AUTOIMMUNITY

A divergent population of autoantigen-responsive CD4$^+$ T cells in infants prior to β cell autoimmunity

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Autoimmune diabetes is marked by sensitization to β cell self-antigens in childhood. We longitudinally followed at-risk children from infancy and performed single-cell gene expression in β cell antigen–responsive CD4$^+$ T cells through pre- and established autoimmune phases. A striking divergence in the gene signature of β cell antigen–responsive naïve CD4$^+$ T cells from children who developed β cell autoimmunity was found in infancy, well before the appearance of β cell antigen–specific memory T cells or autoantibodies. The signature resembled a pre–T helper 1 (T_H1)/T_H17/T follicular helper cell response with expression of *CCR6*, *IL21*, *TBX21*, *TNF*, *RORC*, *EGR2*, *TGFB1*, and *ICOS*, in the absence of *FOXP3*, *IL17*, and other cytokines. The cells transitioned to an *IFNG*-T_H1 memory phenotype with the emergence of autoantibodies. We suggest that the divergent naïve T cell response is a consequence of genetic or environmental priming during unfavorable perinatal exposures and that the signature will guide future efforts to detect and prevent β cell autoimmunity.

INTRODUCTION

Autoimmunity results from the chronic activation of T and/or B lymphocyte clones, which recognize self-antigens in the absence of sufficient regulatory signals (*1*). Type 1 diabetes is an autoimmune disease in which patients become sensitized to β cell antigens before the clinical symptoms appear (*2*). Sensitization is marked by the appearance of autoantibodies against β cell proteins such as insulin (*3*) and glutamic acid decarboxylase (GAD65) (*4*). Sensitization is recognized as the start of the disease process and is most frequent at around 1 to 3 years of age in genetically susceptible children (*5*, *6*). (Pro)insulin is the major early target in this age period (*7*, *8*), and the emergence of insulin autoantibodies is followed by a relatively rapid spread of autoimmunity to other antigens, including GAD65 (*9*), before the development of clinical diabetes (*10*).

Sensitization against β cell antigens requires the activation of antigen-responsive CD4$^+$ T cells. Their activation and conversion to memory T helper 1 (T_H1) (*11*) and T_H17 (*12*, *13*) cells is thought to be initiated by ill-defined environmental cues encountered around the time of β cell autoantibody appearance. However, the search for environmental exposures, which temporally coincide with sensitization, has largely been uneventful (*14*) and has mainly provided evidence regarding its associations with perinatal and very early life events (*15*–*19*).

Since 2000, we have longitudinally followed a birth cohort of children with strong genetic susceptibility for type 1 diabetes and regularly obtained blood samples for immune cell profiling (*20*). This has provided us with a rare opportunity to trace the emergence, priming, and fate of human autoreactive CD4$^+$ T cells relative to the development of β cell autoimmunity. Here, we investigated the transition of β cell antigen–responsive CD4$^+$ T cells from naïve cells to sensitized memory autoreactive cells, and describe their transcription profiles at the single-cell level during this transition. We discovered unique β cell antigen–responsive naïve CD4$^+$ T cells with an unusual proinflammatory pre–T_H1/T_H17/T follicular helper (T_{FH}) cell–like gene expression profile early in life in infants who subsequently developed β cell autoimmunity. We suggest that this is the result of genetic susceptibility or early priming by specific perinatal exposures and that such priming leads to a latent immunosusceptible phase of undefined duration before fully sensitized autoimmunity.

RESULTS

We examined multiple peripheral blood mononuclear cell (PBMC) samples collected longitudinally over the first years of life from children with a family history and human lymphocyte antigen (HLA) susceptibility for type 1 diabetes (Fig. 1A and table S1). These included samples from 12 children who did not develop β cell autoimmunity (group 1) and 16 children who developed autoimmunity against the β cell antigens insulin and/or GAD65 (group 2). The frequencies of lymphocyte subpopulations matured as expected in these children, and there were no differences in the T and B lymphocyte subpopulations observed between the two groups until 5 years of age (fig. S1).

The appearance of β cell antigen–responsive memory CD4$^+$ T cells is synchronous to β cell autoantibody production

We sought to identify memory CD4$^+$ T cells reactive to β cell antigens. Memory proinsulin- and GAD65-responsive cells were defined as CD4$^+$ T cells, which proliferated vigorously (CFSE^{superdim}), became activated (CD25$^+$), and were CD45RO$^+$ after exposing the PBMCs to the antigen for 5 days (Fig. 1B). This definition was based on analyses of naïve and memory CD4$^+$ T cell responses to a vaccine antigen (fig. S2, A and B) and the responses observed after depletion of the CD45RO compartment of PBMCs (fig. S2C). The memory CD4$^+$ T cell responses to proinsulin and GAD65 were confined to group 2 (Fig. 1C, left and

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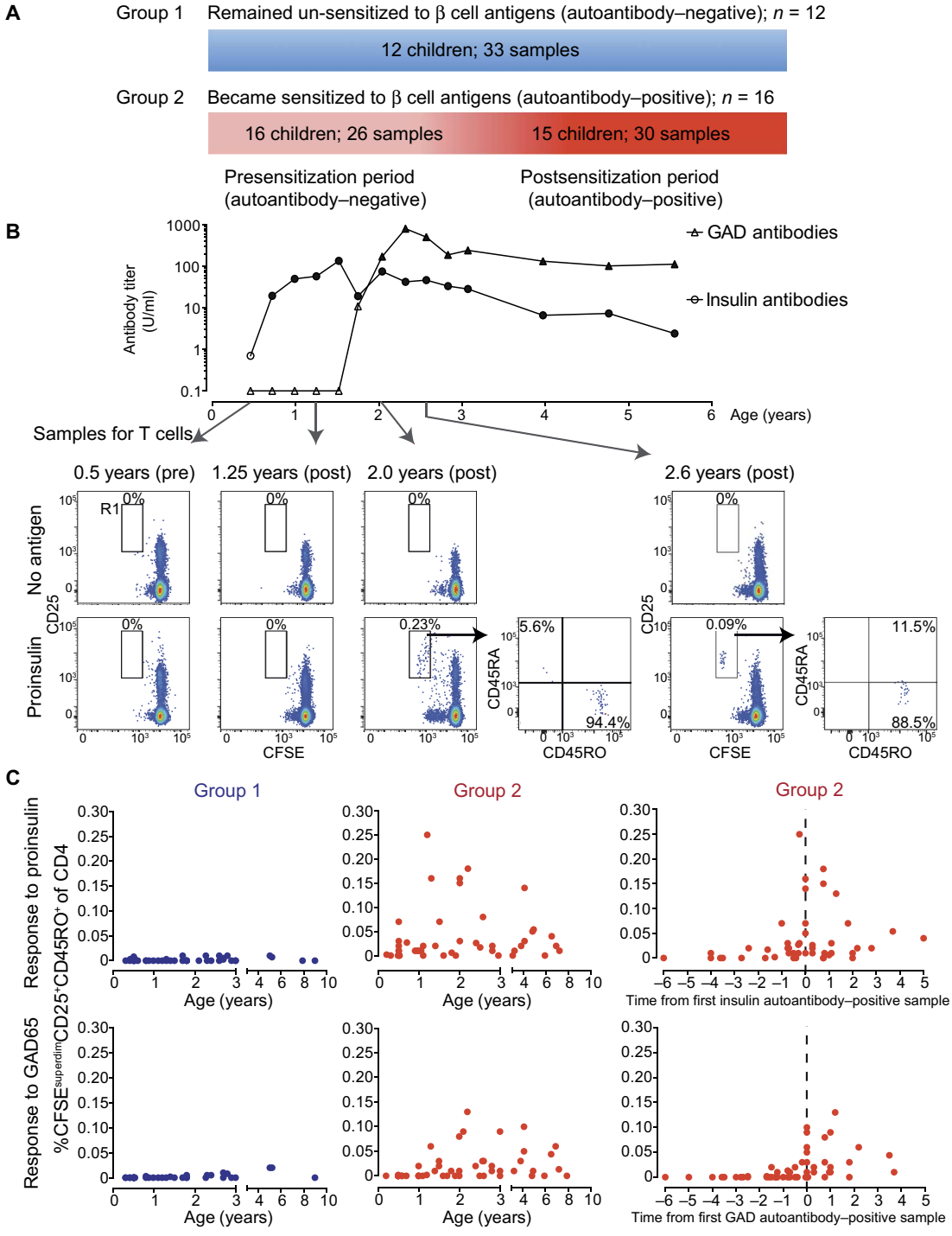

Fig. 1. Identification and emergence of autoreactive memory CD4$^+$ T cells. (**A**) Schematic representation of PBMC sampling relative to sensitization, which was defined by β cell autoantibody production (group 1, no sensitization; group 2, sensitization and production of β cell autoantibodies). The numbers of children and samples tested are given for each group and for pre- and postsensitization periods. Child details are provided in table S1. (**B**) Example of a sensitized child. Top panel shows autoantibody production against insulin (○) and GAD65 (Δ) relative to the child's age. Positive antibodies are shown as filled symbols. Bottom panels show the CD4$^+$ T cell response to proinsulin or no antigen for the same child before (0.5 years) and after (1.25, 2, and 2.6 years) sensitization. Lymphocytes were gated using forward and side scatter (SSC), doublets excluded, dead cells excluded by 7-aminoactinomycin D (7AAD) staining, and the live (7AAD$^-$) CD3$^+$CD4$^+$ lymphocytes gated for analysis of CD25 up-regulation and proliferation [carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution]. The R1 gate containing the most proliferative (more than three dilution cycles) and most activated CD4$^+$ T cell population (CFSE^{superdim}CD25$^+$) was defined as the memory CD4$^+$ T cell response. CD45RO and CD45RA staining for cells within the R1 gate are shown in the adjacent fluorescence-activated cell sorting (FACS) plot as indicated by the arrow. (**C**) Memory CD4$^+$ T cell responses to proinsulin (top panels) or GAD65 (bottom panels) from all tested samples in children who remained unsensitized (n = 12; group 1; left panel, blue symbols) and in children who became sensitized and produced insulin (n = 13) or GAD65 (n = 14) autoantibodies (group 2; right panels, red symbols). The responses are shown relative to the age of the child (left and middle panels) and for the children who developed autoantibodies relative to autoantibody development (right panel).

middle panels). Ten of 16 children who became sensitized and produced β cell autoantibodies had >0.02% proinsulin-responsive memory $CD4^+$ T cells or GAD65-responsive memory $CD4^+$ T cells in at least one sample versus 0 of 12 children who remained β cell autoantibody–negative ($P = 0.006$). There were also differences between samples from children in group 1 and group 2 in the magnitudes of $CD4^+$ T cell responsiveness to proinsulin (mean, 0.002% versus 0.04%; $P < 0.0001$) and GAD65 (mean, 0.002% versus 0.019%; $P = 0.001$). The frequencies of proinsulin- and GAD65-responsive memory $CD4^+$ T cells in group 2 children were low and, on the basis of average CFSE dilution (four to five cycles), correspond to precursor frequencies of <10 per 100,000 $CD4^+$ T cells (Fig. 1B).

Synchronicity was observed between the presence of memory $CD4^+$ T cells and the timing of the corresponding autoantibody appearance (Fig. 1C, right panels). The mean T cell response to proinsulin was 0.043% greater in insulin autoantibody–positive samples than in insulin autoantibody–negative samples ($P < 0.0001$), whereas the age of the child did not affect the proliferative responsiveness to proinsulin (−0.003% per year, $P = 0.31$). Similarly, the GAD65 T cell response was 0.034% greater in GAD65 autoantibody–positive samples ($P < 0.0001$), whereas the age of the child did not affect the proliferative responsiveness to GAD65 (0.000% per year, $P = 0.82$). This synchronicity indicates that β cell autoimmunity is marked by a classic adaptive immune response with concurrent $CD4^+$ T cell and B lymphocyte activation and autoantibody production.

Naïve β cell antigen–responsive $CD4^+$ T cells are present in the absence of β cell autoantibody production

We previously reported that naïve β cell antigen–responsive $CD4^+$ T cells are present in neonates (*21*), but we could not examine their relationship to β cell autoimmunity. Therefore, we searched for these cells in our current cohort of children. We defined naïve β cell antigen–responsive cells as proliferative ($CFSE^{dim}$, with dim defined as dye dilution up to a maximum of three proliferation cycles), activated ($CD25^+$), $CD45RA^+$ $CD45RO^-CD4^+$ T cells on the basis of the naïve and memory T cell responses to a vaccine antigen (fig. S2, A and B) and their responses to β cell antigens (fig. S3A). Naïve proinsulin- and GAD65-responsive $CD4^+$ T cells with these characteristics were identified in samples from both groups of children, with no marked quantitative differences between the two groups (fig. S3B).

Gene expression profiling of naïve β cell antigen–responsive $CD4^+$ T cells revealed unique clusters consistent with subsequent sensitization

We next investigated how the naïve $CD4^+$ T cells respond to presented β cell antigens and determined the gene expression profiles of the β cell antigen–responsive $CD4^+$ T cells obtained from 14 children at 6 months of age using single-cell polymerase chain reaction (PCR) (Figs. 2 and 3).

The proinsulin-responsive $CD4^+$ T cells could be segregated into three major clusters (cluster 1, 350 cells; cluster 2, 82 cells; cluster 3, 137 cells) (Fig. 2A). Cluster 1 was characterized by a low expression of all genes or the expression of *FOXP3*, *TGFB1*, *CTLA4*, *CCR7*, and/or *CCR5* in the absence of cytokine genes. Clusters 2 and 3 were distinguished from cluster 1 by the expression of *CCR6* and *EGR2* and a relative absence of *FOXP3*, and cluster 3 was further distinguished from cluster 2 by the expression of *IL21*, *TGFB1*, *TNF*, *TBX21*, *RORC*, *ICOS*, and *CTLA4* (Fig. 2B). The profile of cluster 3 was unusual and had features of a proinflammatory pre-$T_H1/T_H17/T_{FH}$ phenotype, whereas the profile of cluster 1 had features of a regulatory phenotype.

The proinflammatory cluster 3 was dominated by cells from children in group 2 who later developed β cell autoantibodies (97.8% of cells versus 37.4% in cluster 1 and 42.7% in cluster 2; $P < 10^{-10}$) (Fig. 2C). Cluster 3 proinsulin-responsive T cells were dominant (>90% of responsive cells) in three of the six group 2 children tested. Heat maps (Fig. 2D) and Hurdle model analysis (Fig. 2, D and E, and table S2) (*22*) showed that the proinsulin-responsive $CD4^+$ T cells from children who developed β cell autoantibodies were biased toward increased expression of *IL21*, *CCR6*, *EGR2*, *TBX21*, *TGFB1*, *RORC*, *ICOS*, *CXCR3*, *TNF*, *CTLA4*, and *CCR5* and decreased expression of *FOXP3* and *IL4*.

The GAD65-responsive $CD4^+$ T cells could also be segregated into clusters (Fig. 3A), with regulatory (cluster 1, 210 cells) and proinflammatory (cluster 4, 194 cells) profiles (Fig. 3B). Again, the proinflammatory cluster was dominated by cells from children in group 2 who subsequently developed β cell autoimmunity (90.2% of cells in cluster 4 versus 39.5%, 50%, and 50% in clusters 1, 2, and 3, respectively; $P < 10^{-10}$) (Fig. 3C). Of the six group 2 children tested, three had a dominant (>90% of responsive cells) cluster 4 $CD4^+$ T cell response to GAD65, and a fourth had 24% of responsive cells in cluster 4. The proinflammatory cluster was characterized by increased expression of *IL21*, *CCR6*, *EGR2*, *TBX21*, *TGFB1*, *RORC*, *ICOS*, *TNF*, *CXCR3*, *IFNG*, *IL4*, *CTLA4*, and *CCR5* genes and decreased expression of *FOXP3* (Fig. 3, D and E).

Gene expression profiles of naïve antigen-responsive $CD4^+$ T cells can predict β cell autoimmunity

We developed an algorithm from the gene expression profile of the proinsulin-responsive $CD4^+$ T cells at 6 months of age to obtain autoimmunity likelihood scores that predict future β cell autoimmunity. To obtain unbiased estimates of the predictive power of our algorithm, we divided the cells into 50 approximately equally sized sets to perform 50-fold cross-validation using 49 of the sets for training and the remaining set for testing with 50 repetitions of the procedure. A random forest classifier based on all genes expressed in ≥5% of the cells yielded discrimination area under the curves (AUCs) of 0.79 ($P < 1 \times 10^{-5}$) for proinsulin-responsive $CD4^+$ T cells and 0.80 ($P < 1 \times 10^{-5}$) when it was applied to GAD65-responsive $CD4^+$ T cells, suggesting high predictive power for subsequent β cell autoimmunity (Fig. 4A). Using recursive feature selection, the best-fit model was derived from a subset of genes that included *CTLA4*, *CCR7*, *CCR6*, *TGFB1*, *CCR5*, *EGR2*, *ICOS*, and *IL21* (all associated with higher expression) and *FOXP3* (associated with lower expression) for proinsulin-responsive $CD4^+$ T cells. This model resulted in cross-validated AUCs of 0.82 ($P < 1 \times 10^{-5}$) for proinsulin-responsive $CD4^+$ T cells and 0.80 ($P < 1 \times 10^{-5}$) for GAD65-responsive $CD4^+$ T cells.

The autoimmunity likelihood scores were used to examine the response profiles in individual children (Fig. 4B). None of the children in group 1 and 83% of the children in group 2 had median scores of >0.5 for proinsulin- or GAD65-responsive $CD4^+$ T cells ($P = 0.01$). Thus, the scores generated from samples obtained as early as 6 months of age were predictive of future β cell autoimmunity. This early response profile suggests that the immune system is preprimed in infancy for a proinflammatory response to β cell antigens.

The gene expression profiles of naïve tetanus toxoid–responsive $CD4^+$ T cells were not associated with β cell autoimmunity

To determine whether there was generalized prepriming in infants, we examined the naïve $CD4^+$ T cell responses to a non–β cell antigen, tetanus toxoid, in samples obtained before the first vaccination at 3 months of age in a validation group of children (fig. S4). We observed no discernable clusters of the gene profile from naïve tetanus toxoid–responsive

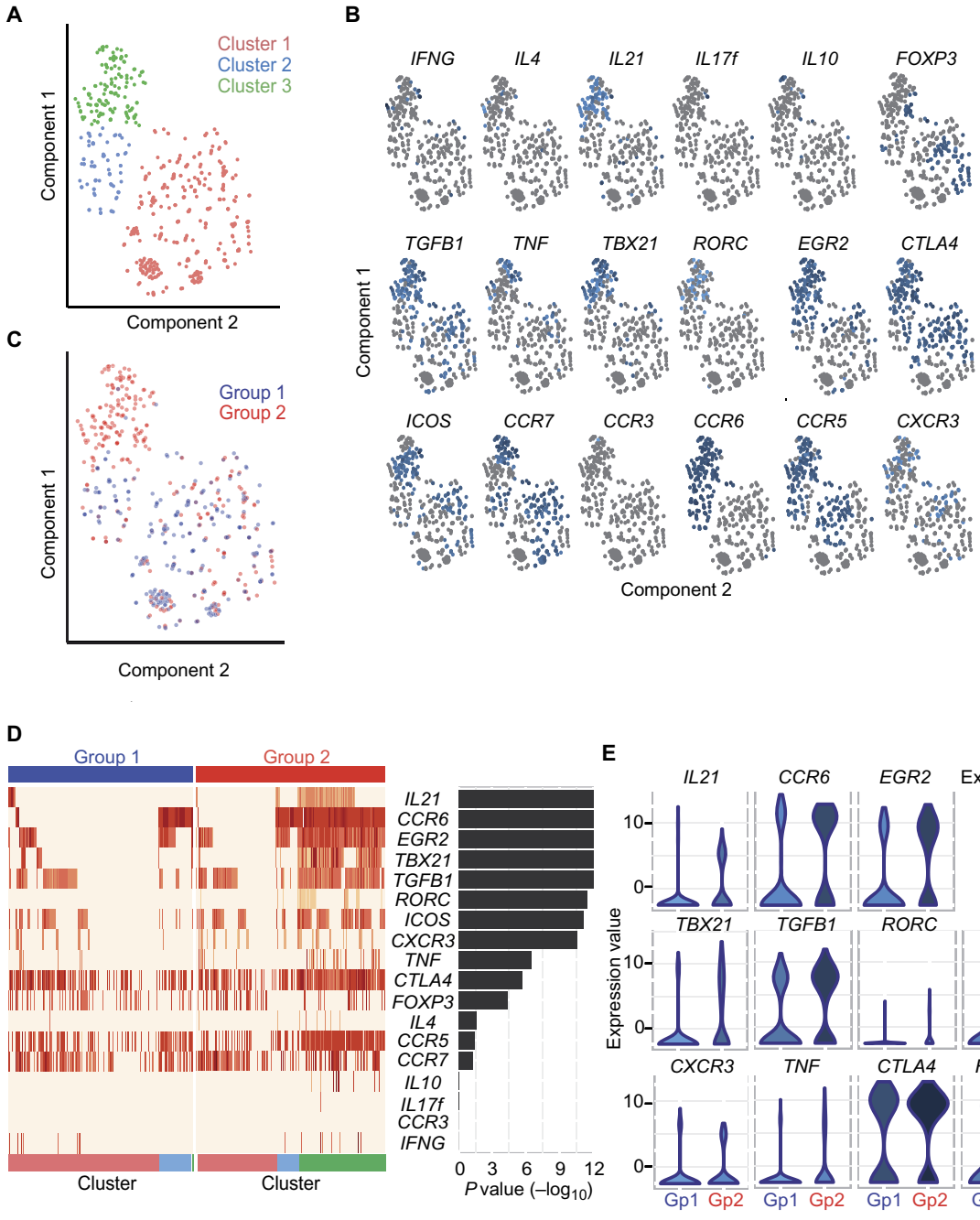

Fig. 2. Gene transcription profiles of proinsulin-responsive CD4$^+$ T cells from children at 6 months of age. Proinsulin-responsive cells were single cell–sorted and processed for multiparameter gene expression. The profiles from 569 proinsulin-responsive cells are shown. (**A**) *t*-distributed stochastic neighbor embedding (t-SNE) visualized three major clusters, defined as clusters 1 (red, 350 cells), 2 (blue, 82 cells), and 3 (green, 137 cells). (**B**) Expression intensities (gray, no expression; dark blue, highest expression) of all 15 immune function genes analyzed in each cell in the t-SNE. (**C**) t-SNE showing cells from children in group 1 (eight children; 270 cells shown in blue) and group 2 (six children; 299 cells shown in red). (**D**) Heat maps of the genes examined in the proinsulin-responsive CD4$^+$ T cells. Each bar represents an individual cell from children in group 1 (left, blue) and group 2 (right, red). Cells are ordered according to their cluster, and genes are ordered according to their overall significance for comparisons between cells from groups 1 and 2 using the Hurdle model and are shown in the right panel (likelihood ratio test). (**E**) Violin plots showing the distribution of C_t expression values for each gene showing significance at $P < 0.0001$ (likelihood ratio test) in cells from group 1 (Gp1, blue) and group 2 (Gp2, red). The proportion of cells expressing the gene ranged from light blue (0%) to dark blue (80%). See table S2 for additional information.

CD4$^+$ T cells. When we applied the gene expression algorithm, which could segregate β cell autoimmunity in both proinsulin- and GAD65-responsive naïve CD4$^+$ T cells, to the naïve tetanus toxoid–responsive CD4$^+$ T cells, we found no relationship with the development of β cell autoimmunity ($P = 0.81$). In contrast, the scores obtained for proinsulin-responsive CD4$^+$ T cells in samples obtained at 3 months of age using the same algorithm were higher in the children who developed β cell autoimmunity ($P = 0.01$); median scores of >0.5 were found in none of the children who

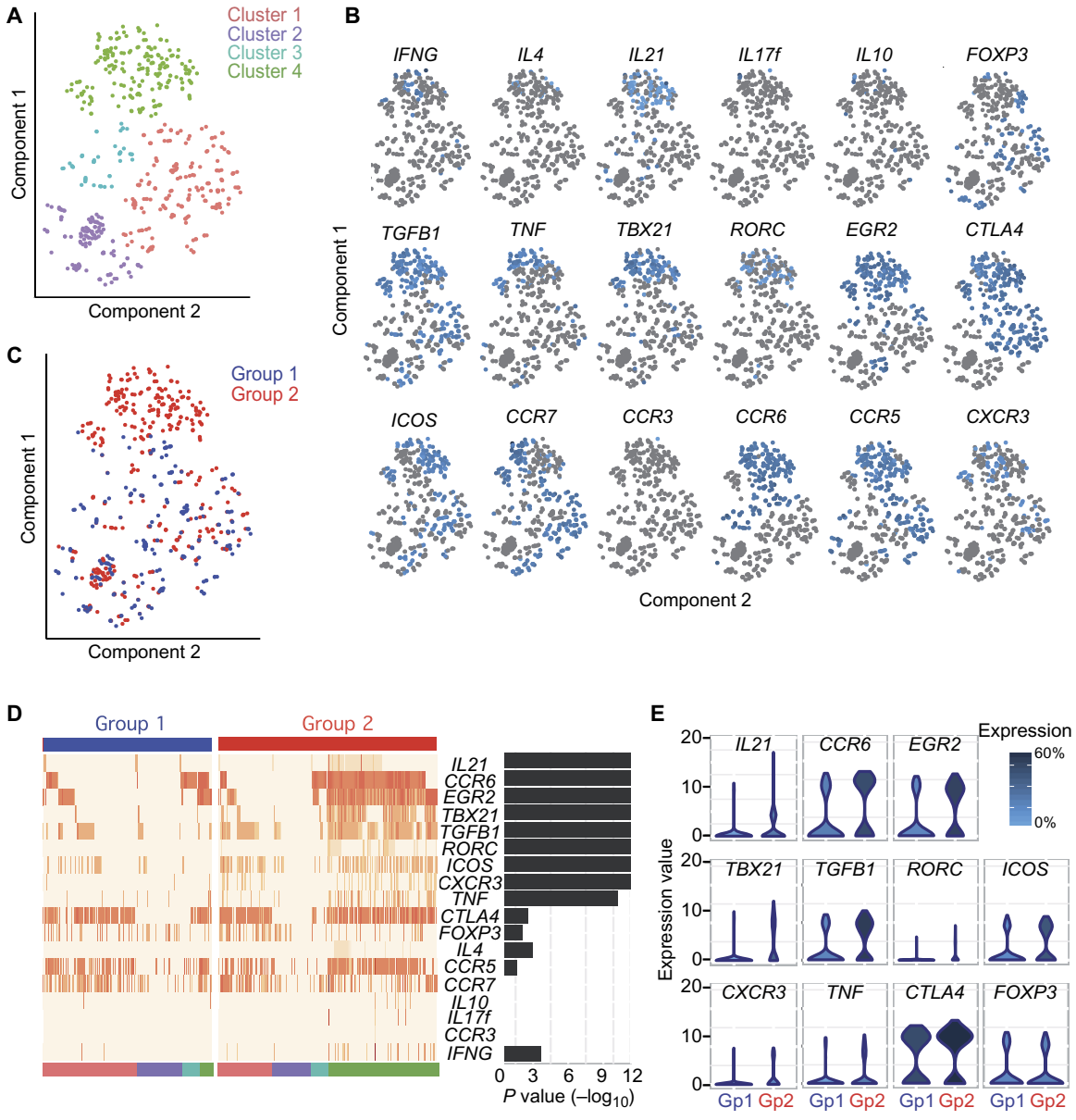

Fig. 3. Gene transcription profiles of GAD65-responsive CD4$^+$ T cells from children at 6 months of age. GAD65-responsive cells were single cell–sorted and processed for multiparameter gene expression. The profiles from 578 GAD65-responsive cells are shown. (**A**) t-SNE visualized four major clusters defined as clusters 1 (red, 210 cells), 2 (purple, 124 cells), 3 (blue, 50 cells), and 4 (green, 194 cells). (**B**) Expression intensities (gray, no expression; dark blue, highest expression) of all 15 immune function genes analyzed in each cell in the t-SNE. (**C**) t-SNE showing cells from children in group 1 (eight children; 233 cells shown in blue) and group 2 (six children; 345 cells shown in red). (**D**) Heat maps of the genes examined in the GAD65-responsive CD4$^+$ T cells. Each bar represents an individual cell from children in group 1 (left, blue) and group 2 (right, red). Cells are ordered according to their cluster, and genes are ordered according to their overall significance for comparisons between cells from groups 1 and 2 using the Hurdle model and are shown in the right panel (likelihood ratio test). (**E**) Violin plots showing the distribution of C_t expression values for each gene showing significance at $P < 0.0001$ (likelihood ratio test) in cells from group 1 (Gp1, blue) and group 2 (Gp2, red). The proportion of cells expressing the gene ranges from light blue (0%) to dark blue (60%). See table S2 for additional information.

remained β cell autoantibody–negative and in 50% of the children who developed β cell autoimmunity (fig. S4C). These data suggested that the fate of naïve antigen-responsive CD4$^+$ T cells in children who develop β cell autoimmunity was not systematically biased for all antigens.

Autoantigen-responsive CD4$^+$ T cells shift toward a T_H1 signature upon sensitization

We examined the changes in the gene expression profiles of β cell antigen–responsive CD4$^+$ T cells during the preclinical period in group 2 (Fig. 5). Distinct gene expression clusters were observed when the pre- and postsensitization naïve and memory CD4$^+$ T cells were analyzed collectively (Fig. 5A for proinsulin and Fig. 5D for GAD65). Both the proinsulin- and the GAD65-responsive cells had clusters dominated by presensitization cells (clusters 1, 2, and 3 for proinsulin; clusters 2 and 4 for GAD65) or postsensitization cells (clusters 6, 7, 8, and 9 for proinsulin; clusters 1, 9, and 10 for GAD65). For proinsulin (Fig. 5A), the postsensitization-dominated clusters were marked by the expression of *IFNG* (clusters 6 and 7), *TNF* (clusters 6, 7, 8, and 9), and

CCR7 (clusters 6, 7, and 8). Similarly, the postsensitization clusters for GAD65 (Fig. 5D) were marked by the expression of *IFNG* (cluster 1), *TNF* (clusters 1, 9, and 10), and *CCR7* (cluster 9).

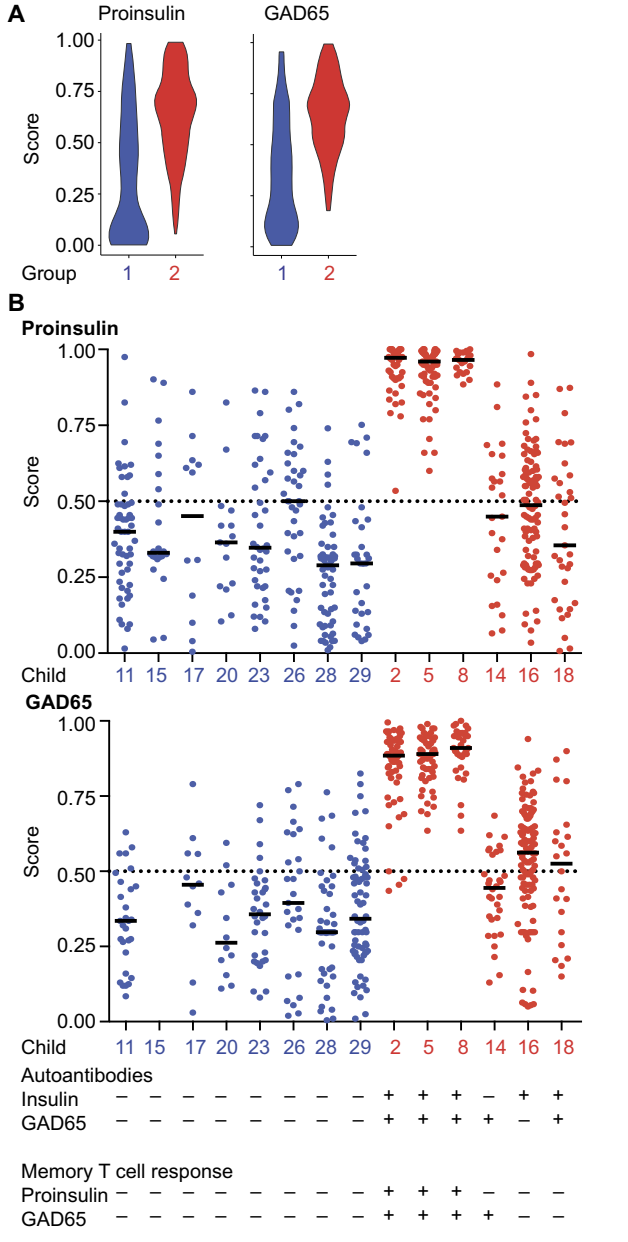

Fig. 4. Composite gene expression profile autoimmunity likelihood scores of β cell antigen–responsive single cells obtained at 6 months of age. (**A**) Distribution of autoimmunity likelihood scores of proinsulin-responsive $CD4^+$ T cells (left panel) from children in group 1 (eight children; 270 cells shown in blue) and group 2 (six children; 299 cells shown in red). The scores are cross-validated prediction scores obtained from the random forest predictor trained to identify cells from group 2 children. The predictor trained on proinsulin-responsive cells was also used to score the GAD65-responsive $CD4^+$ T cells [bottom panel of (B)] to validate the predictive model. (**B**) The scores for proinsulin-responsive $CD4^+$ T cells (top) and GAD65-responsive $CD4^+$ T cells (bottom) obtained from individual children at 6 months of age are indicated by the child's ID number used in table S1. For each child, the subsequent outcome in follow-up samples with respect to β cell autoantibodies and memory $CD4^+$ T cell response is indicated under the child's ID number, where + for the $CD4^+$ T cell response is >0.02%.

A comparison of all pre- and postsensitization β cell antigen–responsive cells revealed decreased expression of *CCR6* and *IL21* ($P < 1 \times 10^{-10}$) and increased expression of the T_H1 genes *IFNG* ($P = 5 \times 10^{-4}$) and *TNF* ($P = 1 \times 10^{-7}$) in the postsensitization cells for both proinsulin (Fig. 5, B and C, and table S3) and GAD65 (Fig. 5, E and F, and table S3). The frequencies of cells expressing *IL17*, *IL4*, or *IL10* and cytokine gene–negative/$FOXP3^+$ cells were low in these children. Notably, the gene expression profiles of postsensitization β cell antigen–responsive cells were different to those of the postvaccination tetanus toxoid–responsive $CD4^+$ T cells, which dominated a single cluster marked by increased expression of *IL21*, *IFNG*, *TNF*, *IL4*, *CXCR3*, *CCR7*, *TBX21*, and *ICOS* (fig. S5).

Finally, we examined the T cell receptor (TCR) α and β sequences of single cell–sorted proinsulin- or GAD65-responsive cells in pre- and postsensitization samples from children in group 2 to determine whether expanded clones from the presensitization period could be identified. However, none of the 596 TCR sequences identified were found in more than one sample or in more than one child (table S4). These data suggest that there is large diversity in the autoreactive $CD4^+$ T cell repertoire and that sensitization is not a result of the expansion of a few dominant clones.

DISCUSSION

Here, we examined the features of lymphocytes obtained during infancy and early childhood in children with genetic susceptibility for type 1 diabetes. We identified a divergent naïve $CD4^+$ T cell response to β cell antigens that preceded the appearance of β cell antigen–responsive memory T cells and β cell autoantibodies. This response was seen in 6-month-old infants and was not observed for an irrelevant non–β cell antigen. These findings indicate that β cell autoimmunity may be preprimed in infancy.

The children in this study had neither autoantibodies nor memory $CD4^+$ T cell responses to β cell antigens at 6 months of age. However, we found evidence of an incomplete priming of β cell antigen–responsive $CD4^+$ T cells at this age. The evidence for this included the presence of naïve $CD4^+$ T cells, which could acquire a semidifferentiated profile in response to β cell antigens. These cells were in 50% or more of children who subsequently developed β cell autoimmunity. The cells were marked by the expression of *CCR6*, *IL21*, *TBX21*, *TNF*, *RORC*, *EGR2*, *TGFB1*, and *ICOS*, in the absence of *FOXP3*, *IL17*, and other cytokines. This divergent response included genes that are induced during naïve T cell activation (*23*) and during differentiation toward T_{FH} (*24*, *25*), T_H1 (*26*, *27*), and T_H17 immunity (*24*, *25*, *28*–*30*). T_{FH}-, T_H1-, and T_H17-biased responses are believed to contribute to the pathogenesis of type 1 diabetes (*11*–*13*, *31*, *32*).

Incomplete priming of $CD4^+$ T cells has been reported for T_{FH} cell differentiation (*33*) and by virus-infected antigen-presenting cells (*34*). Neonates are known to have inefficient antigen presentation that ineffectively primes T cells (*21*, *35*). We suggest that perinatal encounters between $CD4^+$ T cells and β cell antigen–loaded antigen-presenting cells could lead to preprimed, semicommitted T cells, as exemplified by our data. Because we did not see a similar divergent response to a non–β cell antigen, we suggest that this phenomenon shows specificity. We further suggest that a preprimed state could be genetically programmed or that diabetogenic environmental exposures encountered during the perinatal period [for example, early viral infections (*16*, *36*, *37*)] may favor partial priming to semicommitted β cell antigen–responsive $CD4^+$ T cells. Viral infections induce a type 1 interferon

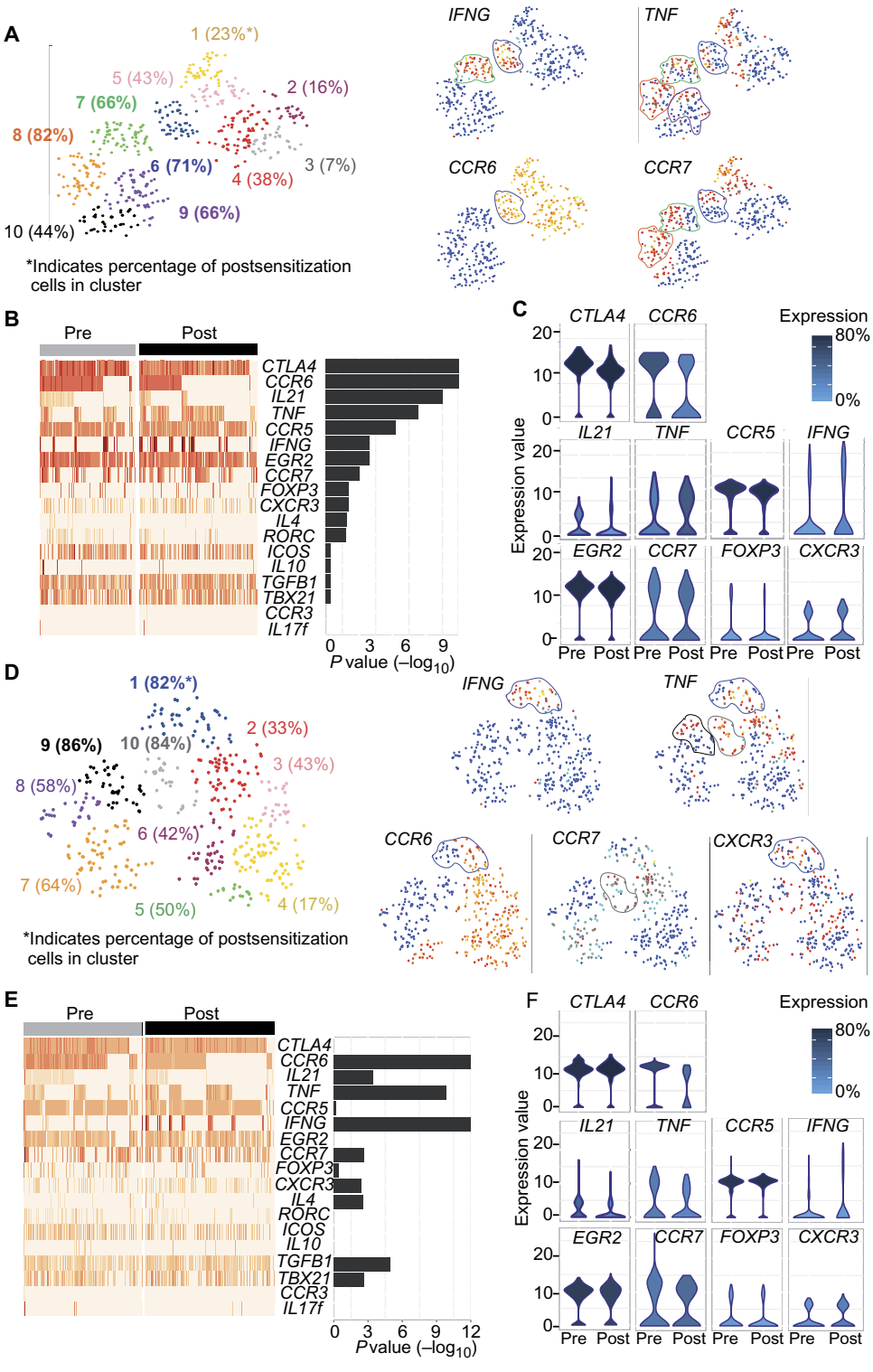

Fig. 5. Transition of β cell antigen–responsive CD4$^+$ T cell gene transcription profiles during the development of β cell autoimmunity. Single proinsulin- and GAD65-responsive cells were sorted as CFSE$^{dim/superdim}$CD25$^+$CD4$^+$ before gene expression analysis. Data for proinsulin- and GAD65-responsive CD4$^+$ T cells from 15 pre- and postsensitization samples in four children from group 2 are shown. (**A**) t-SNE for gene expression of proinsulin-responsive CD4$^+$ T cells. Left panel shows all cells classified into 10 clusters. The proportion of cells derived from postsensitization samples is indicated for each cluster. Clusters with a preference for postsensitization cells (>66%, all $P < 0.01$ compared to the other clusters) are indicated in bold. The expression levels of genes that distinguish the postsensitization clusters are shown in the right panels (postsensitization-associated clusters showing abundant expression of the indicated gene are outlined; dark blue, no expression; yellow, highest expression). (**B**) Heat maps (left panel) of the genes examined in the proinsulin-responsive CD4$^+$ T cells separated into the presensitization (282 cells) and postsensitization (280 cells) periods. The genes are ordered from top to bottom according to their overall significance for comparisons between cells from pre- and postsensitization samples using the Hurdle model (right panel) (likelihood ratio test). (**C**) Violin plots of the genes showing significant changes ($P < 0.0001$) (likelihood ratio test) in expression between the pre- and postsensitization periods for proinsulin-responsive CD4$^+$ T cells, where the proportion of cells expressing the gene ranges from light blue (0%) to dark blue (80%). (**D** to **F**) t-SNE (D), heat maps (E), and violin plots (F) for gene expression of GAD65-responsive CD4$^+$ T cells from presensitization (215 cells) and postsensitization (243 cells) periods as described for the proinsulin-responsive cells. See table S3 for additional information.

microenvironment, which increases the potency of antigen presentation (*38*), but also acts directly on activated CD4$^+$ T cells to increase their survival (*39*) and priming of adaptive immune responses (*40*, *41*). Hence, it is conceivable that such microenvironments around sites where β cell antigens may be presented to CD4$^+$ T cells could provide an extended phase of activation that sets the immune system into a state of latent autoreactivity.

A second feature of our study was the ability to observe the transition to a memory T cell response. Memory CD4$^+$ T cell responsiveness to β cell antigens has been previously detected in the late stages of type 1 diabetes (*11*, *42*, *43*). We show that the initial semicommitted naïve CD4$^+$ T cell responsiveness to β cell antigens in infancy was eventually accompanied by memory CD4$^+$ T cell responses, which was synchronous

to the appearance of β cell autoantibodies. We also observed a clear committed fate of the memory β cell antigen–responsive $CD4^+$ T cells, as the semicommitted T_{FH}-T_H1-T_H17 response of naïve T cells was replaced by a T_H1-associated *IFNG* memory response around the time of in vivo sensitization. Very few cells expressed *IL17*, *IL4*, or *IL10* genes. Moreover, the expression of *IL21*, a T_{FH}-associated cytokine gene, decreased after sensitization; this finding distinguished the β cell antigen–responsive cells from the tetanus toxoid–responsive memory $CD4^+$ T cells.

We found no evidence for the selection of dominant or public clones in the memory $CD4^+$ T cell response to β cell antigens, and there was significant diversity in the TCRs found in the responsive cells. Although we have studied small numbers of children, it was also notable that we found no alterations in the frequencies of T and B lymphocyte subpopulations during the development of autoimmunity, suggesting that differences observed at diabetes onset may be acquired during the course of autoimmunity (*44*).

The main findings of the study are based on proliferative responses and have excluded any autoreactive T cells that respond to presented autoantigen in nonproliferative manners. It should also be considered that we did not use purified memory and naïve $CD4^+$ T cells in the response assays and that the findings rely on the assumption that the features used to define naïve and memory $CD4^+$ T cell responses are similar in vaccine (tetanus toxoid)– and autoantigen-specific $CD4^+$ T cells. An important weakness of this study is the absence of a separate validation cohort of infants and children. Thus, the findings and their implication require validation in a further cohort.

Overall, our findings suggest that priming of $CD4^+$ T cell responses to β cell autoantigens may be a multistep process that is initiated in infancy with inefficient presentation of antigens to naïve T cells, which remain in a protracted phase of incomplete differentiation and subsequent transition into competent T_H1 cells.

MATERIALS AND METHODS

Study design

The main research objective was to determine whether children who developed β cell autoimmunity had evidence of autoreactive T lymphocyte engagement in infancy before autoimmunity. The study objective could only be addressed by the availability of stored PBMCs from children who had been prospectively followed from birth to seroconversion to β cell autoantibody–positive. These samples were available in the German BABYDIET study (*20*), which became the sample source of the study. Sample size was determined by the number of children in the German BABYDIET study who developed β cell autoimmunity and who had viable stored PBMCs that had been collected before β cell autoimmunity. The age of sample collection selected for the measurement of $CD4^+$ T cell responses was made on the basis of the availability of stored viable PBMCs and with the intention to include samples before, at, and after β cell autoantibody seroconversion, where possible. A similar number of children who remained autoantibody-negative throughout follow-up in the BABYDIET study were included as controls; samples were chosen at ages that were similar to those of children who developed β cell autoantibodies. All data generated were included in the analysis. Researchers performing the measurements were blinded to the child's autoantibody status and age. Primary data for experiments where $n < 20$ are in the Supplementary Materials (table S6).

Study subjects, samples, and cell isolation

A total of 86 samples of PBMCs from 28 children who participated in the German BABYDIET study (*20*) were analyzed (table S1). Children had at least one first-degree relative with type 1 diabetes, as well as the HLA DRB1*0301-DQB1*0201/DRB1*04-DQB1*0302, DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, or DRB1*03-DQB1*0201/DRB1*03-DQB1*0201 genotypes. The children were prospectively followed from birth, with three monthly blood samples taken for the measurement of β cell autoantibodies. Of the 28 children included in the current analysis, 16 developed β cell antibodies at a median age of 2.1 years (range, 9 months to 7.9 years). A second group of children from the BABYDIET study was used to investigate naïve $CD4^+$ T cell responses to vaccine antigen. These included samples taken from three children aged 3 months who remained β cell autoantibody–negative throughout follow-up and four children who became β cell autoantibody–positive at ages 0.8, 2.7, 3.7, and 4.3 years (table S1). All PBMCs were isolated by density centrifugation over Ficoll-Hypaque from sodium-heparinized peripheral venous blood samples and were frozen in medium containing dimethyl sulfoxide. Tests were done using thawed PBMCs. All samples for one child were tested on the same day. The BABYDIET study was approved by the ethics committee of Ludwig-Maximilians University (protocol no. 329/00) and is registered at ClinicalTrials.gov (NCT01115621). The parents or guardians of each child provided informed consent for participation in the BABYDIET study. In some experiments, adult PBMCs were used and isolated by density centrifugation over Ficoll-Hypaque from sodium-heparinized peripheral venous blood samples obtained from healthy adults. These samples were provided by the Deutsches Rotes Kreuz Blutspendedienst Ost (Dresden, Germany) after obtaining informed consent and with approval from the ethics committee of Technische Universität Dresden (protocol no. EK 240062016).

Autoantibody status of the children

Autoantibodies were measured by radiobinding immunoprecipitation assays with ^{125}I-labeled recombinant human insulin, [^{35}S]methionine-labeled in vitro transcribed and translated human GAD65, [^{35}S]methionine-labeled in vitro transcribed and translated human IA-2, or [^{35}S]methionine-labeled in vitro transcribed and translated human ZnT8 antigens, as previously described (*8*). Children were defined as β cell autoantibody–positive if they were positive for insulin autoantibodies, GAD autoantibodies, or IA-2 autoantibodies in at least two consecutive samples. The age of β cell autoantibody appearance was defined as the age at the first positive sample. To exclude concurrent presence of maternally transferred β cell autoantibodies, all β cell autoantibody–positive children used in this study should have had at least one autoantibody-negative sample before the first positive sample.

Antibodies and flow cytometry

The list of anti-human monoclonal antibodies used for FACS is provided in table S5. Cells were acquired on a Becton Dickinson LSR II flow cytometer or FACSAria II equipped with FACSDiva software. Doublets and clumps were excluded using SSC-A (area) versus SSC-W (width) plots. Live cell populations were gated as 7AAD (BD Biosciences)–negative cells. At least 50,000 gated events were acquired for each sample and were analyzed using FlowJo software version 10 (TreeStar Inc.).

T cell proliferation analysis and isolation of antigen-responsive $CD4^+$ T cells

Thawed PBMCs were stained with CFSE (Invitrogen), by incubating 10^7 cells/ml in phosphate-buffered saline (PBS) at 37°C for 10 min with 0.5 μM CFSE. Staining was terminated by adding RPMI 1640/5% human AB serum (Invitrogen) at 4°C, followed by a cold wash in PBS.

Then, 2.0×10^5 stained cells per well were cultured in round-bottom 96-well microtiter plates in RPMI 1640 supplemented with 5% human AB serum (Invitrogen), 2 mM glutamine, and penicillin/streptomycin (100 U/ml). PBMC cultures were stimulated without antigen or with GAD65 (10 μg/ml) (Diamyd), proinsulin (10 μg/ml) (Lilly), or tetanus toxoid (1 μl/ml) (Sanofi Pasteur MSD). Each condition was performed in two to eight replicate wells. After incubation for 5 days (37°C, 5% CO_2), the cells were harvested, washed in PBS with 1% human serum, and stained for surface molecules. The addition of phorbol 12-myristate 13-acetate (5 ng/ml) (Sigma) and ionomycin (500 ng/ml) 4 hours before cell harvesting was performed for gene expression. Using FACS, $CD4^+$ T cells were analyzed on the basis of CFSE dilution, CD25 up-regulation, CD45RO expression, and CD45RA expression. $CD25^+CD4^+$ T cells that had diluted CFSE were identified as responding cells and were single cell–sorted into 96-well PCR plates containing 5 μl of PBS (prepared with diethyl pyrocarbonate H_2O) and frozen at −80°C for real-time PCR analysis. Cells were sorted as $CFSE^{superdim}$ (CFSE intensity corresponding to more than three dilution cycles) and/or $CFSE^{dim}$ (one to three dilution cycles).

Gene expression analysis

Gene expression of the single cell–sorted T cells was performed as previously described with some modifications (*45*). Complementary DNA (cDNA) was synthesized by applying qScript cDNA Supermix (Quanta Biosciences Inc.) directly to the cells. Total cDNA was preamplified for 16 cycles [1 × 95°C for 8 min, 16 × (95°C for 45 s, 49°C for 1 min, increasing by 0.3°C for each cycle, and 72°C for 1.5 min), and 1 × 72°C for 7 min] with TATAA GrandMaster Mix (TATAA Biocenter) and 19 primer pairs (table S5) at a final concentration of 25 nM in a total reaction volume of 50 μl. Ten microliters of preamplified DNA was treated with 1.2 U of exonuclease I. To quantify gene expression, real-time PCR was performed using a BioMark HD System (Fluidigm Corporation) with the 96.96 Dynamic Array IFC, GE 96 × 96 Fast PCR^+ Melt protocol, and SsoFast EvaGreen Supermix with Low-ROX (Bio-Rad) and 5 μM of the primer in each assay. Raw data were analyzed using KNIME 2.9.4 (*46*) and R version 3.2.2 (Vienna). C_t values were preprocessed using a linear model to correct for potential confounding effects, which can mask biological variability, as previously described (*47*). Briefly, the plate and group (or stage) effects were modeled jointly, thereby regressing out plate effects on each individual gene while controlling for group (or stage) effects to obtain a corrected gene expression data set.

TCR α- and β-chain PCR

The TCR sequences in single cells were amplified and analyzed as previously described (*45*). The TCR α and β sequences and the junction peptide amino acid sequence were analyzed with reference to the IMGT (ImMunoGeneTics) database (*48*). The junction peptides were analyzed using KNIME 2.5.2.

Statistical analysis

Results are presented as means and SD or median and interquartile range. The expression of cell surface molecules is shown as the percentage of positive cells of the tested population. The number of children included in the study was based on the availability of PBMC samples from children who developed β cell autoimmunity ($n = 16$). All were included plus an additional 12 children who did not develop β cell autoimmunity. This was the number required for >80% power to detect a difference in the proportion of children with memory T cell responses to proinsulin or GAD65 between the groups that corresponded to 50% with a two-tailed α of 0.05. To assess the longitudinal behavior of leukocyte populations after birth, we calculated the first-, second-, and third-order polynomial growth models (*49*) for each parameter in each group and performed model selection according to the Akaike information criterion (*50*). The associations of antigen responses to insulin and GAD with the respective autoantibody status at the time the sample was taken were assessed in linear mixed models with random intercepts for each subject and adjustment for age. To identify biologically meaningful gene expression patterns, we performed t-SNE analysis (*51*) using Rtsne (*52*). To model the bimodal expression of single cells, the Hurdle model, a semicontinuous modeling framework, was applied to the preprocessed data (*22*). This allowed us to assess the differential expression profiles with respect to the frequency of expression and the positive expression mean via a likelihood ratio test (*53*). Random forests were trained using the scikit-learn framework with standard settings (*54*). Feature selection was performed in scikit-learn using recursive feature elimination based on the area under the receiver operator characteristic curve. For all tests, a two-tailed P value of <0.05 was considered to be significant. Analyses were performed using GraphPad Prism 6 (GraphPad Software), SPSS 19.0 (IBM Inc.), and R Project for Statistical Computing.

SUPPLEMENTARY MATERIALS

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Fig. S1. Phenotypic characterization of the circulating T and B cell subsets relative to age in children who became sensitized with the production of autoantibodies to insulin or GAD65 (group 2) and children who remained unsensitized to these antigens (group 1).

Fig. S2. Discrimination between naïve and memory $CD4^+$ T cell responses to antigens.

Fig. S3. Low proliferative β cell antigen–responsive $CD4^+$ T cells derive from naïve T cells.

Fig. S4. Gene expression profiles of tetanus toxoid–responsive $CD4^+$ naïve T cells in children prior to tetanus vaccination at 3 months of age.

Fig. S5. Comparison of gene expression profiles between β cell antigen–responsive memory $CD4^+$ T cells and tetanus toxoid–responsive memory $CD4^+$ T cells.

Table S1. Characteristics of the children and samples.

Table S2. Gene expression in proinsulin- and GAD65-responsive $CD4^+$ T cells from children at age 6 months.

Table S3. Gene expression in proinsulin- and GAD65-responsive $CD4^+$ T cells from group 2 children pre and post sensitization.

Table S4. T cell receptor sequences in GAD65- and proinsulin-responsive $CD4^+$ T cells.

Table S5. Genes and primers used for single-cell gene expression analysis and a list of antibodies used in flow cytometry.

Table S6. Primary source data.

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A divergent population of autoantigen-responsive CD4$^+$ T cells in infants prior to β cell autoimmunity

Anne-Kristin Heninger, Anne Eugster, Denise Kuehn, Florian Buettner, Matthias Kuhn, Annett Lindner, Sevina Dietz, Sibille Jergens, Carmen Wilhelm, Andreas Beyerlein, Anette-G. Ziegler and Ezio Bonifacio (February 22, 2017)

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Editor's Summary

Baby β cells in danger

Susceptibility to type 1 diabetes involves genetic risk factors and potential environmental triggers, making it harder to predict which individuals will actually develop disease. Longitudinal samples collected starting shortly after birth from children at risk for type 1 diabetes were examined by Heninger *et al*. to scrutinize early immune responses. Some, but not all, of these children eventually developed autoimmunity; CD4$^+$ T cells from those that progressed had a distinct phenotype after presentation of autoantigen, suggesting that they had already been somewhat activated, even before autoantibodies were detectable. These exciting findings indicate that cues that dictate type 1 diabetes development may be happening even earlier than expected, in infancy.

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