Activation of Islet Autoreactive Naïve T Cells in Infants Is Influenced by Homeostatic Mechanisms and Antigen-Presenting Capacity

Anne-Kristin Heninger,[1] Paolo Monti,[1] Carmen Wilhelm,[1] Petra Schwaiger,[2] Denise Kuehn,[1] Anette-G. Ziegler,[2,3] and Ezio Bonifacio[1]

Islet autoimmunity precedes type 1 diabetes onset. We previously found that islet autoimmunity rarely starts before 6 months of age but reaches its highest incidence already at ~1 year of age. We now examine whether homeostatic expansion and immune competence changes seen in a maturating immune system may account for this marked variation in islet autoimmunity risk in the first year of life. We found naïve proinsulin- and GAD65-responsive T cells in cord blood (CB) of healthy newborns, with highest responses observed in children with type 1 diabetes-susceptible HLA-DRB1/DQB1 genotypes. Homeostatic expansion characteristics with increased IL-7 concentrations and enhanced T-cell responsiveness to IL-7 were observed throughout the first year of life. However, the ability of antigen-presenting cells to activate naïve T cells was compromised at birth, and CB monocytes had low surface expression of CD40 and HLA class II. In contrast, antigen presentation and expression of these molecules had reached competent adult levels by the high incidence age of 8 months. We propose that temporal changes in islet autoimmunity seroconversion in infants are a consequence of the changing balance between homeostatic drive and antigen presentation competence. These findings are relevant for early prevention of type 1 diabetes. ***Diabetes*** **62:2059–2066, 2013**

Type 1 diabetes is an autoimmune disease resulting from the destruction of insulin-producing pancreatic islet β-cells. The disease is associated with the presence of autoantibodies and antigen-experienced T cells against islet autoantigens (1). Autoreactive T cells can be identified in patients with type 1 diabetes and nondiabetic control subjects (2). Patients have naïve and memory autoantigen-responsive $CD4^+$ and $CD8^+$ T cells, whereas autoreactive T cells identified in control subjects appear to be confined to the naïve T-cell compartment (3).

The presence of autoimmunity against islet antigens is detected by measuring autoantibodies in the blood and it is assumed that seroconversion to islet autoantibody positivity is accompanied by activation of islet autoreactive T cells. Seroconversion is rare prior to 6 months of age but rapidly reaches a peak incidence at ~1 year of age in genetically susceptible children (4,5). We postulated that a switch from relative protection to susceptibility to islet autoimmunity may be driven by physiological changes in immune competence and homeostatic mechanisms in the first years of life. T cells at birth are mostly naïve, thereby requiring strong signals through the T-cell receptor and costimulation for priming. On the other hand, neonates have active homeostatic mechanisms, including a high cell cycle rate and an increased serum IL-7 concentration favoring clonal expansion (6–8). Here, we compare responsiveness and immune competence of circulating T cells and antigen-presenting cells (APCs) during periods of relative protection (birth) and susceptibility (8 months) to islet autoimmunity. Both periods are characterized by the presence of autoantigen-responsive naïve T cells and T cells that are highly sensitive to homeostatic expansion mechanisms. However, the activation of naïve T cells is compromised at birth and appears fully competent at 8 months of age. We predict, therefore, that the combination of an HLA genotype–determined islet autoreactive naïve T-cell population, homeostatic expansion mechanisms, and immune competence provides a highly favorable environment for islet autoimmunity and, in part, determines the high incidence of seroconversion observed at ~1 year of age. This has implications for early prevention of islet autoimmunity.

RESEARCH DESIGN AND METHODS

Subjects. Cord blood (CB) from 20 healthy, full-term newborns, acquired immediately after delivery from the clamped umbilical cord, was collected in citrate-phosphate-dextrose. Samples were provided through the DKMS Cord Blood Bank of the University Hospital Dresden (Germany) with informed consent and ethics committee approval. For the comparison of antigen presentation efficiency at birth versus infancy, a second group of nine healthy newborns was recruited at the Institute of Diabetes Research (Klinikum rechts der Isar, University of Technology Munich) and prospectively followed until 1 year of age, with informed consent and ethics committee approval. CB follow-up of peripheral venous blood at a median infant age of 8.1 months (range, 7–10 months) was obtained. Sodium-heparinized peripheral venous blood samples from 10 healthy nondiabetic adults >18 years of age were provided by the German Red Cross GmbH Dresden (Germany).

Cell isolation. CB mononuclear cells (CBMCs) and peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Ficoll-Hypaque. $CD4^+$ T cells (>96% purity) and $CD14^+$ monocytes (>95% purity) were enriched by negative (CD4) or positive isolation (CD14) using magnetic beads according to the manufacturer's instructions (Miltenyi-Biotech, Inc., Auburn, CA). Subsequently, $CD4^+CD25^+$ T cells were depleted from the $CD4^+$ T-cell fraction by positive selection (resultant purity >98% $CD25^-$ of $CD4^+$). For the newborn versus infant autologous mixed leukocyte assay, $CD14^+$ monocytes (>95% purity) and $CD3^+$ T cells (>97% purity) were obtained from CBMCs and PBMCs by negative magnetic bead isolation.

From the [1]DFG Research Center and Cluster of Excellence, Center for Regenerative Therapies Dresden, Dresden University of Technology, Dresden, Germany; the [2]Forschergruppe Diabetes e.V., Neuherberg, Germany; and the [3]Institute of Diabetes Research, Helmholtz Zentrum München, and Forschergruppe Diabetes, Klinikum rechts der Isar, Technical University of Munich, Neuherberg, Germany.
Corresponding author: Ezio Bonifacio, ezio.bonifacio@crt-dresden.de.
Received 16 July 2012 and accepted 11 January 2013.
DOI: 10.2337/db12-0942
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-0942/-/DC1.
P.M. is currently affiliated with the Diabetes Research Institute, San Raffaele Scientific Institute, Milan, Italy.

Cell staining and flow cytometry. The following monoclonal antibodies were used for FACS staining: BD Biosciences: anti-CD45 APC (HI30), anti-CD4 APC (SK3), anti-CD4 Pacific Blue (RPA-T4), anti-CD3 APC (HIT3a), anti-CD127 PE (M21), anti-CD25 PE, APC-H7 (M-A251), anti-CD45RA APC (HI100), anti-CD45RO PE-Cy7 (UCHL1), anti-Helios PE (22F6), anti-CD14 FITC (M5E2), anti–HLA-DR PE (G46-6), anti-CD86 Pacific Blue (2331), and the Annexin V FITC apoptosis detection kit II; Bioscience: anti-CD127 eFlour450 (eBioRDR5); BioLegend (San Diego, CA): anti-CD40 AF647 (5C3) and anti-FOXP3 Alexa Fluor 488 (259D). After staining, cells were washed twice with FACS buffer and fixed with 1% paraformaldehyde in PBS. To detect intracellular protein expression, cells were fixed and permeabilized after surface staining using the FOXP3 Fix/Perm buffer set (BioLegend). Cells were acquired on a Becton Dickinson LSR-II flow cytometer with FACS Diva. Doublets and clumps were excluded based on SSC-A versus SSC-W plots. Live cell populations were gated as 7-AAD (BD Biosciences)–negative cells. At least 50,000 gated events were acquired for each sample and analyzed using FlowJo software version 7.6.1 (TreeStar, Inc., Ashland, OR).

Dendritic cell maturation. Monocytes from CBMCs were cultured in six-well plates at 10^6 cells/well in 3 mL dendritic cell (DC) medium (CellGenix, Freiburg, Germany) supplemented with 3% heat-inactivated FCS, 50 ng/mL GM-CSF, and 10 ng/mL IL-4 (R&D Systems, Minneapolis, MN) at 37°C. After 6 days, immature DCs were pulsed without antigen or with 100 μg/mL GAD65 (Diamyd, Stockholm, Sweden), proinsulin (Lilly, Indianapolis, IN), keyhole limpet hemocyanin (Calbiochem, Darmstadt, Germany), thyroglobulin (Lee Biosolutions, St. Louis, MO), or 10 μL/mL tetanus toxoid and diphtheria toxoid (TT/DPT; Sanofi-Pasteur MSD, Leimen, Germany) for 6 h and subsequently matured overnight with 100 units/mL IFN-γ (R&D Systems) and 10 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO). The mature antigen-loaded DCs were washed three times with PBS. DC phenotype was determined via FACS analysis to ensure maturation.

CFSE proliferation assay. For CB T-cell responses, T cells ($CD4^+$ or $CD4^+CD25^-$) were labeled with CFSE (Invitrogen, Carlsbad, CA) as previously described (9). CFSE-labeled T cells (1.5×10^5) were added to autologous mature DCs loaded with or without antigen at a ratio of 1 DC:40 T cells in round-bottom 96-well plates. IL-7 (0.1 ng/mL; R&D systems) was added to the culture medium to increase the survival of T cells. For comparison of infant peripheral blood (PB) versus CB T-cell responses, 2×10^5 CFSE-labeled $CD3^+$ T cells were added to 2×10^4 autologous monocytes previously pulsed without antigen or with 10 μg/mL GAD65, 10 μg/mL proinsulin, or 1 μL/mL TT for 3 h. In all assays, each condition was performed in triplicate. Cells were cultured in RPMI 1640 supplemented with 2 mmol/L glutamine, penicillin (50 units/mL), streptomycin (50 μg/mL), and 5% (volume/volume) heat-inactivated human AB serum (Invitrogen). After 6 days, the cell cultures were stained for CD4, CD25, and CD45RO and processed for FACS analysis.

[^{3}H]thymidine incorporation assay. To determine specificity of responses in the CFSE dilution assay, antigen-responsive $CD4^+CFSE^{dim}CD25^+CD45RO^+$ and nonproliferating $CD4^+CFSE^{hi}CD25^-$ T cells were sorted on a FACSAriaII cell sorter and subsequently stimulated with autologous antigen-pulsed or unpulsed matured DCs (ratio 1:20 DC/T cell) in the presence of 0.1 ng/mL IL-7 for 3 days. [^{3}H]thymidine (1 μCi/mL) was added for the last 16 h. Cells were subsequently harvested and collected onto a 96-well 1.2-μm pore size filter plate. [^{3}H]thymidine incorporation was measured as counts per minute (cpm) using a Top Count Microplate Scintillation β-particle counter.

IL-7 concentration. Plasma IL-7 was quantified by enzyme-linked immunosorbent assay using the commercial Duoset ELISA system (R&D Systems). The assay detection range was between 3.5 and 2,000 pg/mL.

HLA genotyping and *INS* VNTR PCR. HLA typing was performed by the DKMS Life Science Laboratory GmbH (Dresden, Germany) using sequence-based typing. *INS* gene typing was performed by *Hph*I digestion of PCR products amplified from the *INS* promoter region of interest as previously described (10).

Statistical analysis. Results are presented as mean and SD or median and interquartile range (IQR) as indicated. Expression of cell surface molecules on cell subsets was assessed as median fluorescence intensity (MFI). For normally distributed data, Student *t* test for unpaired values was used to compare means between groups and Student *t* test for paired values was used to compare values for the same sample or subject tested under different conditions. Comparisons of other nonnormally distributed variables between paired groups were made using the nonparametric Wilcoxon matched pairs test. Multivariate analysis of covariates determining responsiveness of $CD4^+$ T cells to antigen was performed using linear regression. For all tests, a two-tailed *P* value of <0.05 was considered to be significant. Statistical significance is shown as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, or not significant (ns) $P > 0.05$. Analyses were performed using the programs GraphPad Prism 5 (La Jolla, CA) and the Statistical Package for the Social Sciences (SPSS 19.0; SPSS Inc., Chicago, IL).

RESULTS

GAD65- and proinsulin-responsive T cells in $CD4^+CD25^-$ T cells from healthy newborns. We searched for unprimed islet autoreactive naïve T cells in CB using autoantigen-stimulated T-cell proliferation. To increase sensitivity, assays were performed using $CD4^+CD25^+$-depleted $CD4^+$ T cells as responder cells (Supplementary Fig. 1*A*) and autologous DCs matured from CB monocytes as APCs. $CD45RO^+$ memory T cells were not depleted since they were infrequent in CB (Supplementary Fig. 1*B*). Antigen-responsive cells (Fig. 1*A*) were identified as proliferative ($CD4^+CFSE^{dim}$), activated (upregulation of CD25), and memory-like ($CD45RO^+$) phenotype. Significant T-cell responses against GAD65 (stimulation index [SI]: median 3.4 [IQR 1.7–6.2], $P = 0.0002$) and proinsulin (2.1 [1.2–2.7], $P = 0.002$) as compared with medium were observed (Fig. 1*B* and Table 1). Responses to GAD65 and proinsulin were also increased when excluding the two highest responders for GAD65 or proinsulin ($P = 0.0005$ and $P = 0.005$, respectively). Little or no response was observed against the exogenous antigens TT/DPT (0.8 [0.3–2.1], $P = 0.8$). No response was detected against the vehicle in which the antigen GAD65 was solubilized (Supplementary Fig. 1*C*). Consistent with previous findings (11), significant proliferative responses in CB $CD4^+CD25^-$ T cells were observed to the highly immunogenic large antigen keyhole limpet hemocyanin (3.2 [1.5–8.4], $P = 0.01$). Some newborns also had responses to the thyroid autoantigen human thyroglobulin (Supplementary Fig. 1*C*), and CB T cells had vigorous responses to the superantigen Staphylococcal enterotoxin B (Supplementary Fig. 1*D*).

Some newborns had robust responses to GAD65 and/or proinsulin with up to 3% of cells having undergone proliferation at the end of culture. Although this is not the precursor frequency, it is substantial and likely includes bystander-type responsive cells as shown for the influenza vaccine (12,13). Therefore, to confirm that proliferating cells were enriched for antigen-specific T cells, GAD65-responding $CFSE^{dim}CD25^+CD45RO^+CD4^+$ T cells were FACS sorted and rechallenged with antigen in a 3-day proliferation assay (Fig. 1*C*). The sorted cells responded specifically to GAD65 (mean ± SD, 15,737 ± 7,335 cpm) but not proinsulin (254 ± 267 cpm), and initially nonresponding T cells remained nonresponsive to autoantigen.

HLA class II genotypes are associated with islet autoantigen responsiveness in CB T cells. Naïve antigen-specific T cells are rare within the T-cell repertoire (11). We expected that the range of responses observed between children may partially reflect the naïve precursor frequency of antigen-specific T cells. Responses to the type 1 diabetes-associated autoantigens were >20-fold over background in three of the children tested (Table 1), suggesting a higher precursor frequency of T cells specific for these antigens in those children. These three children uniquely had two type 1 diabetes high-risk HLA-DRB1*HLA-DQB1* haplotypes, and responses to both GAD65 and proinsulin were higher in these neonates as compared with the remaining neonates ($P = 0.006$ for GAD65; $P = 0.0004$ for proinsulin).

Polymorphisms in the insulin gene (*INS*) promoter region are associated with inefficient deletion of proinsulin-reactive T cells in the thymus (14) and associated with responses to insulin (15). Consistent with this, all CB with

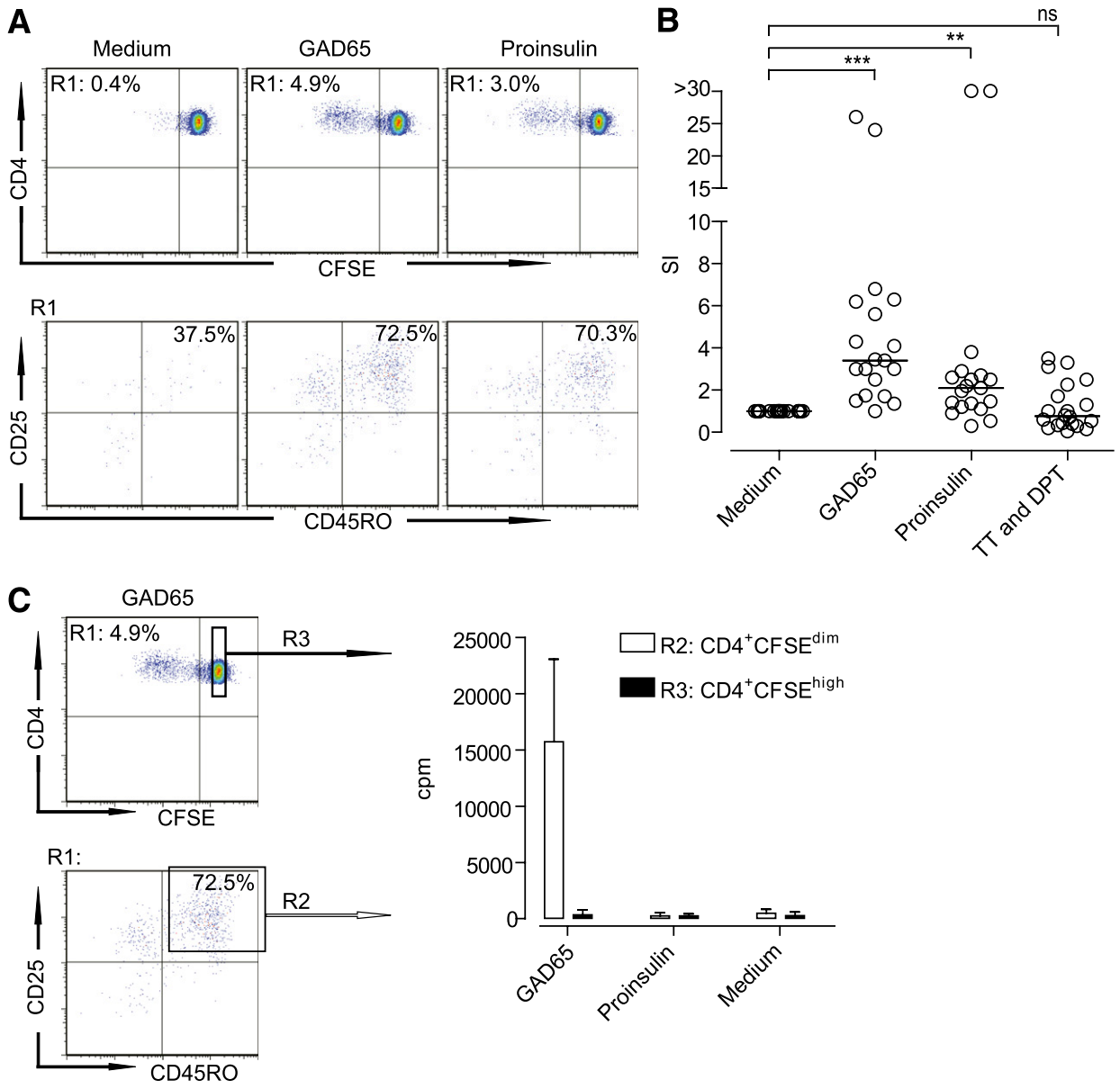

FIG. 1. Detection of GAD65- and proinsulin-responsive CD4$^+$CD25$^-$ T cells in CB. *A*: Representative FACS plots of CFSE-labeled CB T cells from a healthy newborn of 20 tested, stimulated for 6 days with autologous DC loaded with no antigen (medium) or with GAD65 or proinsulin. Proliferating CD4$^+$CFSE^{dim} of CD4$^+$ T cells (*top*, R1), which also upregulate CD25 and convert to a CD45RO$^+$ phenotype (*bottom panel*, *top right quadrant*), were considered responding T cells. *B*: Summary of CB CD4$^+$CD25$^-$ T cells from 20 healthy newborn responses responding to GAD65, proinsulin, or TT/DPT shown as the SI over the control (medium) and represents for each condition and sample the mean of triplicate wells. One child responded strongly (SI >20) to both GAD65 and proinsulin, one child only to GAD65, and a third child only to proinsulin. The bar for each antigen indicates the median SI. **$P < 0.01$; ***$P < 0.001$; $^{ns}P > 0.05$. *C*: CD4$^+$CD25$^-$ T cells were first stimulated with GAD65 and the responding cells (CFSE^{dim}CD25$^+$CD45RO$^+$; R2) or nonresponding cells (CFSE^{high}; R3) were sorted and restimulated with DC pulsed with GAD65, proinsulin, or no antigen (medium) for 3 days. During the last 16 h of incubation, [^{3}H]thymidine was added to the culture. The graph on the right shows [^{3}H]thymidine incorporation in each condition as mean ± SD from three different CB samples.

an SI >3.0 for proinsulin (CB 1, 3, and 17) had the type 1 diabetes-susceptible *INS* VNTR I/I genotype. Multivariate analysis that included the HLA genotype (children with two susceptible haplotypes vs. others), *INS* VNTR genotype (children with I/I vs. others), and background proliferation in medium confirmed that the HLA genotype was a determinant of CB CD4$^+$ T-cell responses to GAD65 (P = 0.006) and proinsulin (P = 0.002). Responses to GAD65 or proinsulin were not associated with the proportion of CD4$^+$CD25$^+$CD127^{hi} T cells or the proportion of CD4$^+$CD25$^+$CD127^{low} T regulatory cells (Tregs) present in the original CB sample (Supplementary Fig. 2), or the MFI of CD127 on either population (data not shown).

CD4$^+$ T-cell survival and responsiveness are IL-7 sensitive in infants. Having established that the newborn is not devoid of autoreactive T cells, we examined factors that may modify the susceptibility of these T cells to be activated at ages relevant to low (birth) and high (8 months of age) islet autoimmunity seroconversion periods. T-cell expansion in infants is high and is maintained by homeostatic mechanisms, including IL-7 (8,16). Consistent with this, serum concentrations of IL-7 were increased in CB (mean 6.4 ± 5.0 pg/mL) and PB of 8-month-old infants (8.6 ± 6.1 pg/mL) as compared with concentrations in adults (0.3 ± 0.7 pg/mL; P = 0.007 vs. CB; P = 0.003 vs. 8 months) (Fig. 2*A*). Furthermore, IL-7R α-chain surface

TABLE 1
Newborn HLA class II haplotypes, *INS* VNTR alleles, and response to autoantigen

Subject number	HLA-DRB1		HLA-DQB1		SI: GAD65*	SI: proinsulin*	*INS* VNTR alleles
1	**0301**	**1302**	**0201**	**0604**	**24.0**	**47.0**	I/I
2	1101	1104	0301	0301	6.2	NT	I/III
3	**0401**	**0401**	**0302**	**0302**	1.0	**>20.0**	I/I
4	0101	1501	0501	0602	6.8	1.5	I/I
5	13	1501	0603	0602	4.1	2.2	I/I
6	**0401**	1301	**0302**	0603	3.4	2.7	I/I
7	1101	1301	0301	0603	5.6	0.5	I/I
8	0101	12	0501	0301	3.5	0.3	I/III
9	**0301**	12	**0201**	0301	3.0	2.0	I/I
10	**0401**	0701	**0302**	0303	2.5	2.1	III/III
11	1010	1010	0501	0501	1.5	1.1	I/I
12	1104	1301	0301	0603	1.7	1.2	I/I
13	0101	1501	0501	0602	NT	2.9	I/III
14	**0301**	1301	**0201**	0602	1.4	2.6	III/III
15	**0301**	**0401**	**0201**	**0302**	**26.0**	2.5	I/I
16	0102	1502	0501	0601	1.7	1.4	I/III
17	0701	1104	0202	0301	3.0	3.8	I/I
18	0103	1301	0501	0603	4.3	2.5	III/III
19	**0404**	1303	**0302**	0301	3.0	0.9	I/I
20	**0405**	1301	**0302**	0603	6.3	1.4	I/I

Summary of HLA class II haplotypes, *INS* VNTR alleles, and corresponding responses to autoantigens GAD65 and proinsulin (SI over background) in the CB tested from 20 healthy newborns. Haplotypes associated with type 1 diabetes susceptibility (boldface) were identified based on previous publications (44,45). The subjects with the highest responses to autoantigen are marked in boldface. These responses were in neonates with two type 1 diabetes-susceptible HLA-DRB1* HLA-DQB1* haplotypes as compared with the remaining neonates ($P = 0.006$ for GAD65; $P = 0.0004$ for proinsulin). *NT, not tested.

expression was increased on CB $CD4^+$ T cells (MFI 2,491 ± 162) as compared with adult naïve T cells (MFI 1,509 ± 244, $P < 0.0001$) (Fig. 2*B*).

Functionally, low concentrations of IL-7 were sufficient to prevent the marked CB T-cell apoptosis normally observed during in vitro culture (live $CD4^+$ CB T cells: 60 ± 9.8% with 0.1 ng/mL IL-7 vs. 32 ± 17.9% without IL-7, $P = 0.001$) (Fig. 2*C*). Moreover, both CB and 8-month T cells were highly responsive to IL-7 in the absence of antigen as compared with adult T cells and showed strong proliferation already at 1.0 ng/mL ($CFSE^{dim}$ of $CD4^+$ T cells: 17.5 ± 3.3% CB; 13.8 ± 4.1% 8 month vs. 1.1 ± 0.4% adult PB naïve T cells, $P < 0.0001$) (Fig. 2*D*). Of note, exposing CB and 8-month T cells to high IL-7 (10 ng/mL) induced proliferation of both $CD45RO^-$ (naïve) and $CD45RO^+$ (memory-like) T cells, with the naïve proliferating T cells remaining $CD45RO^-$ (Fig. 2*E*). In adult PB, however, proliferation in response to IL-7 was predominantly limited to the $CD45RO^+$ T cells ($CFSE^{dim}$ of $CD45RO^-CD4^+$ T cells at 10 ng/mL of IL-7: 5.8 ± 2.5% vs. 24.2 ± 6.6% in $CD45RO^+$ $CD4^+$ T cells, $P < 0.0001$).

Exposure to autoantigen and IL-7 in vitro does not induce a Treg phenotype. The immune system at birth is biased toward tolerance (17,18), and IL-7 may affect the Treg pool (19,20). We therefore examined whether culture of naïve CB $CD4^+CD25^-$ T cells with autoantigen presented by DCs with and without IL-7 leads to cells with Treg phenotypes, indicated as $CD4^+CD25^+CD127^{low}$-$FOXP3^+$ and the Helios-negative subset of these cells. Helios negativity was used as a potential marker of peripherally induced Tregs (21), and it is noted that the large majority of uncultured CB Tregs were Helios positive (Supplementary Fig. 3*A*). We found that the maximum proportion of $CD4^+CD25^+CD127^{low}FOXP3^+$ cells among $CD4^+$ T cells present at the end of culture was <2% with no influence by antigen stimulation or IL-7 (Supplementary Fig. 3*B*). Similarly, the proportion of Helios-negative Tregs among $CD4^+$ T cells at the end of culture was <0.2% and not influenced by antigen or IL-7 (Supplementary Fig. 3*B*, *right*).

Functional impairment of APCs at birth. High responsiveness to IL-7 could facilitate islet-reactive T-cell activation (19) but would not explain the difference in low and high islet autoimmunity incidence periods seen in the first year of life. We therefore hypothesized that differences in immune competence may play a role, and separately compared APC competence and T-cell responsiveness in CB and at 8 months of age in healthy children.

Monocytes (APCs) and T cells from the same child isolated at birth and at 8 months of age were studied in mixed leukocyte assays (Fig. 3*A*). Responses to TT were similar to background when unprimed CB $CD4^+$ T cells were stimulated by autologous CB monocytes (median SI, 1.0 [IQR 1.0–1.1]), whereas, as expected, tetanus vaccination–primed 8-month $CD4^+$ T cells responded strongly to TT when stimulated by 8-month monocytes (191 [34–650], $P = 0.007$). The vaccine-primed 8-month $CD4^+$ T cells also responded when CB monocytes were used for antigen presentation (132 [31–386]), consistent with an ability of CB monocytes to activate memory $CD4^+$ T cells. In contrast, priming of naïve T cells against autoantigen by CB monocytes was impaired (2.7 [1.5–63]; $P = 0.04$). CB and 8-month $CD4^+$ T cells from none of the five children were responsive to GAD65 when CB monocytes were used as APCs. However, both CB and 8-month $CD4^+$ T cells from three children consistently responded to GAD65 when monocytes from the 8-month samples were used for presentation, and responses to GAD65 were increased when 8-month monocytes were used for presentation (median SI all five children, 2.95 [1.5–4.3]) as compared with CB monocytes (median SI, 1.05 [1.0–1.6]; $P = 0.004$).

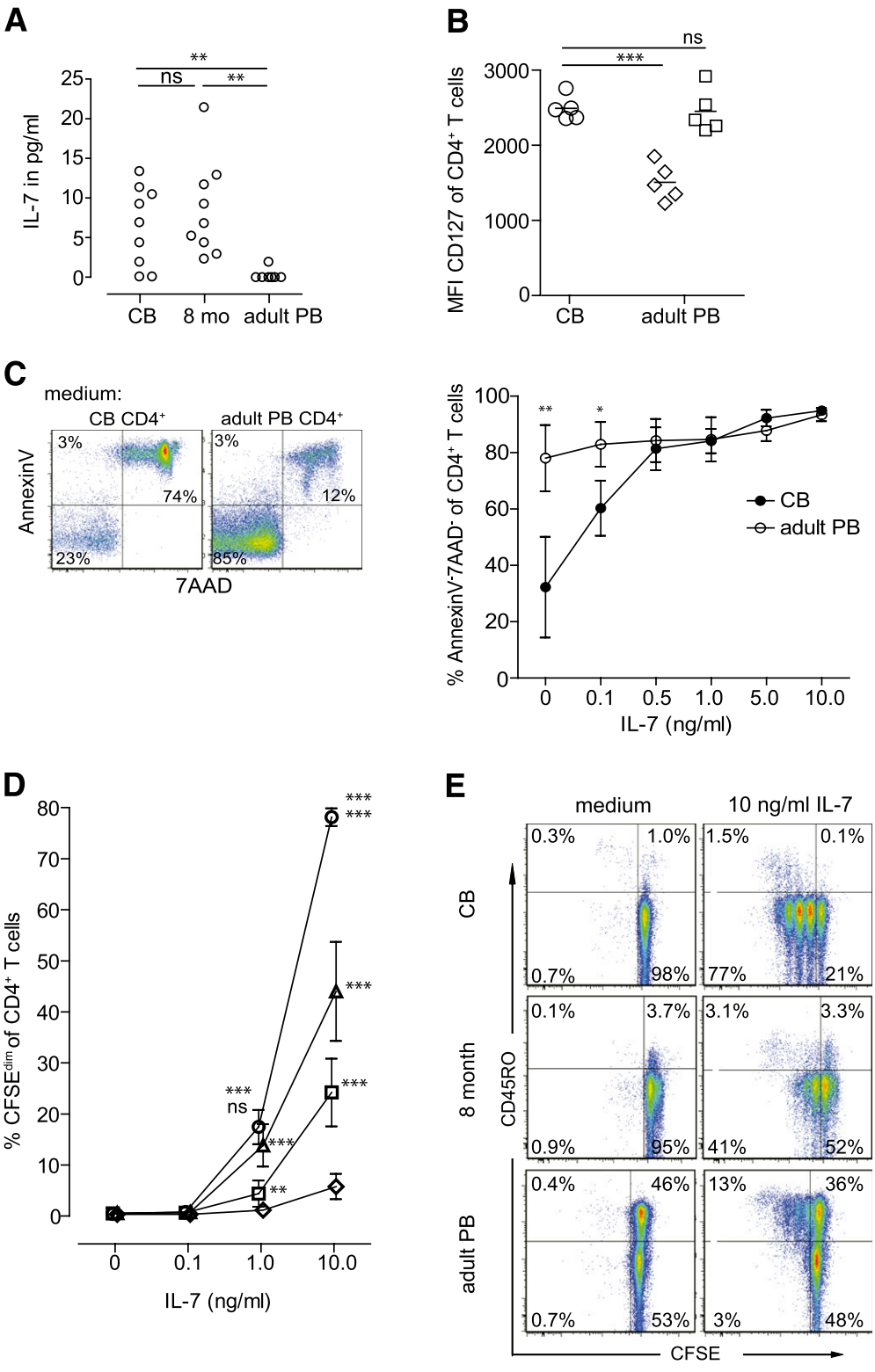

FIG. 2. IL-7 responsiveness of CD4⁺ T cells. *A*: Concentration of IL-7 (pg/mL) in human plasma samples at birth (n = 9) or 8-month-old infant (n = 9) or adult (n = 9) samples. $^{**}P < 0.01$; $^{ns}P > 0.05$. *B*: CD127 surface expression (MFI) of CB total CD4⁺ T cells (circle, n = 5) compared with adult PB CD4⁺CD45RA⁺CD45RO⁻ (naïve, diamond, n = 5) and CD4⁺CD45RA⁻CD45RO⁺ (memory, square, n = 5) T cells. $^{***}P < 0.001$. *C*: Representative FACS plots of an apoptosis assay showing the survival (negative for Annexin V and 7AAD; *bottom left quadrant*) of CD4⁺ T cells from five CB and five adult PB samples after 6 days of culture. Percentages in each quadrant are of CD4⁺ T cells. The right panel shows the summary of the survival (% of CD4⁺ T cells, mean ± SD) of CB vs. adult PB CD4⁺ T cells without or with increasing concentrations of IL-7. $^{*}P < 0.05$; $^{**}P < 0.01$. *D*: Proliferation (mean ± SD of CFSE dilution) of CD4⁺ T cells in CBMCs (circle, n = 6), 8-month-old PBMCs (triangle, n = 6), or adult PBMCs (n = 6) gated on CD45RO⁺ memory T cells (square) or CD45RA⁺ naïve T cells (diamond) when cultured for 6 days without or in the presence of increasing concentrations of IL-7. For all samples, statistical significance refers to the comparison against naïve adult CD4⁺ T cells, except in CB, where the significance is shown with comparison to naïve adult CD4⁺ T cells (*top*) and 8-month CD4⁺ T cells (*bottom*). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{ns}P > 0.05$. *E*: Representative FACS plots showing CFSE dilution vs. CD45RO expression of CB, 8-month, and adult PB CD4⁺ T cells cultured without (medium) and in the presence of 10 ng/mL IL-7 and absence of antigen. The number in each quadrant shows % of CD4⁺ T cells.

Moreover, variation in response to GAD65 was associated with the CB versus 8-month APCs ($P = 0.001$) and not the $CD4^+$ T-cell source ($P = 0.3$) in a multivariate analysis. Analogous differences were also observed for proinsulin (data not shown). These data suggest that naïve T cells at birth can be activated by antigen but that the APC compartment at birth may be inefficient in its ability to activate and expand naïve T cells.

Priming of naïve $CD4^+$ T cells requires strong costimulatory signals. Therefore, the expression of costimulatory molecules was analyzed on monocytes in CB, at 8 months, and in adults (Fig. 3*B*). Consistent with the impaired ability to activate naïve T cells, monocytes at birth had significantly lower surface expression of CD40 (MFI: 740 ± 409) and HLA-DR (MFI: 302 ± 154) than monocytes obtained at 8 months of age (MFI CD40: 3,663 ± 805, $P = 0.0009$; HLA-DR: 1,302 ± 408, $P = 0.007$) and from adults (MFI CD40: 3,500 ± 1,560, $P = 0.0007$; HLA-DR: 1,316 ± 549, $P = 0.0005$).

DISCUSSION

The presence of autoreactive T-cell clones in the T-cell repertoire is determined in the thymus, but activation and expansion of naïve clonal populations is regulated in the periphery by antigen encounter, homeostatic mechanisms, and the inhibitory network of Tregs. Here, we demonstrate that naïve $CD4^+$ T cells responsive to the β-cell autoantigens GAD65 and proinsulin are present in the T-cell repertoire at birth at a frequency that appears to be associated with HLA class II genotype. Neonatal naïve T cells were highly sensitive to IL-7, but antigen presentation was inefficient at birth and rescued by 8 months of age. We predict that these factors collectively influence the likelihood of type 1 diabetes-related autoimmunity, and in part explain the initial rarity of autoimmune seroconversion prior to 6 months of age and subsequent high incidence of seroconversion seen at ~1 year of age (4,5).

Autoreactive T cells in man are difficult to consistently and reliably demonstrate in vitro (22,23). We examined naïve CB $CD4^+$ T cells from healthy newborns and chose conditions that should enhance activation and proliferation of naïve T cells, including the use of matured DCs for antigen presentation and removal of $CD4^+CD25^+$ Tregs. Importantly, we used stringent criteria for T-cell responsiveness. First, only proliferating cells ($CD4^+CFSE^{dim}$) that expressed high levels of the IL-2Rα chain ($CD25^+$) and were $CD45RO^+$ at the end of culture were considered responsive. Second, we showed that isolated GAD65-responsive CB $CD4^+$ T cells could be successfully and specifically rechallenged. Although we cannot be certain that in vitro responsive naïve autoreactive T cells have the potential to be activated by autoantigen in vivo, our findings strongly suggest that naïve T cells that can specifically respond to islet autoantigen are found in the T-cell repertoire of CB. This is not unreasonable since the thymus is active already in fetal life (24), and negative selection appears to allow the passage of low-affinity self-reactive T cells (25,26).

There was substantial heterogeneity between neonates in the responsiveness of their CB $CD4^+$ T cells to islet autoantigens. This heterogeneity is informative with respect to mechanisms controlling autoreactive T-cell frequency. By examining the HLA genotype, we could show an association between the presence of high type 1 diabetes risk HLA class II genotypes and responsiveness, consistent with the role of HLA class II in thymic selection of $CD4^+$ T cells. The striking response observed in the three neonates with full HLA class II risk genotypes suggests inefficient deletion of islet autoreactive T cells in the thymus and, as a consequence, a relative abundance in the periphery. This was observed for both proinsulin and GAD65. The limitation of these data is the small number of neonates studied, and therefore these data need to be confirmed on a larger set of samples and in neonates who subsequently develop islet autoimmunity. A further limitation is that we are unable to determine precursor frequencies from the in vitro assays since limiting dilution was not performed. Moreover, since some responses were robust, it is possible that the responsive cells also included bystander cells that proliferated to stimuli other than autoantigen.

Our study is potentially informative with respect to the early islet autoantibody seroconversion observed in children who develop type 1 diabetes (4). Homeostatic proliferation is discussed as a mechanism of autoreactive T-cell expansion (27–30). The immune system of an infant must adapt to rapid growth, and, consequently, neonatal T cells are exposed to active homeostatic signals (8,31). These include increased concentrations of circulating IL-7 and, as shown in our study, high sensitivity of naïve infant T cells to IL-7. Increased IL-7 concentrations also promote the activation and expansion of T cells in the presence of antigen (32), as well as Tregs (19,20). We hypothesize, therefore, that the increased homeostatic expansion pressure and the increased sensitivity to IL-7 increase the number of naïve autoreactive T-cell clones in the periphery of the type 1 diabetes-susceptible infants, and favor their activation during events of β-cell antigen exposure. In the current study, we were unable to find in vitro evidence for substantial conversion of naïve $CD4^+$ T cells to Tregs in the presence of IL-7. However, it remains possible that this occurs and could be a preferred in vivo pathway in neonates, since IL-7 is essential for peripheral Treg survival and expansion in vivo (33).

Homeostatic T-cell expansion mechanisms in early childhood could help explain the higher incidence of islet autoimmunity observed in this period. However, such stimuli appear to be strongest in the first months of life where islet autoantibody seroconversion is rare. Thus, mechanisms that prevent very early autoimmunity must be ongoing. Tregs at birth appeared functional as judged by the ability to suppress the response to antigen in vitro in this (Supplementary Fig. 1) and previous studies (34). Equally potent as an autoimmune brake is the state of immune competence of APCs at birth. We demonstrated that the monocyte compartment of CB is functionally impaired to prime naïve T cells. This is consistent with previous findings that mononuclear cells at birth are not readily activated (35), APCs in neonates are immature (36,37), and that early after birth, the immune system is relatively tolerant (38). Relevant to the poor priming, CB monocytes had decreased expression of key molecules involved in antigen presentation as compared with adult monocytes. It is noted that we have not demonstrated that DCs circulating at birth also have similar deficiencies in antigen presentation, but others have reported such defects (39,40). Relevant to the heightened incidence of seroconversion after 6 months of age, monocytes obtained from blood samples at 8 months strongly expressed HLA-DR, CD40, and CD86 and appeared to be fully competent in their ability to prime naïve $CD4^+$ T cells. It is also demonstrated that Toll-like receptor signaling in the first

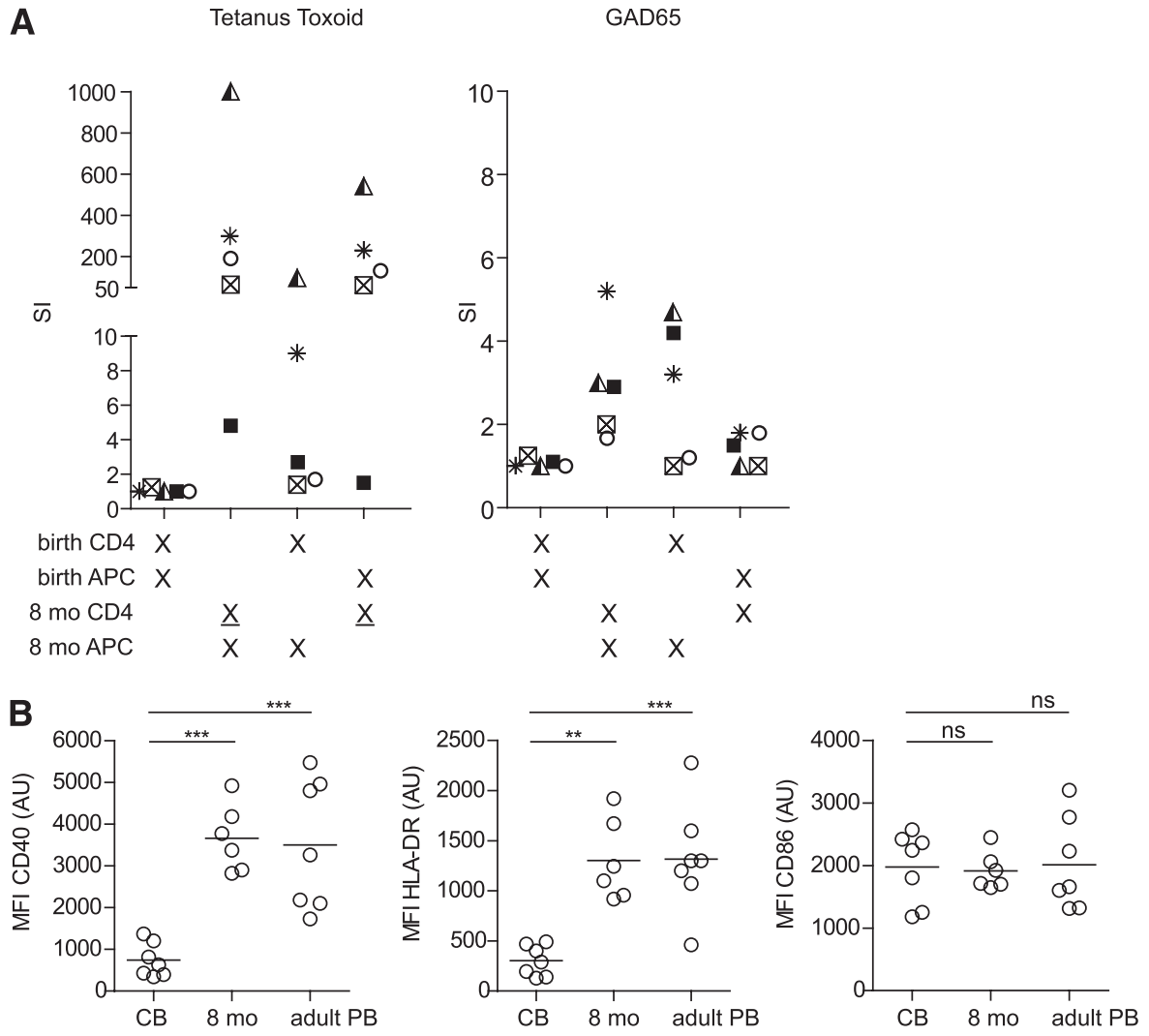

FIG. 3. Impaired APC function in CB. *A*: SI over background (no antigen) of CD4$^+$ T cells from birth vs. 8-month infants in response (CFSE^{dim}CD25$^+$CD45RO$^+$) to the antigens TT and GAD65. Autologous monocytes isolated either at birth or at 8 months (8 mo) were used as APCs. Cell subsets that were cultured together are indicated below the *x*-axis (conditions in which primed memory T cells are expected are underlined). Five children were tested, each shown by unique symbols. For TT, significant differences were observed between birth and 8-month monocyte stimulation of birth CD4$^+$ T cells ($P = 0.043$). For GAD65, multivariate analysis showed significantly increased responses when 8-month monocytes were used for antigen presentation ($P = 0.001$) and no difference for birth vs. 8-month CD4$^+$ T-cell responders ($P = 0.33$). *B*: CD40, HLA-DR, and CD86 surface expression (median fluorescence intensity) of CB ($n = 7$), 8-month ($n = 6$), and adult PB ($n = 7$) CD14$^+$ monocytes. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{ns}P > 0.05$.

months of life favor IL-10 production, potentially reducing the risk to activate autoreactive T cells (18,41). In addition, it is possible that maternal transfer of APCs may further modify the risk of activating autoreactive T cells (42). Although our findings are consistent with a strong role of APCs in compromising T-cell responsiveness in neonates, we cannot exclude additional deficiencies in birth T cells as have been recently reported (43).

In conclusion, we demonstrate that naïve islet autoantigen-responsive CD4$^+$ T cells are present from birth in healthy newborns and that their frequency appears to be increased in neonates with type 1 diabetes-susceptible HLA genotypes. Our findings predict that homeostatic mechanisms of T-cell expansion in infants will increase the likelihood of islet autoimmunity, and that inefficient antigen presentation will operate to reduce this risk in the first months of life. We propose that these features are relevant to the timing of islet autoantibody seroconversion in the pathogenesis of type 1 diabetes, and provide an opportunity when designing intervention trials to prevent the development of autoimmunity against β-cells.

ACKNOWLEDGMENTS

This work was supported by funding from the Competence Network for Diabetes Mellitus funded by the Federal Ministry of Education and Research (KFZ 01GI0807 and 01GI1102), the DFG Research Center and Cluster of Excellence, Center for Regenerative Therapies Dresden, (FZ 111), and the Helmholtz Center Munich. A.-K.H. was enrolled in the International PhD program "Metabolism and Endothelium" as part of the Medical Faculty of the Dresden University of Technology. P.M. is supported by an advanced postdoctoral fellowship from the Juvenile Diabetes Research Foundation (10-2009-284).

No potential conflicts of interest relevant to this article were reported.

A.-K.H. designed and performed experiments, analyzed data, conceived ideas, and wrote the manuscript. P.M. conceived ideas, contributed to discussion, and edited and

reviewed the manuscript. C.W. provided technical assistance. P.S. organized the CB and 8-month blood samples from healthy children. D.K. performed all experiments documenting the Treg phenotype. A.-G.Z. provided blood sample collection, contributed to discussion, and edited and reviewed the manuscript. E.B. conceived ideas, oversaw research, and helped write the manuscript. E.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Dr. A. Platz (DKMS Cord Blood Bank) for providing CB samples, the midwives of the Technical University Hospital Rechts der Isar (Munich, Germany) for sample collection, K. Ruhner (DKMS Life Science Laboratory GmbH) for HLA typing, and all subjects for their blood donation.

REFERENCES

1. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001;358:221–229
2. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. J Immunol 2004;172:5967–5972
3. Monti P, Scirpoli M, Rigamonti A, et al. Evidence for in vivo primed and expanded autoreactive T cells as a specific feature of patients with type 1 diabetes. J Immunol 2007;179:5785–5792
4. Ziegler AG, Bonifacio E; BABYDIAB-BABYDIET Study Group. Age-related islet autoantibody incidence in offspring of patients with type 1 diabetes. Diabetologia 2012;55:1937–1943
5. Parikka V, Näntö-Salonen K, Saarinen M, et al. Early seroconversion and rapidly increasing autoantibody concentrations predict prepubertal manifestation of type 1 diabetes in children at genetic risk. Diabetologia 2012; 55:1926–1936
6. Bolotin E, Annett G, Parkman R, Weinberg K. Serum levels of IL-7 in bone marrow transplant recipients: relationship to clinical characteristics and lymphocyte count. Bone Marrow Transplant 1999;23:783–788
7. Marchant A, Goldman M. T cell-mediated immune responses in human newborns: ready to learn? Clin Exp Immunol 2005;141:10–18
8. Szabolcs P, Park KD, Reese M, Marti L, Broadwater G, Kurtzberg J. Coexistent naïve phenotype and higher cycling rate of cord blood T cells as compared to adult peripheral blood. Exp Hematol 2003;31:708–714
9. Mannering SI, Morris JS, Jensen KP, et al. A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. J Immunol Methods 2003;283:173–183
10. Walter M, Albert E, Conrad M, et al. IDDM2/insulin VNTR modifies risk conferred by IDDM1/HLA for development of type 1 diabetes and associated autoimmunity. Diabetologia 2003;46:712–720
11. Geiger R, Duhen T, Lanzavecchia A, Sallusto F. Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. J Exp Med 2009;206:1525–1534
12. Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. J Clin Invest 1999;104:R63–R67
13. Novak EJ, Masewicz SA, Liu AW, Lernmark A, Kwok WW, Nepom GT. Activated human epitope-specific T cells identified by class II tetramers reside within a CD4high, proliferating subset. Int Immunol 2001;13:799–806
14. Pugliese A, Zeller M, Fernandez A Jr, et al. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet 1997;15:293–297
15. Durinovic-Belló I, Wu RP, Gersuk VH, Sanda S, Shilling HG, Nepom GT. Insulin gene VNTR genotype associates with frequency and phenotype of the autoimmune response to proinsulin. Genes Immun 2010;11:188–193
16. Schönland SO, Zimmer JK, Lopez-Benitez CM, et al. Homeostatic control of T-cell generation in neonates. Blood 2003;102:1428–1434
17. Mold JE, Venkatasubrahmanyam S, Burt TD, et al. Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. Science 2010;330:1695–1699
18. Belderbos ME, van Bleek GM, Levy O, et al. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clin Immunol 2009;133:228–237
19. Heninger AK, Theil A, Wilhelm C, et al. IL-7 abrogates suppressive activity of human CD4+CD25+FOXP3+ regulatory T cells and allows expansion of alloreactive and autoreactive T cells. J Immunol 2012;189:5649–5658
20. Simonetta F, Gestermann N, Martinet KZ, et al. Interleukin-7 influences FOXP3+CD4+ regulatory T cells peripheral homeostasis. PLoS ONE 2012; 7:e36596
21. Thornton AM, Korty PE, Tran DQ, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 2010;184:3433–3441
22. Mannering SI, Wong FS, Durinovic-Belló I, et al.; Immunology of Diabetes Society T-Cell Workshop Committee. Current approaches to measuring human islet-antigen specific T cell function in type 1 diabetes. Clin Exp Immunol 2010;162:197–209
23. Herold KC, Brooks-Worrell B, Palmer J, et al.; Type 1 Diabetes TrialNet Research Group. Validity and reproducibility of measurement of islet autoreactivity by T-cell assays in subjects with early type 1 diabetes. Diabetes 2009;58:2588–2595
24. Haynes BF, Heinly CS. Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into the fetal thymic microenvironment. J Exp Med 1995;181:1445–1458
25. Zehn D, Bevan MJ. T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. Immunity 2006;25:261–270
26. Bouneaud C, Kourilsky P, Bousso P. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. Immunity 2000;13:829–840
27. Monti P, Scirpoli M, Maffi P, et al. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest 2008;118:1806–1814
28. Monti P, Heninger AK, Bonifacio E. Differentiation, expansion, and homeostasis of autoreactive T cells in type 1 diabetes mellitus. Curr Diab Rep 2009;9:113–118
29. Hartgring SA, van Roon JA, Wenting-van Wijk M, et al. Elevated expression of interleukin-7 receptor in inflamed joints mediates interleukin-7-induced immune activation in rheumatoid arthritis. Arthritis Rheum 2009;60:2595–2605
30. Traggiai E, Biagioli T, Rosati E, et al. IL-7-enhanced T-cell response to myelin proteins in multiple sclerosis. J Neuroimmunol 2001;121:111–119
31. Min B, McHugh R, Sempowski GD, Mackall C, Foucras G, Paul WE. Neonates support lymphopenia-induced proliferation. Immunity 2003;18:131–140
32. Calzascia T, Pellegrini M, Lin A, et al. CD4 T cells, lymphopenia, and IL-7 in a multistep pathway to autoimmunity. Proc Natl Acad Sci USA 2008;105: 2999–3004
33. Kim GY, Ligons DL, Hong C, et al. An in vivo IL-7 requirement for peripheral Foxp3+ regulatory T cell homeostasis. J Immunol 2012;188:5859–5866
34. Wing K, Larsson P, Sandström K, Lundin SB, Suri-Payer E, Rudin A. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. Immunology 2005;115:516–525
35. Giannoni E, Guignard L, Knaup Reymond M, et al. Estradiol and progesterone strongly inhibit the innate immune response of mononuclear cells in newborns. Infect Immun 2011;79:2690–2698
36. Corbett NP, Blimkie D, Ho KC, et al. Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells. PLoS ONE 2010;5:e15041
37. Velilla PA, Rugeles MT, Chougnet CA. Defective antigen-presenting cell function in human neonates. Clin Immunol 2006;121:251–259
38. Mold JE, Michaëlsson J, Burt TD, et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. Science 2008; 322:1562–1565
39. Lee HH, Hoeman CM, Hardaway JC, et al. Delayed maturation of an IL-12-producing dendritic cell subset explains the early Th2 bias in neonatal immunity. J Exp Med 2008;205:2269–2280
40. Langrish CL, Buddle JC, Thrasher AJ, Goldblatt D. Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. Clin Exp Immunol 2002;128:118–123
41. Kollmann TR, Levy O, Montgomery RR, Goriely S. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. Immunity 2012;37:771–783
42. Nelson JL, Gillespie KM, Lambert NC, et al. Maternal microchimerism in peripheral blood in type 1 diabetes and pancreatic islet beta cell microchimerism. Proc Natl Acad Sci USA 2007;104:1637–1642
43. Walk J, Westerlaken GH, van Uden NO, Belderbos ME, Meyaard L, Bont LJ. Inhibitory receptor expression on neonatal immune cells. Clin Exp Immunol 2012;169:164–171
44. Schenker M, Hummel M, Ferber K, et al. Early expression and high prevalence of islet autoantibodies for DR3/4 heterozygous and DR4/4 homozygous offspring of parents with type I diabetes: the German BABYDIAB study. Diabetologia 1999;42:671–677
45. Lambert AP, Gillespie KM, Thomson G, et al. Absolute risk of childhood-onset type 1 diabetes defined by human leukocyte antigen class II genotype: a population-based study in the United Kingdom. J Clin Endocrinol Metab 2004;89:4037–4043