Targeting CD22 reprograms B cells and reverses autoimmune diabetes

Paolo Fiorina MD PhD1,2, Andrea Vergani MD1,2, Shirine Dada MD1, Mollie Jurewicz BA1, Masie Wong BS1, Kenneth Law BS3, Erxi Wu PhD4; Ze Tian PhD4, Reza Abdi MD1, Indira Guleria, PhD1, Scott Rodig MD PhD3, Kyri Dunussi-Joannopoulos MD PhD5, Jeffrey Bluestone, PhD6 and Mohamed H. Sayegh, MD1

1Transplantation Research Center (TRC), Children's Hospital and Brigham and Women's Hospital, Harvard Medical School, Boston, USA. 2Medicine, San Raffaele Scientific Institute, Milan, Italy. 3Department of Pathology, Division of Hematopathology, Brigham & Women's Hospital. 4Children’s Hospital Informatics Program at the Harvard–MIT Division of Health Sciences and Technology, Boston, USA. 5Inflammation, Wyeth Research, Cambridge, MA 02140. 6University of California San Francisco (UCSF) Diabetes Center, San Francisco, CA.

Address for correspondence:  
Mohamed H. Sayegh, M.D.  
Transplantation Research Center (TRC)  
221 Longwood Avenue  
Boston, MA 02115  
E-mail: msayegh@rics.bwh.harvard.edu

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ABSTRACT

Rationale: A B cell-depleting strategy to reverse diabetes has not been fully investigated in naïve NOD mice.

Objectives: We targeted the CD22 receptor on B cells of naïve NOD mice to deplete and reprogram B cells to effectively reverse autoimmune diabetes.

Findings: Anti-CD22/calcimAb therapy resulted in early and prolonged B cell depletion and delayed disease in prediabetic mice. Importantly, when new onset hyperglycemic mice were treated with the anti-CD22/calcimAb, 100% of B cell-depleted mice became normoglycemic by 2 days, and 70% of them maintained a state of long-term normoglycemia. Early therapy after onset of hyperglycemia and complete B cell depletion are essential for optimal efficacy. Treated mice showed an increase in percentage of regulatory T cells in islets and pancreatic lymph nodes, as well as a diminished immune response to islet peptides in vitro. Trascriptome analysis of re-emerging B cells showed significant changes of a set of pro-inflammatory genes. Functionally, re-emerging B cells failed to present autoantigen and prevented diabetes when co-transferred with autoreactive CD4+ T cells into NOD.SCID hosts.

Conclusions: Targeting CD22 depletes and reprograms B cells and reverses autoimmune diabetes, thereby providing a blueprint for development of novel therapies to cure autoimmune diabetes.
Although B cells have been primarily considered antibody-producing cells, recent studies demonstrate that they participate in the priming of autoimmune responses (1; 2). Many investigations have examined the role of B lymphocytes as antigen-presenting cells (APCs) in the generation of autoreactive T cell responses (3; 4). The role of B cells in one of the most classical autoimmune disorders, type 1 diabetes (T1D), a disease characterized by insulin deficiency resulting from the autoimmune destruction of β cells, is controversial (5). Indeed, most individuals affected by T1D exhibit multiple features associated with impaired B cell function, including autoantibodies against a variety of islet cell antigens (6; 7). Data from different groups using NOD mice, the best animal model for the study of T1D, have confirmed the importance of B cells in the onset of diabetes (2-4; 8; 9). NOD mice that are deficient in B cells have been shown to be protected from autoimmune diabetes (3; 10; 11) and are deficient in the development of a T cell response to major autoantigens (such as 65-kDa glutamate decarboxylase-GAD65) (3; 10; 11). In humans, the production of autoantibodies to islet antigens is well-documented to be an early indicator of disease onset (12). These observations render B cell-targeting a particularly attractive and novel strategy for the treatment of T1D (13-15).

Unfortunately, this strategy has not been fully described in naïve NOD mice. Only recently did a publication show the positive effects of an anti-CD20-based B cell-depleting strategy in transgenic NOD mice expressing the humanized CD20 receptor on B cells (8). Interestingly, use of B cell depletion as a therapy for human autoimmune disease (16-20) including in patients with new onset T1D is ongoing (21; 22).

We made use of a newly-developed reagent [anti-CD22 calicheamicin-conjugated mAb (anti-CD22/cal mAb)] that efficiently depletes mature B cells in mice (13) to establish a therapeutic approach for T1D. Our main hypothesis was that depleting B cells by targeting CD22 should prevent diabetes onset and restore normoglycemia in newly hyperglycemic NOD mice. Furthermore, we hypothesize that our approach will generate a pool of re-emerging B cells that may function to regulate the autoimmune response in vivo, establishing a state of long-term tolerance toward autoantigens.

MATERIALS AND METHODS

A complete description of our methods can be found in the online supplement.

RESULTS

**CD22 is widely expressed on mature B cells in NOD mice.** We first examined CD22 expression on B cells in NOD mice. No differences were observed in terms of CD19 and CD22 expression on B220+ cells (B220+CD19+ cells=82.0±2.5 vs. B220+CD22+ cells=83.1±2.7 %, ns) (Figure 1A-1B). Furthermore, CD22 is expressed on CD138+ cells (Figure 1C).

**B cells represent the majority of infiltrating cells in the pancreata of NOD mice.** The kinetics of CD45+CD19+ (B cells) infiltration in the pancreata of NOD mice showed a significant increase at 8 weeks, when the mice began to exhibit islet peri-infiltration (from 22.8±7.8 at 4 weeks up to 65.1±5.0% at 8 weeks of age, p<0.01) (Figure 1D). This increase was not observed for CD45+CD3+ cells (T cells) (Figure 1E). Indeed, the percentage of CD45+CD19+ cells (B cells) was significantly higher than CD45+CD3+ cells (T cells) in the pancreata of 8-week-old NOD mice (B cells=65.1±5.0 vs. T cells=30.2±3.2% p=0.004), (Figure 1E).

**Anti-CD22/cal mAb produces a profound depletion of B cells in NOD mice.** We first treated NOD mice with anti-CD22/cal mAb to evaluate whether our antibody can successfully deplete B cells in NOD mice.
Two injections (160 µg/Kg i.p. 5 days apart, D0-D5) of anti-CD22/cal mAb elicited a quick and profound depletion of B cells in the peripheral blood of 10-week-old NOD mice; the effect appears at 1 week and lasts for 5 to 7 weeks (Figures 1F and 1H). Control NOD mice did not appear to be depleted of B cells (Figures 1F and 1G), while the group treated with an equivalent dose of unconjugated anti-CD22 mAb (0.2 mg/injection i.p. 5 days apart, D0-D5) showed transient and partial B cell depletion (Figures 1F and 1I). B cell recovery was complete by 8-10 weeks after therapy (Figures 1F and 1H). No B cell depletion was evident when using the control mAb, a mouse IgG1 anti–rat very late antigen 4 (VLA-4) mAb which does not bind to mouse cells and is conjugated to calicheamicin (GG5/cal), (data not shown).

**Anti-CD22/cal mAb delays diabetes onset in prediabetic NOD mice.**

The effect of anti-CD22/cal mAb treatment on diabetes onset was evaluated in female 10-week-old NOD mice. NOD mice were treated with 2 injections of 160 µg/Kg of anti-CD22/cal mAb 5 days apart and were monitored for diabetes development. As shown in Figure 2A, we observed a significant delay in diabetes onset in the anti-CD22/cal mAb-treated mice (n=20 mice, 50% protected in the long-term) compared to untreated controls (n=30 mice, p<0.01, 10% protected in the long-term) and to the group treated with calicheamicin alone (GG5/cal) (n=10 mice, p<0.01), (Figure 2A).

We also treated 10-week-old female NOD mice with the unconjugated anti-CD22 mAb that only partially depletes B cells (n=20), (Figures 1F and 1I). Diabetes onset was slightly delayed compared to controls (p=0.06 Figure 2A). This indicates that complete B cell depletion is required to induce long-term protection from diabetes as well as stable tolerance toward autoantigens.

**Anti-CD22/cal mAb treatment in prediabetic mice is associated with an increase in the percentage of CD4^+CD25^+FoxP3^+ cells in the pancreatic lymph nodes (PLn).** We also examined the effect of B cell depletion on T cell phenotype in NOD mice.

A significant increase in the percentage of CD4^+CD25^+FoxP3^+ cells is evident at 35 weeks of age (but not at 15 weeks) in the PLn of normoglycemic treated mice compared to both untreated hyperglycemic and normoglycemic untreated control NOD mice (anti-CD22/cal mAb-treated = 20.3±3.1 vs. normoglycemic control = 8.1±0.6, p=0.02 and vs. hyperglycemic control = 7.7±3.1%, p=0.009, Figure 2B).

**Hyporesponsiveness of CD4^+ T cells towards autoantigen in anti-CD22/cal mAb-treated NOD mice.** We sought to determine whether B cell depletion can modify BDC2.5 peptide-driven IFN-γ production of T cells, which can be considered an index of the T cell anti-islet response (23). CD4^+ T cells extracted from splenocytes of normoglycemic anti-CD22/cal mAb-treated and normoglycemic control NOD mice were isolated at 15 and 35 weeks of age and were challenged with the BDC2.5 peptide and syngeneic DC in an ELISpot assay to evaluate IFN-γ production. Only at 35, but not at 15, weeks of age, was the frequency of CD4^+ T cells extracted from normoglycemic treated animals responding to autoantigen significantly reduced as compared to responding CD4^+ T cells of normoglycemic 10-week-old and hyperglycemic untreated NOD mice (Figure 2C). Interestingly, CD4^+ T cells extracted from splenocytes obtained from a normoglycemic anti-CD22/cal mAb-treated NOD mice at 15 and 35 weeks of age are capable of mounting an immune response to alloantigen similar to the response by CD4^+ T cells extracted from normo- or hyperglycemic untreated control NOD mice (at 35 weeks: anti-CD22/cal mAb-treated = 15210±5524 vs. normoglycemic control = 11863±2470, ns and vs. hyperglycemic control = 12389±897 ³H-thymidine incorporation counts per minute,
p=ns), indicating that the CD4⁺ T cells are indeed immunocompetent. **Lack of B cells prevent expansion of autoreactive T cells in an adoptive transfer model.** We then tracked the effect of B cell depletion on survival and proliferation of autoreactive CD4⁺ T cells in vivo (24-26). NOD.SCID mice were reconstituted with splenocytes from normoglycemic 10-week-old NOD mice. After 7 days (thereby allowing reconstitution of the immune system), mice were either treated with anti-CD22/cal mAb or were left untreated. After another 7 days, in order to allow ample time for B cell depletion, isolated BDC2.5 TCR Tg⁺ CD4⁺ cells extracted from splenocytes were transferred into B cell-depleted or untreated NOD.SCID mice. After 72 hours mice were euthanized and examined for autoreactive CD4⁺ cells frequency in the spleen of recipients (easily tracked using the anti-ideotypic antibody against the Vβ4 chain of the TCR receptor), (26). Interestingly, when B cells are absent, fewer BDC2.5 TCR Tg⁺ CD4⁺ cells can be recovered from the host (reduction of 50%), (Figure 2D and Figure 2E, lower quadrant) compared to the anti-CD22/cal mAb-treated NOD mice (Figure 2D and Figure 2E, upper quadrant). **Islets in anti-CD22/cal mAb-treated mice showed reduced infiltration and preserved morphology even after complete B cell recovery.** In the anti-CD22/cal mAb-treated NOD mice at 15 weeks of age, infiltrates were reduced compared to untreated control NOD mice and baseline untreated 10-week-old normoglycemic NOD mice (Figures 3A1, 3B1 and 3C1). As expected, B220⁺ cells were very few in the anti-CD22/cal mAb-treated NOD mice, but not in the control and in the baseline group (Figures 3A2, 3B2 and 3C2). Surprisingly, very few CD3⁺ cells were apparent in the anti-CD22/cal mAb-treated NOD mice, but not in the control and in the baseline group (Figures 3A3, 3B3 and 3C3). Insulin (Figures 3A5, 3B5 and 3C5) and glucagon (Figures 3A6, 3B6 and 3C6) staining showed well preserved islets in all the 3 groups. An increase in FoxP3⁺ cells was evident within the islets of anti-CD22/cal mAb-treated compared to the baseline and the untreated control group (Figures 3A4, 3B4 and 3C4).

At 35 weeks of age, despite the complete recovery of the B cell pool in the originally anti-CD22/cal mAb-treated group, pancreatic islets appeared to contain much less infiltrate than untreated control hyperglycemic NOD mice (Figures 3D1 and 3E1). Again, neither the B220⁺ nor the CD3⁺ cells infiltrated the islets but instead remained at the islet border in the anti-CD22/cal mAb-treated group, but not in the control group (Figures 3D2, 3E2 and 3D3, 3E3). Insulin and glucagon staining, confirmed the presence of many well-preserved islets in the anti-CD22/cal mAb-treated, but not in the control group (Figures 3D5, 3E5 and 3D6, 3E6). FoxP3 staining of islet infiltrate revealed reduced FoxP3 expression, particularly when compared with the massive presence of T cells, in the untreated control but not in the anti-CD22/cal mAb-treated NOD mice (Figure 3D4 and 3E4).

Finally, insulitis score revealed more well-preserved islets (0-50% of infiltration) in the anti-CD22/cal mAb-treated compared to untreated control NOD mice (Figure 2G). **Anti-CD22/cal mAb treatment restores normoglycemia in newly hyperglycemic NOD mice.** Newly hyperglycemic female NOD mice (defined on the basis of glucose levels higher than 250 mg/dl for 3 consecutive days) were treated with a protocol identical to what is outlined above using the anti-CD22/cal mAb. A rapid reversal of hyperglycemia (within 2 days) was observed in all the B cell-depleted NOD mice (10 out of 10; Figure 4A). 6 out of 10 remained normoglycemic for 20-40 days and then
reverted to hyperglycemia (Figure 4A). 1 mouse remained normoglycemic for more than 50 days and then reverted to hyperglycemia (Figure 4A). No consistent correlation was evident between glucose levels at baseline and the ability to restore or maintain normoglycemia after treatment (data not shown). None of the control NOD mice (n=10) ever reverted from hyperglycemia spontaneously after 3 consecutive days of hyperglycemia (Figure 4B). When hyperglycemic NOD mice were treated with anti-CD22/cal mAb after 5 days (n=6) from hyperglycemia onset, a transient return to normoglycemia was evident in 5 out of 6 NOD mice, which lasted for 20 days (Figure 4B); subsequently, all mice then reverted to hyperglycemia. These data suggest that for treatment to be most effective, it must be initiated early after onset of hyperglycemia. We then treated newly hyperglycemic NOD mice with GC5/cal (n=5), and no effect was observed in 3 out of 5 mice treated, while in 2 NOD mice treated some glycemic oscillations were observed before a return to stable hyperglycemia (Figure 4B). Finally, B cell depletion appeared to be mandatory for the restoration of normoglycemia. In fact, when B cells composed more than 3-5% of the blood, normoglycemia was not restored. Only 2 out of 10 partially depleted (with unconjugated CD22 treatment) hyperglycemic NOD mice showed a transient return to normoglycemia with a quick reappearance of hyperglycemia (Figure 4B). Taken together, these data clearly indicate that optimal therapeutic efficacy for reversal of diabetes requires early initiation of therapy that effectively depletes B cells in hyperglycemic animals.

**Anti-CD22/cal mAb treatment reduces pro-inflammatory peripheral cytokine levels in hyperglycemic NOD mice.** Remarkably, a change in peripheral cytokine levels was observed during the restoration of normoglycemia (Figure 4C). Most pro-inflammatory cytokines were reduced 10 days after treatment, when normoglycemia was restored (Figure 4C). Particularly, 10 days after injection in the normoglycemic anti-CD22/cal mAb-treated mice, IL-17 levels were significantly reduced compared to hyperglycemic untreated controls (p=0.01). Interestingly, while in the long-term hyperglycemic untreated control NOD mice, peripheral levels of TNF-α (p=0.06), IL-17 (p=0.03) and IFN-γ (p=0.03) were higher than baseline hyperglycemic NOD mice, those normoglycemic NOD mice treated with anti-CD22/cal mAb showed peripheral levels of pro-inflammatory cytokines similar to newly hyperglycemic NOD mice (Figure 4C).

In the course of reversal anti-CD22/cal mAb treatment is associated with changes in the percentage of CD4^+CD25^+FoxP3^+ cells. The percentage of CD4^+CD25^+FoxP3^+ cells was significantly increased in normoglycemic anti-CD22/cal mAb-treated long-term tolerant mice compared to hyperglycemic untreated control NOD mice, in both the PLn (anti-CD22/cal mAb-treated= 14.3±1.9 vs. normoglycemic 10-week-old=6.4±0.7, p=0.007 and vs. hyperglycemic=8.7±1.1%, p=0.03, Figure 4D) and in the spleen (anti-CD22/cal mAb-treated = 12.8±1.7 vs. normoglycemic 10-week-old = 7.9±0.4, p=0.02 and vs. hyperglycaemic = 8.4±0.2%, p=0.01, Figure 4E).

100 days post-injection Teffs/Tregs ratio in the PLn and spleen was similar between normoglycemic anti-CD22/cal mAb-treated and hyperglycemic untreated control NOD mice (data not shown).

**Islets from anti-CD22/cal mAb-treated NOD mice demonstrated an absence of T and B cell infiltrates long after B cell recovery.** After 3 days of hyperglycemia, islets are extensively infiltrated by lymphoid cells (Figure 5A1) with disrupted structure, marked reduction in insulin staining and a smaller reduction in glucagon staining (Figures 5A5 and 5A6). The lymphoid infiltrate is composed predominately of B220^+ cells with
a smaller population of CD3+ cells (Figures 5A2 and 5A3) and very few FoxP3+ cells (Figure 5A4).

After 10 days, pancreas histology and immunohistochemistry in untreated control NOD mice all the above features worsen (Figures 5B1-5B3 and 5B5, 5B6). Surprisingly, in the anti-CD22/cal mAb-treated NOD mice 10 days after treatment, islets showed very mild infiltrates confined to the borders of β cells (Figure 5C1) with an almost complete absence of B220+ and CD3+ cells (Figures 5C2 and 5C3) and well-maintained and preserved insulin and glucagon staining (Figures 5C5 and 5C6). In the anti-CD22/cal mAb-treated NOD mice, but not in the controls, after 10 days from treatment, more infiltrating cells appeared to be FoxP3+ cells (Figures 5B4 and 5C4).

100 days after treatment, in the anti-CD22/cal mAb-treated NOD mice, two histological patterns were observed; islets still appeared almost completely free of infiltrates (Figure 5D1) with very few B220+ and CD3+ cells inside islets (Figures 5D2 and 5D3). Many small but well-preserved islets were present in the pancreas (Figures 5D5 and 5D6) without a clear increase in FoxP3+ cells (Figure 5D4). A smaller subset of islets showed an abundant infiltrate of B220+ and CD3+ B cells (Figures 5E1, 5E2 and 5E3), which remained however largely confined to the periphery of the islets.

Phenotype of re-emerging B cells in contrast to naïve B cells.

We examined the gene expression profile of re-emerging B cells (obtained from normoglycemic NOD mice treated with anti-CD22/cal mAb 100 days after B cell depletion) and compared it to that of B cells obtained from naïve normoglycemic 10-week-old or hyperglycemic untreated control NOD mice. CD19+ cells were extracted from splenocytes with microbeads. Interestingly, a significant downregulation of inducible gene transcription was observed within the re-emerging B cell pool. Almost 200 genes were downregulated in re-emerging B cells compared to B cells extracted from normoglycemic 10-week-old NOD mice (Figure 6A), and 38 genes were downregulated in re-emerging B cells compared to B cells extracted from hyperglycemic NOD mice (Figure 6B). When all 3 groups of B cells were compared (naïve normoglycemic 10-week-old NOD mice, naïve hyperglycemic NOD mice and re-emerging), 21 genes appeared to be downregulated in the re-emerging B cell population (Table 1 and Figure 6C). It should be noted that the downregulation of many extracellular lytic enzyme products (elastase 1 and 2, lysozime, chymotrypsinogen B1, amylase) may be associated with directed islet damage or a sustained pro-inflammatory effect (Table 1 and Figure 6C). Genes of the complement cascade (Fcna, C1qb) and pro-inflammatory (Hebp1, Pilrb1, PPARγ and Hmox-1) genes are downregulated in re-emerging B cells as well. Hebp1 (heme binding protein 1) has been recently shown to be involved in monocyte chemotaxis (27); Pilrb1 (paired immunoglobulin-like type 2 receptor beta 1) is a receptor that can activate NK cells, DC and monocytes (28); PPARγ has been related to DC/platelet activation/function (29); and heme oxygenase-1 (Hmox-1) is an anti-oxidant gene (Table 1 and Figure 6C).

Transcriptome analysis revealed a reprogramming of re-emerging B cells compared to naïve B cells.

We then analyzed by FACS the proportion of different B cell subpopulations before B cell depletion and after B cell reconstitution with respect to expression of CD80, CD86, CD40, IgM and
MHC class II and with respect to the presence of anergic B cells; the latter can be identified as a small population of B220⁺CD93⁺CD23⁻IgMlo cells (30).

No differences were detected in CD80, CD86, CD40, IgM and MHC class II expression between naïve and re-emerging B cells obtained from splenocytes (too few B cells can be recovered from PLn) of normoglycemic treated NOD mice (Figure 7A). No differences were detected in the frequency of marginal zone B cells or B cell subpopulations as well (Figure 7A). A small percentage of anergic B cells is evident in naïve B cells from either normo- or hyperglycemic untreated control NOD mice (Figure 7B), while in the re-emerging B cell population obtained from normoglycemic treated NOD mice an increase of B220⁺CD93⁺CD23⁻IgMlo cells was detected, with a restoration of the original frequency found in naïve NOD mice, which was reduced in hyperglycemic NOD mice (Figure 7B).

**Re-emerging B cells have a reduced ability to present autoantigen in vitro and to reduce pro-inflammatory cytokine production by autoreactive T cells.** We also evaluated the functional ability of re-emerging and naïve B cells to present autoantigen to T cells in vitro. We designed and optimized an in vitro assay in which B cells are used as APCs and autoreactive BDC2.5 TCR Tg⁺ CD4⁺ cells are used as responders in the presence of the BDC2.5 peptide. While B cells from hyperglycemic untreated control NOD mice were capable of presenting autoantigen and stimulating IFN-γ production by CD4⁺ T cells (Figure 7C), re-emerging B cells obtained from normoglycemic treated NOD mice were defective in this capacity (Figure 7C). In the same experiment, we evaluated the ability of re-emerging B cells to modulate cytokines profile of autoreactive BDC2.5 TCR Tg⁺ CD4⁺ cells; supernatant was therefore collected and cytokines evaluated with a Luminex assay. Interestingly, when re-emerging B cells, but not naïve B cells, were used, BDC2.5 TCR Tg⁺ CD4⁺ cells downregulated their production of pro-inflammatory cytokines (Figures 7F-I). Particularly, when re-emerging B cells obtained from normoglycemic treated NOD mice were used, BDC2.5 TCR Tg⁺ CD4⁺ cells produced less TNF-α compared to naïve B cells extracted from normoglycemic and hyperglycemic untreated control NOD mice (p<0.05 vs. both), less IL-17 compared to hyperglycemic untreated control NOD mice (p=0.004), and less IFN-γ compared to hyperglycemic untreated control NOD mice (p=0.02) (Figures 7F-I).

**Re-emerging B cells are regulatory in vivo and halt the transfer of diabetes from diabetogenic CD4⁺ T cells to NOD.SCID recipients.** To compare the regulatory functions of re-emerging and naïve B cells in vivo, diabetogenic CD4⁺ T cells extracted with magnetic beads from splenocytes of normoglycemic anti-CD22/cal mAb-treated mice at 100 days (i.e. re-emerging B cells) or from untreated control NOD mice from our prevention studies at 35 weeks of age. Interestingly, when B cells from untreated controls were transferred, NOD.SCID developed diabetes (particularly when naïve B cells were extracted from hyperglycemic NOD mice) (Figure 7D). Conversely, when re-emerging B cells were used, the onset of diabetes mediated by the transfer of CD4⁺ T cells from hyperglycemic NOD mice was completely abrogated (Figure 7D). In order to determine whether this protection is related to induction/expansion of Tregs in vivo we analyzed the percentage of CD4⁺CD25⁺FoxP3⁺ cells (Tregs) in spleen of the NOD.SCID recipients of the diabetogenic CD4⁺ T cells and re-emerging B cells or
controls (B cells from hyperglycemic animals or no cells) at day 30 post-adoptive transfer. As seen in figure 7E no differences were detected among the three groups. These data suggest that the re-emerging B cells may function to inhibit autoreactivity by a mechanism distinct from induction/expansion of Tregs, although further studies are required to define the exact mechanisms in vivo.

**DISCUSSION**

B cell-depleting strategies are rapidly growing in popularity as a treatment approach for autoimmune diseases, thanks to the availability of an anti-CD20 mAb in humans. A recent paper from Yale University (8) showed that the human anti-CD20 mAb is capable of preventing autoimmune diabetes and reverses established diabetes in transgenic NOD mice expressing the human CD20 receptor on B cells. Another recent paper showed that a murine anti-CD20 protects from diabetes onset when given at an early time point (4 weeks) and delays diabetes onset when given later on (15 weeks) (31). In this paper no data on reversal of diabetes were reported and B cell depletion was not complete (5% of B cells were still found in NOD mice).

No data were Our approach is novel for the following reasons: (i) we established a B cell-depleting protocol in naïve NOD mice and not in transgenic NOD mice; (ii) we targeted a new pathway, CD22, as CD22 expression is found on more mature B cells and plasma cells, and this may also have a positive effect on autoantibody production; (iii) for the first time the complete gene profiling of naïve NOD B cells and re-emerging B cells has been identified; (iv) our therapy not only depletes B cells but also reprograms the entire pool of re-emerging B cells, generating a novel functionally-impaired and regulatory B cell population; and (v) anti-CD22 antibodies are available for human use as well, and this therapy is potentially different from an anti-CD20 approach in humans. While anti-CD20 therapy can efficiently deplete B cells in the blood, a number of reports suggest a potential incomplete depletion in lymphoid organs (32-35). This issue raises the question as to whether pancreatic B cells are depleted, and it is likely that the current ongoing trial will not clarify this issue due to the impossibility of performing pancreatic biopsies. On the contrary, anti-CD22 therapy seems to be more comprehensive regarding B cell depletion (36).

We made use of a novel agent targeting CD22 (13) to develop and study a B cell-depleting approach in naïve NOD mice as a model for human type 1 diabetes. Anti-CD22 treatment has been tested in humans, both for the immunoregulatory properties of CD22 engagement and for the possibility of depleting mature B cells, with promising results in the fields of autoimmune disease (37; 38) and B cell malignancies (39). Surprisingly, the effect of targeting CD22 has never been tested in diabetes.

Our data show that anti-CD22/αmAb treatment is capable of delaying diabetes onset in prediabetic NOD mice and, more importantly, of restoring normoglycemia in new onset hyperglycemic NOD mice. In our experiments, complete B cell depletion is required for restoration of normoglycemia; this is relevant from a clinical point of view, given that inefficient B cell depletion after anti-CD20 mAb therapy (Rituximab) is a well-recognized phenomenon that can result in poor clinical outcome (32). Our data indicate that the absence of B cells increases Tregs and reduces autoreactive T cell proliferation, highlighting the importance of a persistent interaction of B cells and autoreactive T cells in maintaining the autoimmune response.

More than 150 treatments are capable of delaying diabetes in NOD mice (40-42); however, only anti-CD3 (43) and a handful of
other strategies were found to be capable of restoring normoglycemia in NOD mice (44-46). Thus far, only the anti-CD3 regimen has been translated into clinical use in humans (47). Regarding our reversal studies, it is surprising how rapidly B cell depletion rids islets of cellular infiltrates, as even treatment with anti-CD3 mAb appeared to be slower than B cell depletion in restoring normoglycemia, and 20% of anti-CD3 treated animals did not revert from hyperglycemia (48). In our studies, 100% of hyperglycemic NOD mice reverted to normoglycemia within 2-3 days.

Our studies also show for the very first time that re-emerging B cells in NOD mice display a different phenotype confirmed by our transcriptome analysis, are functionally impaired in their ability to present antigen, and can regulate the autoimmune response resulting in long-term tolerance to autoantigens in vivo. Indeed, our adoptive transfer studies show that re-emerging B cells can abrogate the transfer of diabetes in NOD.SCID by diabetogenic CD4\(^+\) T cells.

In conclusion, we have shown for the very first time that anti-CD22 immunotherapy can deplete and reprogram B cells, thereby reversing autoimmune diabetes in naïve NOD mice. Our study provides valuable knowledge to develop an approach using anti-CD22 in patients affected by T1D.

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Table 1. Downregulated genes in re-emerging B cells compared to B cells extracted from naïve normoglycemic or hyperglycemic NOD mice.

| Symbol | Name                                      | Function                          | Process                          | Component                    |
|--------|-------------------------------------------|-----------------------------------|----------------------------------|------------------------------|
| Acp2   | acid phosphatase 2, lysoosomal            | -acid phosphatase hydrolase       | -lysosome organization and biogenesis | -lysosome                    |
| Amy2   | amylase 2                                 | -amylase/hydrolase                | -metabolic process               | -extracellular space         |
| Clqb   | complement component 1, beta polypeptide |                                   | -complement activation, immune response | -cytoplasm                   |
| Ctrb1  | chymotrypsinogen B1                       | -chymotripsin hydrolase/peptidase | -digestion/proteolysis           | -extracellular space         |
| Dgat2  | diacylglycerol O-acyltransferase 2         | -diacylglycerol O-acyltransferase | -glycerol/lipidic metabolic process | -endoplasmic reticulum       |
| Ela1   | elastase 1                                | -hydrolase/peptidase              | -proteolysis/digestion           | -extracellular space         |
| Ela2   | elastase 2                                | -hydrolase/peptidase              | -leukocyte migration, proteolysis, phagocytosis | -extracellular space         |
| Fcna   | ficolin A                                 | -receptor binding, sugar binding  | -signal transduction, complement activation | -cytoplasm                   |
| Hepb1  | heme binding protein 1                    | -heme binding                     | -heme metabolic process, chemotaxis | -cytoplasm                   |
| Hmox1  | heme oxygenase (decycling) 1              | -heme oxygenase, ion binding, oxidoreductase | -heme oxidation, immune response, stress response | -membrane, mitochondrion     |
| Hs3st2 | heparan sulfate                           | -transferase                      | -biological process              | -golgi apparatus, membrane   |
| Igf1   | insulin-like growth factor 1              | -growth factor, hormone            | -anti-apoptosis/metabolic process and cell growth, IGF pathway | -extracellular space         |
| Lyzs   | lysozime                                  | -hydrolase                        | -cell wall catabolic process/cytolysis, host defense | -extracellular space         |
| Nr1h3  | nuclear receptor subfamily 1              | -transcription factor, steroid hormone receptor | -transcription | -nucleus                    |
| Plrb   | paired immunoglobulin-like type 2 receptor beta 1 | -molecular function               | -biological process              | -membrane                   |
| Pparg  | peroxisome-proliferator activated receptor gamma | -transcription factor, receptor activity, metal ion binding | -transcription, inflammatory response, fat cell differentiation | -cytosol, nucleus            |
| Tgfb1  | transforming growth factor, beta induced   | -protein binding                  | -cell adhesion                   | -extracellular space         |
| Tgm1   | transglutaminase 1                        | -acyltransferase, ion binding      | -morphogenesis, peptide cross linking, protein metabolic process | -adherens junction, membrane |
Figure Legends

Figure 1. Depletion studies. Splenocytes were extracted from normoglycemic 10-week-old NOD mice (n=5) and were analyzed by flow cytometry for CD19 and CD22 expression on B220⁺ cells and CD138⁺ cells (plasma cells). CD19 and CD22 were similarly expressed on B220⁺ cells (Panels A and B), and CD22 is expressed on CD138⁺ cells (Panel C). We then examined by flow cytometry the infiltrating cells in the pancreata of 4, 8, 12-week-old and hyperglycemic NOD mice (> 14-weeks-old) (n=5 mice/group). Most of the infiltrate is constituted by CD45⁺CD19⁺ cells (B cells), (Panel D). B cell pancreatic infiltration in NOD mice peaks around 8 to 10 weeks (p<0.05; Panel D), while CD45⁺CD3⁺ cells (T cells) remained stable over time (Panel E). Indeed, the percentage of CD45⁺CD19⁺ cells (B cells) was significantly higher than CD45⁺CD3⁺ cells (T cells) in the pancreata of 8-week-old NOD mice (B cells=65.1±5.0 vs. T cells=30.2±3.2% p=0.004), (Panel E). Two injections (160 µg i.p. 5 days apart, day 0 and day 5) of anti-CD22/ cal mAb elicits a quick and profound depletion of B cells in the peripheral blood of 10-week-old NOD mice (n=6 mice/group) by 1 week that lasts for 6-7 weeks (Panels F and H). Control NOD mice did not appear to be depleted (Panels F and G), while the group treated with unconjugated anti-CD22 mAb shows a transient and partial B cell depletion (Panels F and I). At 8-10 weeks after depletion, B cells recovered almost completely (Panels F and H).
Figure 2. Diabetes prevention studies. We observed a significant delay in diabetes onset in anti-CD22/cal mAb-treated female 10-week-old NOD mice (n=20) compared to controls (n=30, p<0.01), (Panel A). The calicheamicin alone-treated group developed diabetes similarly to untreated controls (n=10, p<0.01 vs. anti-CD22/cal mAb-treated NOD mice), (Panel A). Anti-CD22 unconjugated treatment slightly delayed diabetes onset (n=10, p=0.06 vs. untreated controls), (Panel A). At 35 weeks of age an increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells was evident in the pancreatic lymph nodes (PLn) of anti-CD22/cal mAb-treated NOD mice (n=4) compared to 10-week-old untreated control NOD (n=4, p=0.02) and compared to hyperglycemic >14-week-old NOD mice (n=4, p=0.009), (Panel B). CD4<sup>+</sup> cells extracted from splenocytes of anti-CD22/cal mAb-treated NOD mice at 35 weeks of age produced less IFN-γ when challenged with the BDC2.5 peptide compared to CD4<sup>+</sup> cells extracted from splenocytes of untreated age-matched control hyperglycemic NOD mice (p=0.001) and 10-week-old NOD mice (p=0.04), (n=4 mice/group), (Panel C).

Isolated autoreactive BDC2.5 TCR Tg<sup>+</sup> CD4<sup>+</sup> cells were transferred into NOD.SCID mice previously reconstituted with NOD splenocytes and then treated with anti-CD22/cal mAb or left untreated. Fewer autoreactive BDC2.5 TCR Tg<sup>+</sup> CD4<sup>+</sup> cells were recovered (Panel D) in the anti-CD22/cal mAb-treated NOD.SCID hosts (Panel E, upper quadrant) compared to the untreated controls (Panel E, lower quadrant). Insulitis score analysis revealed better-preserved islets in the anti-CD22/cal mAb-treated NOD mice at 15 and 35 weeks of age (Panel F).
Figure 3. Histology of prevention studies. At baseline, NOD mice showed mild perinsulitis (Panel A1) with many B220⁺ cells (Panel A2) and some CD3⁺ cells (Panel A3), but still with well-preserved insulin and glucagon staining (Panels A5 and A6). FoxP3⁺ cells are merely present at baseline (Panel A4). Interestingly, at 15 weeks of age, treated NOD mice showed reduced infiltrate (Panel B1) with no B220⁺ cells (Panel B2) and fewer CD3⁺ cells (Panel B3), while in the control, B220⁺ and CD3⁺ cells are abundantly represented with increased infiltrate (Panels C1-C3). At 35 weeks of age, the treated group showed cleaner pancreata compared to the untreated control hyperglycemic NOD mice (Panels D1 and E1). B220⁺ and CD3⁺ cells did not infiltrate the islets in the treated group (Panels D2 and D3), while in the controls, islets were extensively infiltrated by B220⁺ and CD3⁺ cells (Panels E2 and E3). Islet morphology is well-preserved in the treated group at 15 and 35 weeks of age (Panels B5, B6 and D5, D6), but not in the control group (Panels C5, C6 and E5, E6). FoxP3 staining of islet infiltrate revealed a persistent reduced expression of FoxP3 in untreated compared to treated NOD mice at 15 and 35 weeks of age, particularly when compared with the massive presence of T cells in the control (Panels B4, D4 and C4, E4).
Figure 4. Hyperglycemia reversal studies. A rapid reversal of hyperglycemia was observed in all treated hyperglycemic NOD mice (Panel A). 6 out of 10 remained normoglycemic in the long-term. None of the untreated newly-hyperglycemic control NOD mice reverted from hyperglycemia (Panel B). After 5 days from hyperglycemia onset (n=6) anti-CD22/cal mAb was not able to restore normoglycemia in the long-term (Panel B). Either calicheamicin alone (GG5/cal) (n=5) or CD22 unconjugated treatment failed to restore normoglycemia in the long-term (Panel B). Pro-inflammatory cytokines (IL-17, TNF-α and slightly IFN-γ) are reduced 10 days after treatment compared to untreated controls (Panel C). CD4⁺CD25⁺FoxP3⁺ cell percentage increases in anti-CD22/cal mAb-treated long-term tolerant compared to untreated control NOD mice in the PLn (anti-CD22/cal mAb-treated vs. normoglycemic 10-week-old, p=0.007 and vs. hyperglycemic, p=0.03, Panel D) and in the spleen as well (anti-CD22/cal mAb-treated vs. normoglycemic 10-week-old, p=0.02 and vs. hyperglycemic, p=0.01, Panel E). Insulitis score confirmed that anti-CD22/cal mAb-treated NOD mice showed better preserved and less infiltrated islets compared to untreated control NOD mice both at baseline and 10 days after hyperglycemia onset (Panel F).
Figure 5. Histology of hyperglycemia reversal studies. Untreated, hyperglycemic mice at baseline show islets heavily infiltrated by lymphocytes (Panel A1) predominantly composed of B220$^+$ and CD3$^+$ cells (Panels A2 and A3) with few FoxP3$^+$ Tregs (Panel A4). Few insulin-positive cells and more glucagon-positive cells can be detected (Panels A5 and A6). 10 days following treatment with anti-CD22/cal mAb, islets appeared scarcely infiltrated compared to untreated controls (Panels B1 and C1), with few B220$^+$ and CD3$^+$ cells (Panels B2, B3 and C2, C3), but with an increase in FoxP3$^+$ cells (Panels B4 and C4). In treated animals, but not in the untreated controls, islets show abundant stainable insulin (Panels B5 and C5) and glucagon (Panels B6 and C6). 100 days post-treatment, 2 histological patterns are seen in the treated group: many of the islets show essentially no lymphoid infiltrate at all (Panels D1-D3), and few cells stain for insulin while more for glucagon (Panels D5 and D6). A smaller subset of islets show an abundant B220$^-$/CD3$^+$ infiltrate (Panels E1, E2 and E3). However, the infiltrate remains largely confined to the periphery of the islets, with a greater percentage of FoxP3$^+$ Tregs (Panel E4). Glucagon is easily detected (Panel E6), but insulin staining is low (Panel E5).
Figure 6. Transcriptome analysis of re-emerging B cells. We extracted B cells (using CD19 magnetic beads) from 10-week-old NOD mice, from hyperglycemic NOD mice as well as from the re-emerging B cell pool from age-matched B cell-depleted NOD mice in which the B cell repertoire is recovered. A gene array analysis was performed to evaluate gene expression of more than 40,000 genes. Genes which are differentially expressed in naïve B cells extracted from normoglycemic 10-week-old or hyperglycemic NOD mice and re-emerging B cells are shown in the heat map (Panels A-C). Blue represents lesser expression and red higher expression. 200 genes are downregulated in the re-emerging B cells compared to naïve B cells from 10-week-old NOD mice (Panel A). 38 genes are downregulated in the re-emerging B cells compared to naïve B cells from hyperglycemic NOD mice (Panel B). 21 genes are downregulated similarly in the re-emerging B cells compared to naïve B cells from 10-week-old and hyperglycemic NOD mice (Panel C).
Figure 7. Functional studies of re-emerging B cells. FACS analysis of CD80, CD86, CD40, Class II and IgM did not reveal any differences between re-emerging and naïve B cells extracted from splenocytes (the latter from either normo- or hyperglycemic NOD mice) (representative of 5 mice, Panel A). Interestingly, we observed by FACS analysis a higher percentage of anergic B cells (B220⁺CD93⁺CD23⁻IgM⁻ cells) in the re-emerging B cell pool compared to naïve B cells from hyperglycemic age-matched untreated NOD mice (representative of 5 mice, Panel B, with anergic B cells circled). We customized an in vitro assay in which B cells are used as APCs and autoreactive BDC2.5 TCR Tg⁺ CD4⁺ cells are used as responders in the presence of the BDC2.5 peptide. When re-emerging B cells were APCs, a lower IFN-γ production by BDC2.5 TCR Tg⁺ CD4⁺ cells was evident compared to when naïve B cells were used (Panel C). Supernatant were collected from the experiment described above and cytokine profile was assessed with a Luminex assay. Interestingly, when re-emerging B cells, but not naïve B cells, were used as APCs, BDC2.5 autoreactive CD4⁺ cells down-regulated the production of pro-inflammatory cytokines (IL-2, IL-17, TNF-α and IFN-γ), (Panels F-I).

We then co-adoptively transferred CD19⁺ cells (obtained from re-emerging or from naïve B cell pool) into NOD.SCID recipients with diabetogenic CD4⁺ cells obtained from hyperglycemic NOD mice. When re-emerging B cells were transferred, but not naïve B cells, they completely abrogated the onset of diabetes mediated by the transfer of diabetogenic CD4⁺ cells (Panel D), (n=5 mice/group). We also analyzed the percentage of CD4⁺CD25⁺FoxP3⁺ cells in the spleen of NOD.SCID recipients of diabetogenic CD4⁺ T cells and re-emerging B cells or controls (B cells from hyperglycemic animals or no cells) at day 30 post-adoptive transfer. Panel E shows that there is no difference between the groups (n=5 mice/group).