-term diabetes prevention and reversal, we modeled this effect in the humanized BLT mouse to assess the response of human Tregs. In this model, human T-cell development originates in the bone marrow (B) of immunodeficient NSG mice from human hematopoietic, liver-derived (L) CD34$^+$ stem cells passing through human thymic (T) development (34) (Fig. 7*C*). Treatment of a cohort of BLT mice with human IgM resulted in a doubling of the Treg proportion within the CD4 T-cell compartment (Fig. 7*D*); these expanded Tregs had a Helios$^+$Foxp3$^+$ phenotype that is indicative of thymus-derived Tregs.

DISCUSSION

This study introduces a previously unrecognized pathologic process in T1D: loss of the protective capacity of the natural IgM. These data demonstrate the importance of natural IgM in endogenous regulation of B lymphocytes in T1D and connect circulating IgM to thymic B-cell and Treg development, promoting normal immune homeostasis. Future work will determine how this process fails in NOD mice and how this finding may apply in humans during progression to T1D.

Naturally occurring polyclonal IgM from healthy donor animals and humans was highly effective not only in preventing diabetes occurrence but also in reversing new-onset disease. Although i.v. Ig therapy has been evaluated previously in patients with T1D, these infusions were not successful (35). These products contain relatively low amounts of IgM, which our investigation suggests is the key immunomodulatory component for the treatment of T1D. Of note, some new Ig preparations, such as Pentaglobin, are enriched in IgM, although they are still not purified IgM preparations and contain relatively low amounts of IgM (36). These preparations have not been evaluated in T1D or other autoimmune disorders but have been effectively applied in sepsis, suggesting that this approach would not cause deleterious immunosuppression in patients with T1D.

The current results suggest that IgM therapy in the NOD mouse in part enhances immune function as is evidenced by the substantial increase in B-lymphocyte numbers (Fig. 1*C*). This effect positions therapy with IgM as an important alternative to immune depletion, which has been ineffective in T1D treatment to date but has remained the paradigm for most clinical approaches to autoimmunity (10,11,37). Indeed, enrichment of immune development may be a critical mechanism to address the defective B-lymphocyte selection that allows the emergence of islet-reactive B lymphocytes that drive disease. This interpretation is supported by animal model studies in which animals with lower B-lymphocyte numbers allow more autoreactive cells to escape to maturity (38,39). This escape typically occurs at the stage of development known as the transitional stage, which is when B-lymphocytes emerge from the bone marrow to the periphery and sample circulating antigens and is similar to patients with B-cell immunodeficiency in whom B-cell autoreactivity also is increased (40,41). NOD mice have a loss of the transitional B-cell compartment as they age; restoration of B-lymphocyte numbers has been demonstrated genetically to improve B-lymphocyte selection and reduce autoreactive lymphocyte numbers (42). Similarly, IgM treatment increased transitional B-cell numbers while eliminating insulin-reactive B lymphocytes and IAA production (Fig. 2*C–E*). Individuals with T1D also have a decrease in circulating transitional B-cell numbers in the blood (3). NOD mice have additional B-lymphocyte developmental abnormalities, including an accumulation of marginal zone

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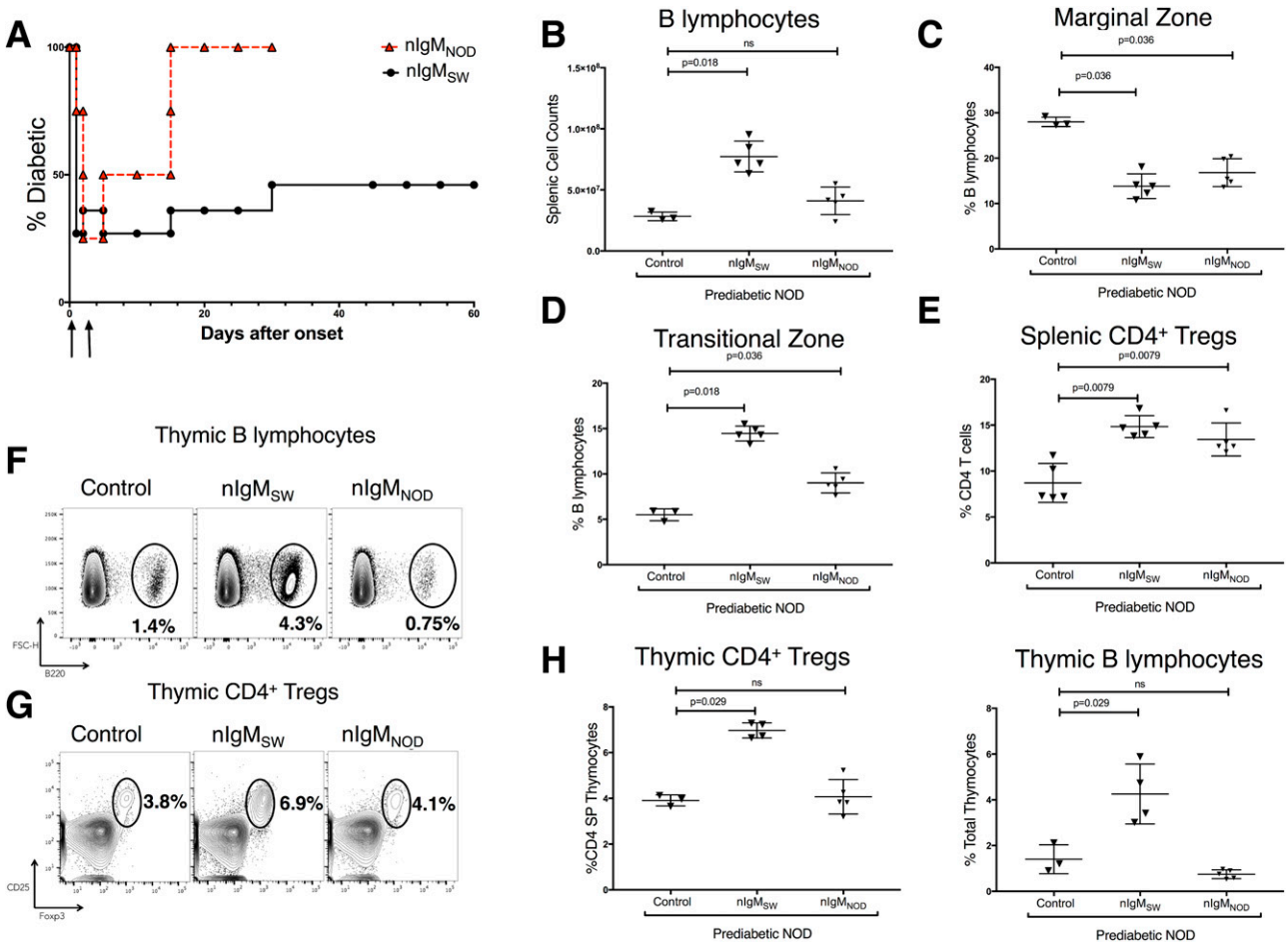

Figure 6— nIgM$_{NOD}$ does not reverse diabetes and lacks some of the immunomodulatory capacity of nIgM$_{SW}$. *A*: Injection of IgM derived from prediabetic NOD donors (IgM$_{NOD}$) did not reverse diabetes in NOD mice. Mice were administered two doses of 100 μg IgM$_{NOD}$. Although all treated mice showed a brief reprieve in high blood glucose, all mice had recurrent and permanent hyperglycemia shortly thereafter. Comparison shown with IgM$_{SW}$-treated mice from Fig. 1. *B–D*: IgM$_{NOD}$ did not increase total B-lymphocyte numbers, although it modestly decreased marginal zone B cells (*C*) and modestly increased transitional B lymphocytes (*D*) but not to the level of treatment with IgM$_{SW}$. *E*: IgM$_{NOD}$ expanded splenic Tregs in NOD mice. *F*: IgM$_{NOD}$ did not expand B lymphocytes in the thymuses of NOD mice. *G* and *H*: IgM$_{NOD}$ also failed to expand Tregs in the thymuses of NOD mice. This is quantified in *H*. FSC-H, forward scatter height; ns, not significant; SP, single positive.

B cells, which have been suspected to contribute to pathogenesis (43,44). Treatment with IgM similarly targeted this differentiation step to produce normal B-lymphocyte frequencies.

B lymphocytes are believed to contribute to T1D pathogenesis primarily through activation of islet-reactive T lymphocytes. The presence of autoreactive B lymphocytes is an absolute requirement for disease pathogenesis in the NOD mouse model (45). Similarly, patients at risk for T1D can be stratified by the presence of autoantibodies in their serum (2). Increasing numbers of serum autoantibodies confer substantial increases in T1D risk, with the presence of two or more autoantibodies now conferring a diagnosis of stage 1 T1D (9). Additional studies in the animal model have demonstrated that B lymphocytes primarily interact with CD4 T cells through MHC class II interactions and drive epitope spreading, leading to diversification of the immune response against the pancreas (46,47). We now establish that treatment with nIgM interferes with this pathologic process by eliminating instigating B lymphocytes in the periphery (Fig. 2*E*). In addition, the current data suggest a previously unrecognized mechanism by which B lymphocytes may control T1D pathogenesis. In this study, we demonstrate that treatment with IgM leads to an expansion of thymic B cells that yields diabetes-preventing Tregs (Fig. 4). Analysis of the thymus of B6 and NOD mice suggested that B lymphocytes in untreated NOD mice reside more in the cortex than in the medulla, which may prevent them from fostering Treg development or could lead to Treg deletion. Studies of Treg development suggest important, but differential interactions at these key locations in the thymus, with the medullary interactions being required for Treg development (31). The role of thymic B lymphocytes is relatively new but has been clearly associated with thymic Treg development (32). The capacity of B lymphocytes to concentrate key antigens may

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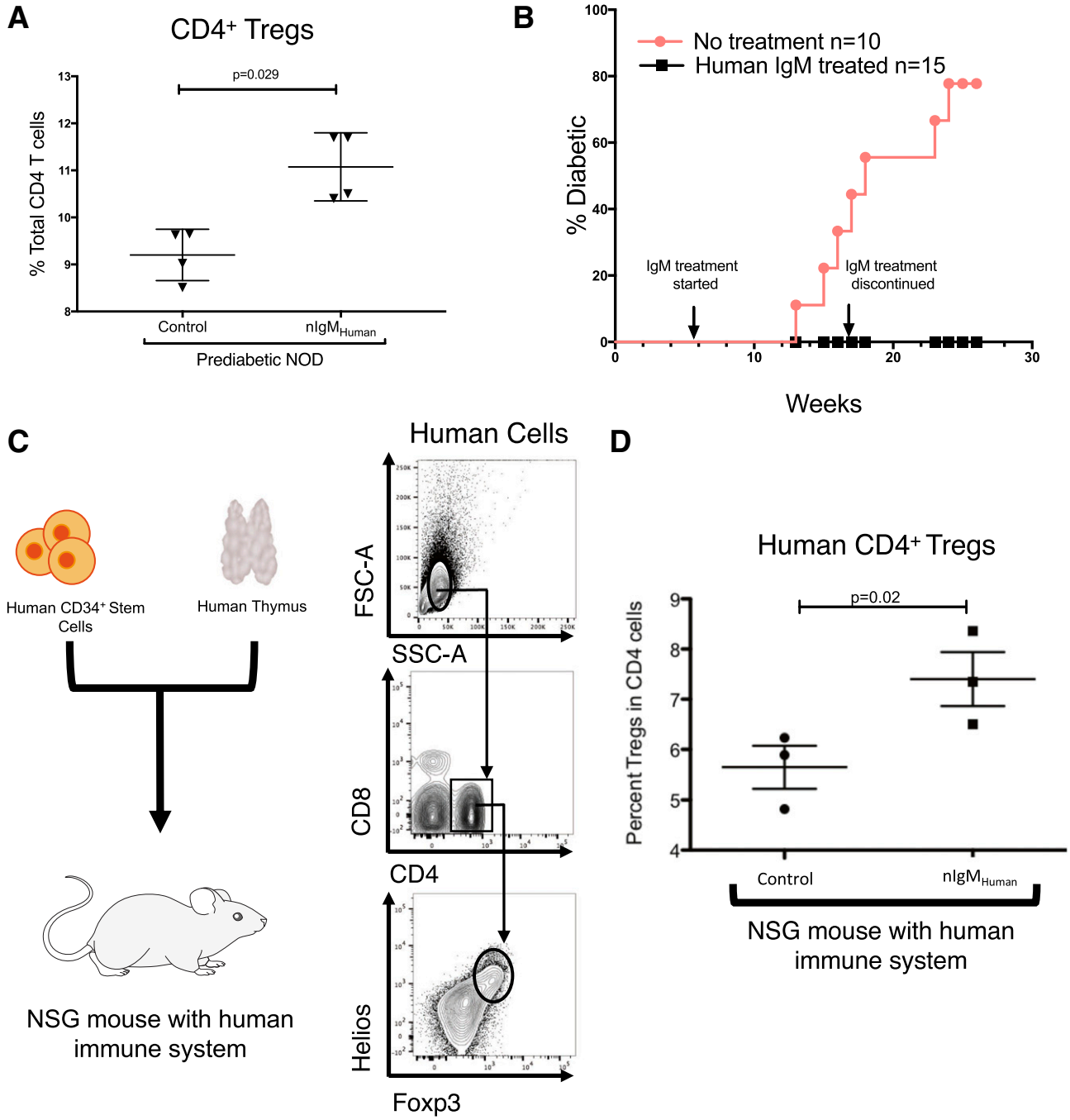

Figure 7—Human IgM expands Tregs to prevent diabetes in NOD mice and expands human Tregs. *A*: Prediabetic NOD mice were treated with human nIgM, and Tregs were measured. Mice treated with human IgM showed an increase in Helios^{+}Foxp3^{+} Tregs compared with untreated controls, demonstrating the therapeutic potential of human IgM (n = 4 in each group). *B*: NOD mice were given human IgM from healthy human donors starting at week 5 and ending at week 15. These mice did not develop overt diabetes at up to 25 weeks of age, whereas 80% of untreated mice developed diabetes by week 25. *C*: Immunodeficient NSG mice were transplanted with fetal thymus and liver CD34^{+} hematopoietic stem cells and allowed for the immune system to reconstitute. Illustrated is the flow cytometry gating scheme to identify human Tregs. *D*: Human IgM expanded human Tregs in the NSG humanized mouse system. FSC-A, forward scatter area; SSC-A, side scatte-A.

further modulate the Treg pool, and this interaction seems amenable to therapeutic correction with IgM. Furthermore, we have established that BAFF plays a complex role in the development of autoimmune disease. Many have found its effect deleterious in progression, whereas we demonstrate in the context of nIgM that it is essential for the expansion of Tregs in the thymus, suggesting that its effect may be both induced and modified by treatment.

Although endogenous IgM from healthy individuals and nonautoimmune mice profoundly improved immune

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function in T1D, IgM derived from prediabetic NOD donors did not reverse diabetes and failed to induce thymic Treg expansion (Fig. 6). This finding suggests that loss of this endogenous regulatory mechanism may be an important contributor to the fundamental pathogenesis of disease. At this point, we do not know whether NOD mice are deficient in this capacity from birth or whether the deficiency develops later. A striking change in B-cell development with loss of the transitional zone occurs between 4 and 8 weeks in NOD mice, which could lead to shifts in antibody production (42). However, many of these IgMs are expected to arise from the innate-like B1 B-cell compartment, which has been associated with diabetes pathogenesis rather than with protection, suggesting that it may be deficient in producing this regulatory component (48). Nonetheless, NOD mice have normal concentrations of circulating IgM (49).

A receptor for the Fc portion of IgM (FcμR or TOSO) is known, and we found it to be expressed on B lymphocytes but not Tregs (data not shown). Animals deficient in TOSO or missing secreted IgM also demonstrate abnormal B-lymphocyte development with loss of the transitional zone and accumulation of autoreactive specificities, which is highly similar to B-lymphocyte biology on the NOD background (24,25,50). Whether other biophysical alterations exist in the NOD IgM, such as changes in glycosylation, folding, or other modifications, is not known but is an important area for future investigation.

Previous studies have focused on the positive effects of IgM on immune function. These studies largely focused on IgM derived from C57BL/6J mice administered at supraphysiologic doses in an autologous manner or, as in our own research, into NOD mice. These results neglect the clinical reality that IgM for therapeutic intervention will most likely be administered from diverse and possibly multiple donors. In the current study, we address this clinical caveat by using IgM from the outbred SW mouse. By using this model, we were able to assess an important control by administering this intervention to both NOD and C57BL/6J mice. In this way, we could appreciate mechanistic biomarkers that may provide clues to therapeutic responsiveness in patients (increase in circulating BAFF) while defining other immunologic reactions that may not be required for the therapeutic activity of nIgM (expansion of MDSCs). The capacity to measure many of these biomarkers, including Tregs in circulation, B-lymphocyte subsets, serum BAFF levels, thymic output through thymic excision circles, and insulin-reactive B lymphocytes through recently described methods, now exists in humans and suggests pathways to support successful clinical translation (51). These effects on immune phenotypes were consistent batch to batch among all IgMs isolated from SW Webster mice. Furthermore, we were able to use autologous transfer of IgM from NOD mice back into NOD recipients to define further the immunologic responses necessary to promote permanent reversal in mice (expansion of thymic Tregs).

Overall, polyclonal IgM represents a new approach to harness endogenous regulatory mechanisms to support rather than deplete immune function to restore normal immune regulation in new-onset T1D. This approach represents a new opportunity to address the central problem of autoreactive B-cell development and function that impedes current approaches. It suggests as well that changing regulatory IgM function over time could contribute to progression toward clinical T1D. Future studies to identify the biologically optimal IgMs and their role in pathogenesis should speed translation to the clinic.

Acknowledgments. The authors thank James W. Thomas (Vanderbilt University) for providing the V_H125^{SD} mice on the NOD and B6 backgrounds and David Serreze (The Jackson Laboratory, Bar Harbor, ME) for providing NOD.μMT mice. The authors also thank Dr. Jonathan Irish and Caroline Maier of the Vanderbilt Mass Cytometry Core for assistance in developing the panels used in this work. Finally, the authors thank Dale Greiner and Michael Brehm (University of Massachusetts, Worcester, MA) for assistance with developing the humanized mouse model.

Funding. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant F31-DK-107321 (to C.S.W.), the Focus to Cure Diabetes Foundation (to K.L.B.), an American Diabetes Association Innovative Basic Science Award (to K.L.B. and D.J.M.), a JDRF Career Development Award (to D.J.M.), National Institute of Allergy and Infectious Diseases grant R21-AI-119224 (to D.J.M.), and a Vanderbilt Digestive Disease Pilot grant (to D.J.M.). This work also used the cores of the Vanderbilt Diabetes Research and Training Center funded by National Institute of Diabetes and Digestive and Kidney Diseases grant DK-020593.

Duality of Interest. P.C. and K.L.B. are listed on patent 20150265704 for use of IgM in Type 1 diabetes. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. C.S.W., P.C., A.F.M., C.V.M., B.T.S., E.M.H., R.H.B., G.P., K.L.B., and D.J.M. designed, executed, and analyzed the experiments. C.S.W., P.C., K.L.B., and D.J.M. wrote the manuscript, which all authors reviewed. D.J.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented at the 77th Scientific Sessions of the American Diabetes Association, San Diego, CA, 9–13 June 2017.

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