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Loss of Anergic B Cells in Prediabetic and New-Onset Type 1 Diabetic Patients

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Although dogma predicts that under normal circumstances, potentially offensive autoreactive cells are silenced by mechanisms of immune tolerance, islet antigen–reactive B lymphocytes are known to play a crucial role in the development of autoimmunity in type 1 diabetes (T1D). Thus, participation of these cells in T1D may reflect escape from silencing mechanisms. Consistent with this concept, we found that in healthy subjects, high-affinity insulin-binding B cells occur exclusively in the anergic naive IgD$^+$, IgM$^-$ B-cell (B_{ND}) compartment. Antigen receptors expressed by these cells are polyreactive and have N-region additions, Vh usage, and charged complementarity-determining region 3 consistent with autoreactivity. Consistent with a potential early role in autoimmunity, these high-affinity insulin-binding B cells are absent from the anergic compartment of some first-degree relatives and all prediabetic and new-onset (<1 year) T1D patients tested, but return to normal levels in individuals diabetic for >1 year. Interestingly, these changes were correlated by transient loss of the entire B_{ND} compartment. These findings suggest that environmental events such as infection or injury may, by disrupting B-cell anergy, dispose individuals toward autoimmunity, the precise nature of which is specified by genetic risk factors, such as HLA alleles.

Although effector T cells mediate islet destruction in type 1 diabetes (T1D), it has become clear that B cells also play an important role in disease development. Rituximab (anti-CD20), a B cell–depleting therapy, has shown efficacy in clinical trials in which newly diagnosed patients had preserved β-cell function 1 year after treatment (1). In nonobese diabetic (NOD) mice, disease development requires B cells specific for islet antigens such as insulin (2).

While autoreactive B lymphocytes play a critical role as producers of pathogenic autoantibodies in diseases such as lupus and rheumatoid arthritis, they appear to function differently in T1D. Although production of high-affinity islet antigen–reactive autoantibodies indicates increased risk, such antibodies appear dispensable for disease, indicating that B cells may instead contribute by antigen presentation and/or cytokine production (3,4).

We hypothesized that insulin-binding B cells (IBCs) that function in T1D are normally silenced by anergy, a mode of B-cell tolerance in which autoantigen-reactive cells populate peripheral lymphoid organs but are antigen unresponsive (5–8). Recent description of the surface phenotype of a cohort of anergic human B cells, termed B_{ND} cells, allowed testing of this hypothesis (7). B_{ND} refers to naive IgD$^+$, IgM$^-$ B cells that normally represent ~2.5% of peripheral blood B cells. More than 75% of cells in the B_{ND} compartment bear autoreactive antigen receptors, are refractile to antigen receptor stimulation in vitro, and thus appear anergic. More recently, Quách et al. (8) extended these findings, showing that the anergic population includes cells that express low membrane IgM but are otherwise B_{ND} in phenotype. This IgM$^{lo/-}$IgD$^+$ phenotype is typical of anergic B cells in the mouse (5,6).

To explore the relationship between development of autoimmunity and integrity of the anergic B-cell compartment, we undertook studies of the affinity, frequency, and surface phenotype of IBCs in the peripheral blood of subjects along the continuum of T1D development. We report that IBCs are present in the anergic B_{ND} B-cell compartment and that antigen receptors expressed by

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these cells are of high affinity and polyreactive. Importantly, IBCs are present in the anergic B-cell compartment of healthy subjects, but absent from this compartment in some first-degree relatives (FDRs), all prediabetic subjects, and all new-onset patients. Interestingly, individuals who are diabetic for >1 year have anergic IBC levels similar to those of healthy control subjects. These findings indicate loss of B_{ND} cells in FDRs, and prediabetic individuals may reflect breach of anergy, predisposing subjects to development of anti-islet antibodies and participation in development of T1D.

RESEARCH DESIGN AND METHODS

Peripheral Blood Processing

Samples were obtained with informed consent at the Barbara Davis Center for Childhood Diabetes using protocols approved by the University of Colorado Institutional Review Board. Eligible subjects were male or female who met the American Diabetes Association criteria for classification of disease. GAD antibody, islet cell antibody, insulin autoantibody, and zinc transporter 8 antibody titer tests were used to confirm diagnosis of prediabetes and T1D. Peripheral blood mononuclear cells (PBMCs) from autoantibody-negative FDRs, autoantibody-positive prediabetics (identified in the Type 1 Diabetes TrialNet Natural History study), new-onset T1D patients, long-standing T1D patients, and healthy age/sex-matched control subjects were isolated from heparinized blood by Ficoll-Hypaque fractionation.

Flow Cytometry Analysis and Enrichment of IBCs

In order to maintain consistency of gating, each day a patient sample was analyzed alongside an age/sex-matched healthy control, and the healthy control cells were used to draw gates, which were then copied to the patient cells. PBMCs were stained in PBS/1% BSA/0.02% Na azide with human FcR Blocking Reagent (Miltenyi Biotec), 0.1 μg/10^6 cells insulin-biotin, and mouse monoclonal anti-human antibodies against CD19-V450 (BD Biosciences), CD27-peridinin chlorophyll (BioLegend), IgM-phycoerythrin (Southern Biotechnology Associates), and IgD-fluorescein isothiocyanate (BD Biosciences) for 20 min at 4°C. For the insulin receptor studies, cells were stained with anti-CD19, CD27, and phycoerythrin–anti-CD220 (BD Biosciences) to detect insulin receptors and fluorescein isothiocyanate–anti-CD14 (BD Biosciences) to detect monocytes. After washing, cells were then fixed with 2% formaldehyde at 4°C, followed by incubation with streptavidin–Alexa Fluor 647 for 20 min at 4°C. Cells were washed, suspended in MACS buffer (PBS/0.5% BSA/2 mmol/L EDTA), and incubated with anti-Cy5/anti-Alexa 647 microbeads (Miltenyi Biotec) for 15 min at 4°C. Samples were then passed over magnetized LS columns (Miltenyi Biotec) and washed three times with 2 mL of MACS buffer, and bound cells were eluted with 6 mL of MACS buffer. Flow cytometry was performed on an LSR II (BD Biosciences) and data analyzed with FlowJo software version 8.8.4.

B-Cell Calcium Flux Analysis

Using a strategy similar to previous reports (7,8), PBMCs freshly isolated from buffy coats were enriched for B cells using a no-touch method (Miltenyi Naive B cell Isolation Kit II). Cells were suspended in warmed 37°C RPMI, 2% BSA, and 1 μmol/L Indo 1-AM (Molecular Probes). Cells were stained with antibodies, as previously described (7), for 30 min and washed two times with warmed RPMI containing 2% BSA. Cells were then placed on an LSRII flow cytometer and after 20 s of baseline readings were stimulated with 20 μg/mL of $F(ab')_2$ goat anti-human IgD (Southern Biotechnology Associates) or 1 μL of 1 mg/mL ionomycin as a control. The mature naive fractions were gated as $CD19^+CD27^-IgG^-$, while the B_{ND} fractions were gated as $CD19^+CD27^-IgM^-$. Calcium mobilization analysis was conducted using FlowJo software.

Phosflow Spleen Tyrosine Kinase Analysis

B cells were enriched using the no-touch B-cell enrichment kit, as described above, and serum starved by incubation in serum-free RPMI medium for 2 h at 37°C. Cells were stimulated with 20 μg/mL rabbit $F(ab')_2$ anti-human IgG (H&L) (Jackson ImmunoResearch Laboratories) for 5 min at 37°C or left unstimulated. Cells were immediately fixed with 2% paraformaldehyde at 37°C for 10 min, spun down, and then resuspended with ice-cold 100% methanol and placed on ice for 30 min. Cells were washed and stained with mouse monoclonal anti-human antibodies against CD19, CD27, IgM, and IgD for 15 min. Cells were also stained with mouse anti–ζ-chain–associated protein 70/spleen tyrosine kinase (Syk) antibody (BD Phosflow) or isotype control simultaneously. Flow cytometry was performed as described above.

Ig Gene Cloning

Cells were stained and enriched for IBCs, as indicated in the flow cytometry section above, before B_{ND} or mature naive cells were single-cell sorted into 96-well plates. Variable region amplification, sequence analysis, and recombinant monoclonal antibody production was completed as previously described (7,9). Briefly, variable region genes were amplified by RT-PCR to identify and clone the Ig heavy and light chain genes. The variable region genes were then cloned into expression vectors and expressed with human IgG constant regions in the 293A human cell line. A total of 11 antibodies from B_{ND} cells and 16 antibodies from mature naive B cells were compared.

Assay of Antibody by ELISA

To screen expressed antibodies for antigen reactivity, ELISA microtiter plates (Costar) were coated with 10 μg/mL of recombinant human insulin (Sigma-Aldrich), lipopolysaccharide (LPS; Sigma-Aldrich), or calf chromatin (kindly provided by L. Wysocki, National Jewish Health) in PBS overnight at 4°C, followed by incubation with blocking buffer solution (PBS with 3% BSA) for 1.5 h at room temperature. Recombinant antibodies or human sera were serially diluted in blocking buffer and incubated

in the 96-well plates for 2 h at room temperature. Among all steps, the plates were washed four times with PBS containing 0.05% Tween-20. HRP goat anti-human IgG (H&L) (Bio-Rad Laboratories), followed by development with TMB single solution (Invitrogen), was used to detect antibody binding. Reactions were stopped using 1 N H_2PO_4 (Sigma-Aldrich). Antibody was measured based on optical density at 450 nm using a VERSAMax plate reader (Molecular Devices), and the data were analyzed with Softmax software.

Statistics

Data were analyzed using Prism software (GraphPad Software). One-way ANOVA followed by a Bonferroni multiple comparison posttest was used to determine significance of differences among the five patient groups. Mann-Whitney nonparametric *t* tests were used to compare differences in ELISA optical density values. Student *t* tests were used to compare differences in variable region sequences. Spearman correlation tests were used to determine correlation between two data sets.

RESULTS

Anticipating that IBCs likely occur in very low frequency in the normal B-cell repertoire, we first developed the magnetic microparticle-adsorbent method described schematically in Fig. 1*A* and *B* to stain and enrich IBCs for subsequent analysis (10). In a typical PBMC sample, ~0.37% of total B cells bound the adsorbent (Fig. 1*C*), and selection resulted in ~20-fold enrichment of IBCs (Fig. 1*C*). Omission of insulin led to a ~90% reduction in IBC yield, indicating that most cells are enriched by virtue of insulin recognition (Fig. 1*D*). We also verified insulin specificity by saturating anti-A647 magnetic beads with free biotin–SA-A647 and saw near complete blocking of cell binding (data not shown). In order to address the possibility that cells are enriched by virtue of insulin receptor (CD220) binding, which seemed unlikely given the

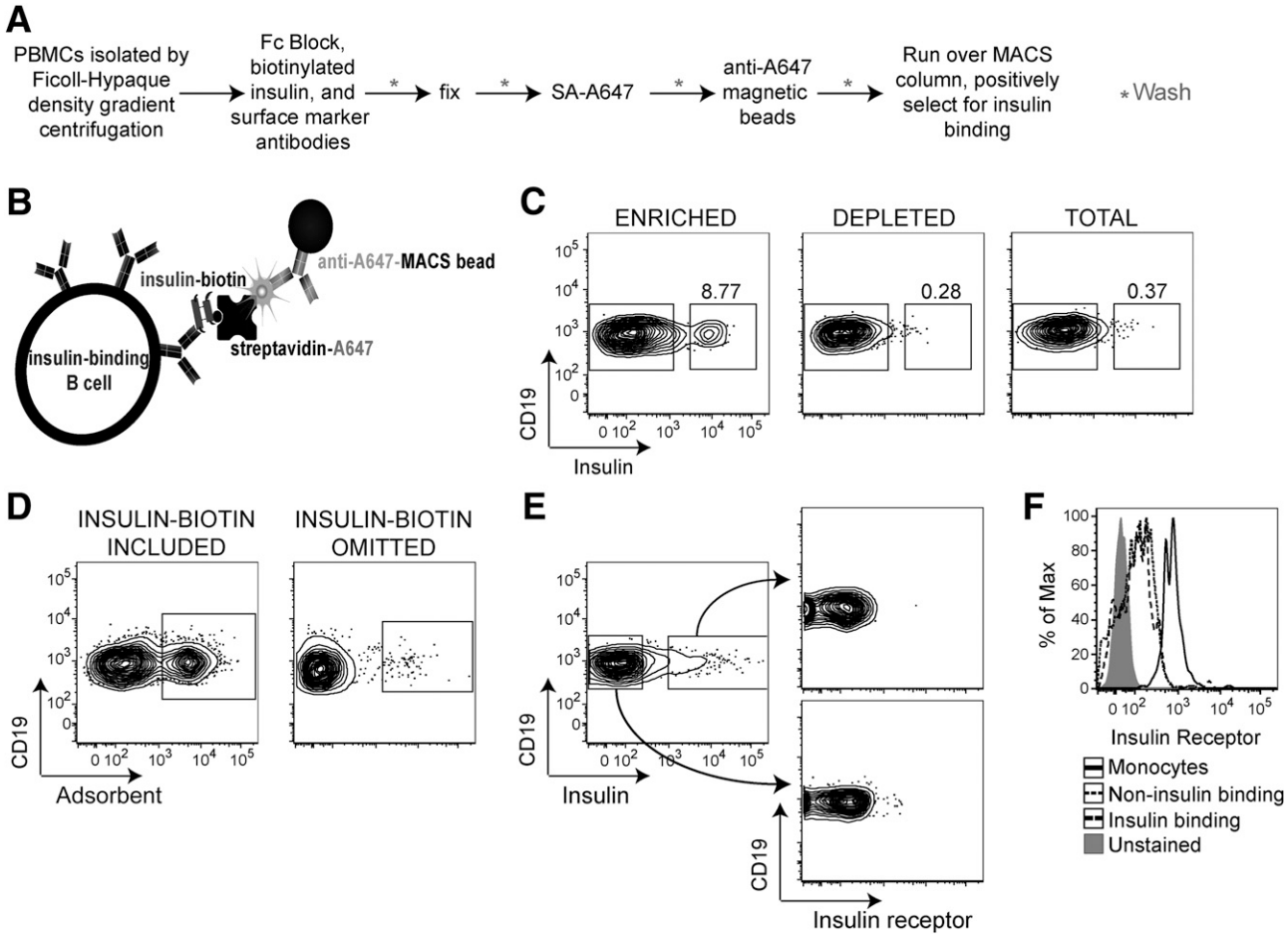

Figure 1—Detection and enrichment of IBCs is dependent on insulin binding to the BCR. *A*: Protocol for enrichment and isolation of IBCs. *B*: Diagram of adsorbent used to identify and isolate IBCs. *C*: Representative cytograms of IBCs enriched from blood of a new-onset patient. Cells that bound to the adsorbent and eluted from magnetic column are termed enriched, nonbinders termed depleted, and unenriched termed total. *D*: Cytograms of PBMCs enriched as in *A* or enriched with insulin-biotin omitted during the procedure. *E* and *F*: Representative cytograms of insulin receptor antibody staining of enriched IBC populations. Monocyte staining is shown as a positive control. All cells were gated on $CD19^+$ lymphocytes.

100-fold lower expression of insulin receptors than antigen receptors on B cells (11), we stained our enriched cells using antibodies against the insulin receptor. As shown in Fig. 1*E* and *F*, enriched IBCs and non-IBCs express equivalent levels of insulin receptors, indicating that IBCs are not selected based on insulin receptor level. Hence, cells isolated by this procedure bind the adsorbent by virtue of antigen receptor recognition of insulin. At ~0.37% of peripheral blood B cells, IBCs occur at higher frequency than those that bind the large exogenous antigen PE (10). This might be explained in part by findings shown in Fig. 3 that some insulin-binding antibodies are polyreactive, binding multiple structurally unrelated antigens (12).

In order to analyze the distribution of IBCs in B-cell subpopulations, we used a gating strategy used by Duty et al. (7) and Quách et al. (8), which allows recognition of B_{ND} cells (Fig. 2*A*). As previously described, cells are $CD19^+CD27^-IgM^{lo/-}IgD^+$, while mature naive B cells are $CD19^+CD27^-IgM^+IgD^+$. In healthy individuals, ~50% of these B_{ND} cells expressed little or no CD21, a phenotype also previously associated with anergy (data not shown) (13). To confirm that B_{ND} cells recognized using these markers are anergic, we assessed their response to B-cell receptor (BCR) stimulation–based Ca^{2+} mobilization and Syk tyrosine phosphorylation (Fig. 2*B* and *C*). Analysis of these parameters using IBC B_{ND}s was not possible due to the low frequency of these cells, and thus, we analyzed total B_{ND}s. As expected based on previous literature (7,8), B_{ND} cells exhibited greatly reduced BCR-mediated Ca^{2+} mobilization and significantly reduced Syk phosphorylation ($P < 0.002$) relative to naive B cells.

Interestingly, we found IBCs in almost all B-cell subpopulations (Fig. 2*A*). We considered the possibility that many of the cells captured by the adsorbent may have such low affinity for insulin that they are effectively ignorant of ambient autoantigen. Studies in other systems have shown that while B cells bearing receptors with moderate to high affinity for self-antigen can be rendered anergic, those bearing a low affinity for the same self-antigen can be effectively ignorant of antigen in their environment (14,15). To test this possibility as well as to confirm the specificity of isolated B_{ND} IBC for insulin, we cloned Ig genes from single naive and B_{ND} IBCs, expressed their variable regions in the context of IgG constant regions, and analyzed the specificity and approximate affinity of these recombinant Igs by ELISA (9). As shown in Fig. 3*A*, while B_{ND} IBCs expressed high-affinity anti-insulin BCR, naive IBC BCR affinity for insulin appears >100–1,000-fold lower. Based on the titration curves, the B_{ND} IBCs have an approximate affinity for insulin of $\sim 6.6 \times 10^{-10}$ mol/L, which is sufficient to maintain anergy in the face of normal blood levels of insulin (6.0×10^{-11} – 6.0×10^{-10} mol/L) when at least 30–40% of BCRs are occupied, which has been reported to sustain anergy (16). However, the mature naive IBCs appear to have an insulin affinity of only $\sim 1.67 \times 10^{-7}$ mol/L, which is too low to maintain anergy, and thus these cells are likely ignorant (i.e., unresponsive to insulin) and therefore fail to be tolerized. Moreover, given that earlier studies have shown that self-reactive B lymphocytes often have polyreactive BCRs (12), we determined the reactivity of our recombinant antibodies to LPS and chromatin. All high-affinity B_{ND}-derived antibodies displayed significant reactivity to LPS and chromatin, indicating polyreactivity (Fig. 3*A*).

We then analyzed heavy chain variable region gene usage by B_{ND} IBCs and found that their sequences showed no signs of somatic mutation, indicating that their auto/polyreactivity is not generated by participation in prior immune responses (data not shown). Auto/polyreactivity has been previously associated with long IgH complementarity-determining region 3 (CDR3) with increased numbers of positive amino acid residues (12,17,18). Comparison of the number of positive-charged amino acid residues in CDR3s of BCR of B_{ND}s and naive IBCs revealed that the B_{ND} cells have more positive-charged amino acids than mature naive cells (mean of 3 vs. 1.5, respectively; $P < 0.001$) (Fig. 3*B*). Further, both B_{ND} and mature naive IBCs had significantly more positive-charged amino acids in their CDR3 regions than seen in the normal human mature B-cell repertoire (12) ($P < 0.01$ for mature naive IBCs and $P < 0.001$ B_{ND} IBCs) (data not shown). In addition, the frequency of IBC B_{ND}s with long CDR3 regions (>17 amino acids) was significantly higher ($P < 0.03$) than the mature naive cells (Fig. 3*C*), consistent with auto/polyreactivity. Lastly, we analyzed variable region gene usage and found the B_{ND} cells use predominantly J_H6 gene segments (Fig. 3*D*), which is indicative of increased receptor editing and autoreactivity ($P < 0.04$) (7,12,19). Together, these data not only confirm that B cells isolated using this adsorbent do indeed bear insulin-binding BCR, but also that B_{ND} IBCs have high insulin affinity consistent with anergy and are polyreactive, and their BCRs have structural features consistent with autoreactivity.

If insulin-reactive B cells that in healthy individuals occur in the anergic B_{ND} fraction participate in autoimmune responses in patients with T1D, one would predict that insulin-reactive antibodies produced by diabetic patients would be polyreactive. If this is the case, sera from new-onset patients should contain antibodies reactive with both insulin and chromatin. We analyzed insulin and chromatin reactivity of serum antibodies from new-onset diabetic, insulin autoantibody–positive (IAA^+) patients who had not yet received exogenous insulin treatment. Interestingly, new-onset diabetic patients have increased reactivity to both insulin and chromatin relative to healthy control subject serum (Fig. 4). LPS was also tested, but reactivity was not statistically different (data not shown). Taken together, these data are consistent with T1D-associated loss of anergy by B_{ND} cells bearing polyreactive antigen receptors with high affinity for insulin.

In order to test further this possibility, we compared the frequency of IBCs captured by our adsorbent,

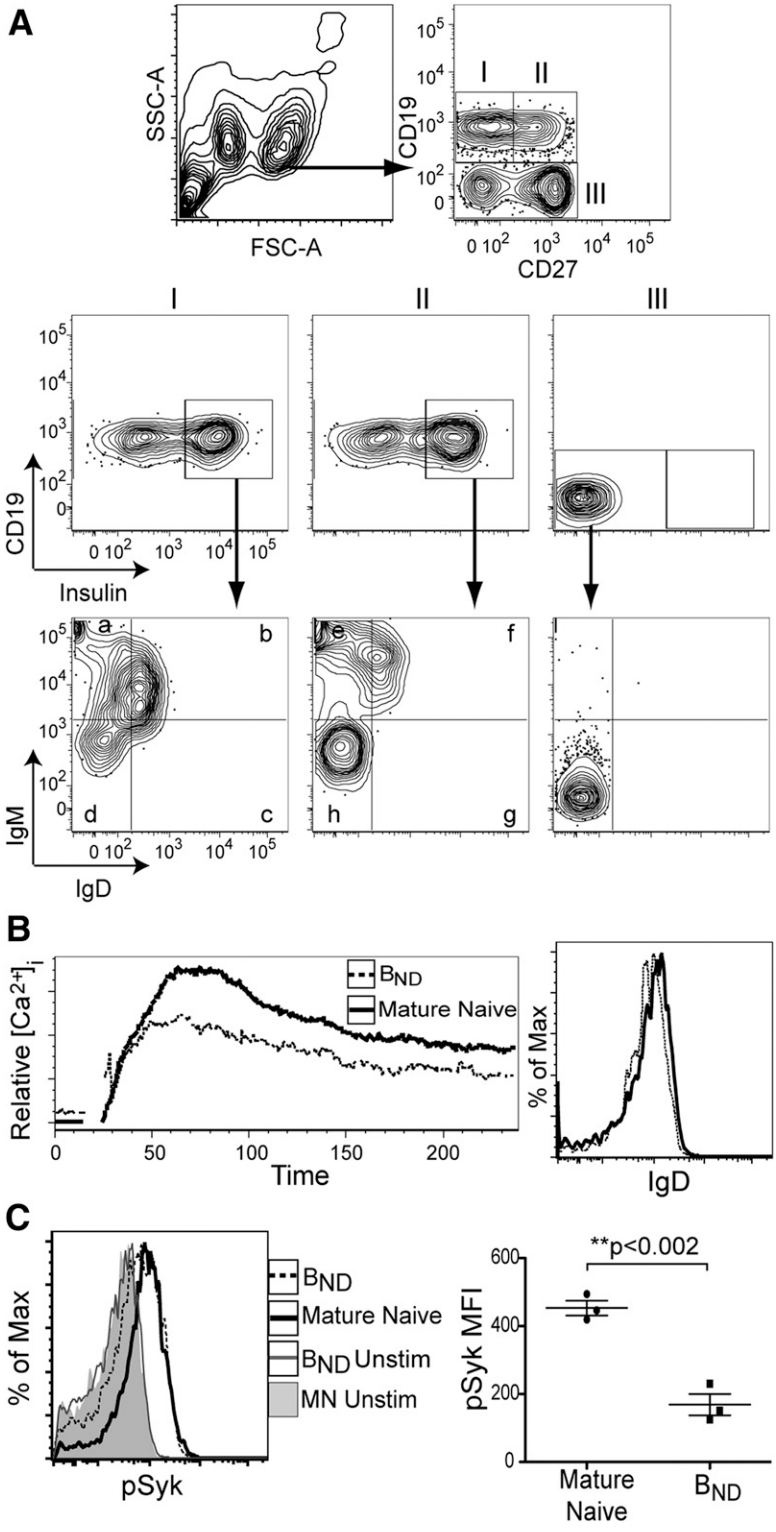

Figure 2—Total B_{ND} cells have reduced Ca^{2+} flux and phosphorylated Syk upon stimulation compared with their mature naive counterparts, suggesting they are anergic. *A*: Gating strategy for identification and analysis of insulin-binding naive and memory B-cell subpopulations illustrated using cells from a healthy control. IgM vs. IgD gates of $CD19^+CD27^-$ IBCs (*I*) produce the following subpopulations: $IgM^+IgD^{lo/-}$ (*a*), mature naive (*b*), B_{ND} (*c*), and $IgM^{lo}IgD^-$ (*d*). IgM vs. IgD gates of $CD19^+CD27^+$ IBCs (*II*) produce the following subpopulations: $IgM^{hi}IgD^{lo/-}$ (*e*), preswitch (*f*), Cδ-CS (*g*), and class-switch (*h*). Staining of T cells ($CD19^-$) (*III*) is shown as verification of gating strategy. *B*: Representative Ca^{2+} mobilization plot over time for B_{ND} and mature naive B cells upon stimulation with 20 μg/mL of goat anti-human IgD $F(ab')_2$. Representative histogram showing the relative expression of IgD on B_{ND} and mature naive B cells is similar. *C*: Representative phosflow Syk histogram for stimulated B_{ND} cells and mature naive cells compared with unstimulated mature naive B cells (gray shaded) and unstimulated B_{ND} cells (gray line). Difference of phosflow Syk (pSyk) median fluorescence intensity (MFI) between unstimulated and stimulated naive and B_{ND} cells in three different experiments (**$P < 0.002$, Student *t* test). FSC-A, forward light scatter area; SSC-A, side scatter area.

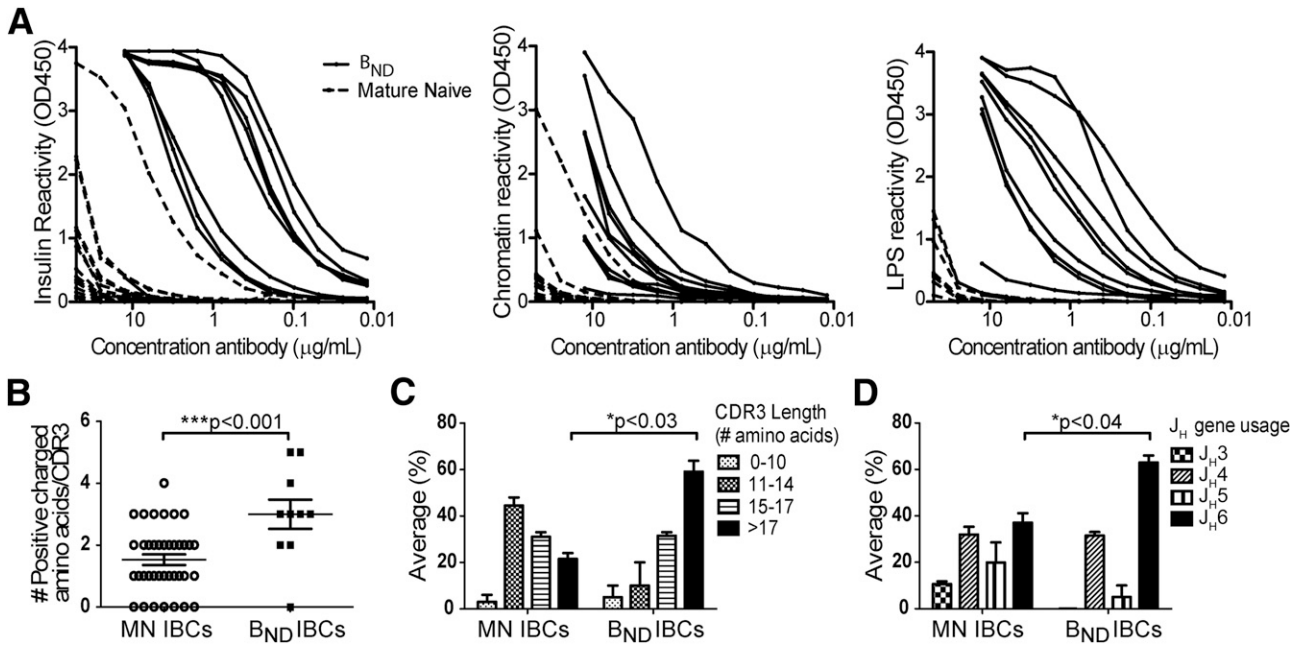

Figure 3—IBCs found in the B_{ND} fraction bear high-affinity, polyreactive antigen receptors and have heavy chain variable regions consistent with auto/polyreactivity. *A*: ELISA data showing the affinity and specificity of recombinant antibodies made from cloned Igs from single-cell B_{ND} (n = 11) and mature naive IBCs (n = 16). Anergic B_{ND} IBCs have higher affinity for insulin and are polyreactive relative to IBCs in the mature naive compartment. *B–D*: Sequencing of the heavy chain variable region genes revealed B_{ND} cells have more positive-charged amino acids in their CDR3 regions (***$P < 0.001$, Student t test), have longer CDR3 regions (*$P < 0.03$ for >17 amino acids and 11–14 amino acids, Student t test), and have increased use of J_H6 (*$P < 0.04$, Student t test), which is consistent with auto/polyreactivity. MN, mature naive.

including anergic IBCs, in blood B-cell subpopulations from various subject groups (Fig. 2*A*). Consistent with the hypothesis, in autoantibody-positive prediabetic and new-onset patients, significantly fewer IBCs were B_{ND} ($CD27^-IgM^{lo/-}IgD^+$) and $CD27^-IgM^{lo/-}IgD^-$ cells relative to healthy control subjects (Fig. 5). Most significant were differences in frequency of IBCs that are B_{ND}, which showed reduction from 2.4% in healthy control subjects to 0.57 and 0.41% in prediabetic and new-onset patients with diabetes, respectively ($P < 0.001$ for both). Parenthetically, this decrease in B_{ND} IBCs was not simply a function of subject age and in follow-up studies appeared stable over >6–12 months (data not shown). Interestingly, some FDRs showed a B_{ND} frequency among IBCs similar to that of unrelated healthy control subjects, while others were similar to those of prediabetic and new-onset patients (Fig. 5*A*). These findings suggest that departure of IBC from the B_{ND} compartment precedes and may therefore predispose individuals to development of insulin autoantibodies and, eventually, T1D.

Loss of the anergic B_{ND} IBC population may reflect entry of these cells into a nonanergic state competent to present antigen to diabetogenetic $CD4^+$ T cells. However, the size of the B_{ND} pool is so small that such a shift would not significantly affect frequency of much larger mature populations. Nonetheless, we examined the relative frequency of IBCs in mature compartments and observed a trend toward increased frequency of IBCs among mature naive (IgM^+IgD^+) B-cell populations in prediabetic and new-onset patients compared with the long-standing T1D and healthy control subjects (Fig. 5*A*). Thus, loss of IBCs from the B_{ND} compartment may reflect upregulation of membrane IgM, consistent with loss of anergy (5). This change may also reflect relocalization of these cells to other anatomical sites, particularly the pancreas and pancreatic lymph nodes that are rich in autoantigen. This possibility would be consistent with reported B-cell accumulation in the pancreata of diabetic individuals (20) and the recent study suggesting that anergic insulin-specific B cells are capable of entering the pancreata of NOD mice (21).

As noted above, we also observed a statistically significant decrease in $CD27^-IgM^{lo/-}IgD^-$ IBCs in prediabetic subjects and new-onset patients relative to healthy control subjects (Fig. 5*A*). In healthy individuals, ~50% of these cells expressed little or no CD21, suggestive of anergy (data not shown) (13). Thus, we suggest that these CD27, IgM, and IgD triple-negative IBCs may represent a second anergic population that is lost in pre- and early T1D. Additionally, these cells may be members of a recently described population that lacks expression of CD27, IgD, and IgM, but bear somatically mutated membrane IgG, and are thought to have class-switched outside a germinal center (22,23).

Perhaps most importantly, some autoantibody-negative FDRs of diabetic patients showed an IBC B_{ND} frequency similar to those of prediabetic and new-onset patients, while

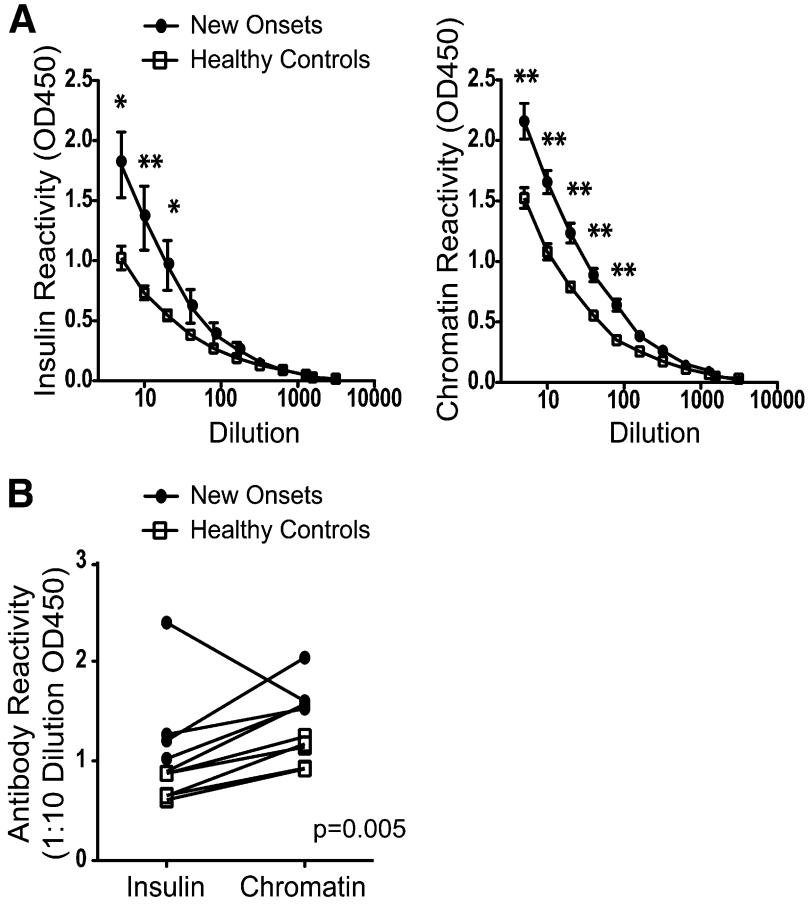

Figure 4—Sera from IAA$^+$ new onsets show increased reactivity to both insulin and chromatin. *A*: ELISA data showing sera from IAA$^+$ new-onset patients have increased reactivity to not only insulin, but also chromatin, compared with healthy control IAA$^-$ samples (significance determined by Student *t* test at each dilution, $^*P < 0.05$, $^{**}P < 0.01$). *B*: Correlation between insulin and chromatin reactivity for each human sample (each bar represents data from one individual) at the 1:10 dilution ($P = 0.005$, Spearman correlation).

others were similar to unrelated healthy control subjects (Fig. 5*A*). These findings suggest that loss of B_{ND} IBC cells precedes and therefore may predispose individuals to develop insulin autoantibodies and, eventually, T1D.

Although the majority (~70%) of the IBCs were found in the CD27$^-$ compartment, some occurred among CD27$^+$ memory B cells. As shown in Fig. 5*B*, prediabetic and new-onset patients exhibit a decrease, though not statistically significant, in the frequency of IBCs among CD19$^+$CD27$^+$IgM$^-$IgD$^+$. Cells in this population were previously shown to be IgD heavy chain class-switched and enriched in autoreactive cells (Cδ-CS) (24). Thus, anergic IBCs that normally occur in this population may also relocalize during onset of diabetes.

Studies in a variety of systems suggest that environmental factors such as infection and/or injury, as well as colonization by certain commensal microbes, may promote development of autoimmunity (25–27). If such triggers act by causing loss of B-cell anergy in diseases such as T1D, they may do so nonspecifically (i.e., irrespective of B-cell autoantigen specificity). If such factors are in play, loss of B_{ND} IBCs may be accompanied by loss of all B_{ND} cells. To test this possibility, we analyzed total B_{ND} frequency and found a correlated reduction in B_{ND} in the IBC and total B-cell population of prediabetic and new-onset patients compared with long-standing T1D and healthy control subjects (Fig. 6). This finding suggests that a generalized loss of anergic B cells may precede development of autoimmunity.

DISCUSSION

We have characterized insulin autoantigen-binding B cells in the previously defined anergic B_{ND} population in peripheral blood of healthy humans. These cells were found to express high-affinity insulin-binding antigen receptors that are cross-reactive with chromatin and LPS. They possess germline-encoded hypervariable regions consistent with origination in the primary repertoire. In addition, they exhibit longer N-region additions and biased usage of J_H6, features previously associated with autoantibodies. Finally, their CDR3 regions contained increased positively charged amino acid residues typical of DNA-binding autoantibodies. These potentially pathogenic anergic B cells were lost or exhibited altered phenotype in some FDRs and all

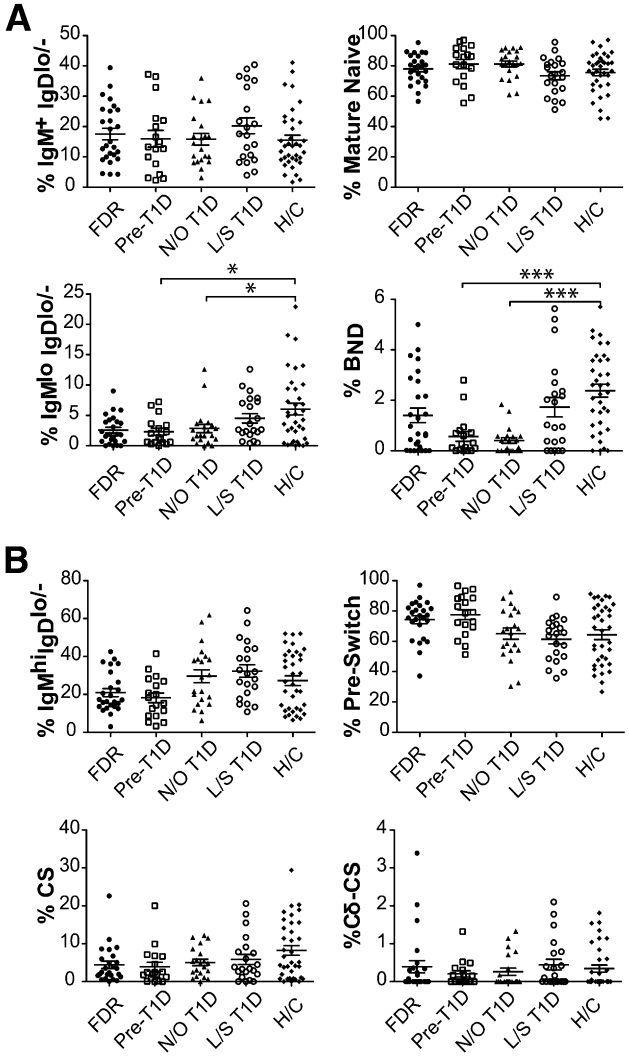

Figure 5—Surface phenotype of naive and memory IBCs shows significant differences between some "at risk" FDRs, prediabetic, and new-onset (N/O) patients compared with long-standing (L/S) T1D patients and healthy control (H/C) subjects. *A*: Percentage of $CD19^+CD27^-$ IBCs captured by the adsorbent that fall in the IgM vs. IgD gates. Percentages reported as percent of total $CD27^-$ IBCs ± SD. *B*: Percentage of $CD19^+CD27^+$ IBC memory subpopulations from subjects reported as percent of total $CD27^+$ IBCs ± SD. Samples include autoantibody-negative FDRs (n = 25), autoantibody-positive prediabetics (Pre-T1D; n = 17), N/O patients with diabetes (<1 year from diagnosis; n = 21), L/S patients with diabetes (>1 year; n = 21), and healthy age-matched control subjects (H/C; n = 36). Statistical significance determined by one-way ANOVA followed by Bonferroni multiple comparison posttest. $^*P < 0.05$, $^{***}P < 0.001$. CS, class-switched.

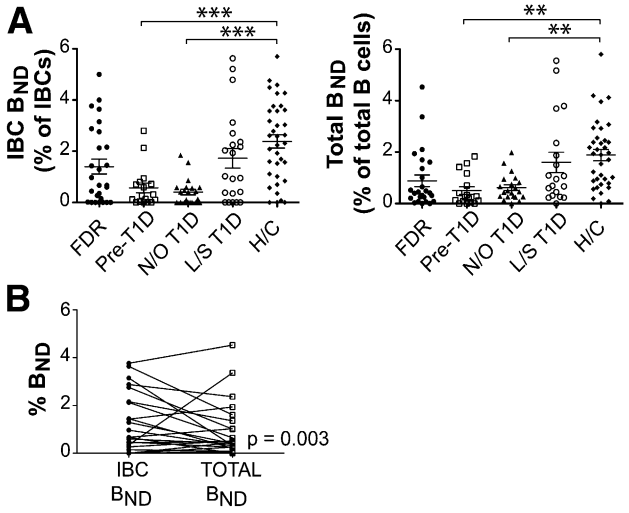

Figure 6—Subjects with low B_{ND} frequency among IBCs also exhibit low frequency of B_{ND} among total B cells. *A*: Percentage ± SD of IBCs that are B_{ND} compared with the percentage of total (noninsulin-binding) $CD27^-$ B cells that are B_{ND} in subjects from various groups. (Statistical significance determined by one-way ANOVA followed by Bonferroni multiple comparison posttest, $^{**}P < 0.01$, $^{***}P < 0.001$.) *B*: Correlation between frequency of total B_{ND} and IBC B_{ND} in individual FDRs (P = 0.003, Spearman correlation test). Each bar represents data from an individual FDR.

prediabetic and new-onset diabetic patients, consistent with an early role in development of T1D.

This is the first reported analysis of alterations in autoantigen-reactive B cells in the context of development of T1D in humans. An initial surprising finding was that IBCs occur at higher frequency in the normal repertoire than cells reactive with the large exogenous antigen phycoerythrin (10). However, given our findings that our IBCs were also reactive to chromatin and LPS, the higher frequency we observed may be explained in part by the polyreactivity of these cells.

Also surprising was the finding that IBCs occur in all major B-cell compartments, suggesting that, in addition to potentially harmful high-affinity receptor-bearing cells, the isolation procedure captures cells that bear antigen receptors of such low affinity that they are ignorant of low-concentration antigen in their surroundings.

Consistent with this, the population containing the most IBCs, the mature naive population, is composed of low-affinity B-cell clones, whereas the anergic IBC population in the healthy individual is almost entirely composed of high-affinity, polyreactive B-cell clones. The chromatin and LPS cross-reactivity of anergic high-affinity IBCs is surprising given previous evidence that polyreactive autoreactive B cells appear to be preferentially purged from the repertoire very early in development (12,28). However, polyreactivity could have functional importance since these cells may be activated by virtue of cross-reactivity with chromatin released by tissue damage and subsequently present insulin also bound by their antigen receptors.

Importantly, this polyreactive anergic population is lost in a subset of FDRs and all prediabetic and new-onset patients, suggesting that a breach in peripheral B-cell tolerance has occurred. Consistent with this, new-onset insulin autoantibody–positive patients have elevated serum antibodies reactive with chromatin relative to healthy control subjects, which is consistent with activation of anergic, polyreactive B cells found in healthy individuals. Although previous studies have found increased anti-DNA antibodies

in the serum of T1D patients, as well as some of their FDRs (29,30), this is the first study to correlate this finding with loss of the anergic polyreactive B-cell population. Moreover, given that T1D patients are prone to development of other autoimmunities (31,32), including autoimmune thyroiditis and Addison's disease, we suspect these polyreactive B cells are also reactive with self-antigens, such as thyroglobulin, as has been observed in the NOD mouse (33). Studies in our laboratory are currently underway to examine this possibility.

In addition to the loss of anergic IBCs, we found a correlated decrease in total anergic B cells, irrespective of their specificity, in prediabetic and new-onset patients, as well as some FDRs. These results suggest that loss of anergy may predispose individuals to multiple types of autoimmunity and that loss of B-cell anergy may be a phenomenon that also precedes other non–T1D-related autoimmune disorders. Consistent with this, Kinnunen et al. (34) recently found that patients with multiple sclerosis, as well as patients with rheumatoid arthritis and long-standing T1D, have increased frequency of auto/polyreactive B cells in their mature naive B-cell pool, suggesting loss of peripheral B-cell tolerance. Given that recent genome-wide association studies have revealed common polymorphisms among multiple autoimmune disorders (35), we suspect the loss of B-cell anergy we observe in T1D patients, and perhaps other autoimmune patients, is associated with combinations of susceptibility alleles that compromise anergy globally. PTPN22 polymorphisms have been shown to dampen B-cell signaling and are associated with increased autoreactive B cells in the peripheral blood of T1D patients (36,37). In addition, susceptibility alleles encoding BLK, Lyn, PTPN2, BANK1, CSK, and FcγRIIb are associated with B-cell signaling and may promote loss of anergy via alterations in thresholds for activation (38). Hence, we speculate that risk alleles that compromise regulation of BCR signaling increase the risk of development of autoimmunity by undermining immune tolerance.

The apparent transience of loss of anergic B cells observed seems inconsistent with a genetic basis, but rather suggests that loss of anergy may be due to an environmental insult, such as infection, injury, or a diet/microbiota change. This seems plausible given that multiple systems have shown that environmental factors can promote development of autoimmunity (25–27) and that it is well established that both genetics and the environment must be playing a role in development of T1D (39).

In conclusion, we have defined changes in the surface phenotype of peripheral blood B cells of patients along the continuum of T1D. Our findings reveal a potential biomarker for increased risk of T1D: a significant decrease in the B_{ND} population of IBCs in peripheral blood. Indeed, we have uncovered a possible biomarker for risk of development of B cell–dependent autoimmunity in general, namely a decrease in total B_{ND}. Longitudinal studies in progress should help establish the utility of these changes as prognostic and diagnostic biomarkers. Finally, findings are consistent with a role for perturbation of B-cell anergy in development of T1D and perhaps other autoimmune diseases.

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