*Original Article*

# Coexpression of CD25 and OX40 (CD134) Receptors Delineates Autoreactive T-cells in Type 1 Diabetes

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**T-cell–mediated loss of pancreatic β-cells is the crucial event in the development of type 1 diabetes. The phenotypic characteristics of disease-associated T-cells in type 1 diabetes have not yet been defined. The negative results from two intervention trials (the Diabetes Prevention Trial–Type 1 Diabetes and the European Nicotinamide Diabetes Intervention Trial) illustrate the need for technologies to specifically monitor ongoing autoimmune reactions. We used fluorescence-activated cell sorter analysis to study surface marker expression on T-cell lines specific for two major type 1 diabetes autoantigens, GAD65 and proinsulin. We then applied this knowledge in a cross-sectional approach to delineate the phenotype of circulating memory T-cells. The autoreactive T-cells of patients could be distinguished from those of control subjects by their coexpression of CD25 and CD134. Autoantigen-specific T-cells that recognized multiple GAD65- and preproinsulin-derived peptides and coexpressed $CD25^+CD134^+$ were confined to patients ($n$ = 32) and pre-diabetic probands ($n$ = 5). Autoantigen-reactive T-cells in control subjects ($n$ = 21) were $CD25^+CD134^-$ and recognized fewer autoantigen-derived peptides. Insulin therapy did not induce $CD25^+CD134^+$ T-cells in type 2 diabetic patients. The coexpression of CD25 and the costimulatory molecule CD134 on memory T-cells provides a novel marker for type 1 diabetes–associated T-cell immunity. The CD134 costimulatory molecule may also provide a novel therapeutic target in type 1 diabetes. *Diabetes* 55:50–60, 2006**

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APC, antigen-presenting cell; FACS, fluorescence-activated cell sorter; IA-2, insulinoma-associated protein 2; ICA, islet cell autoantibody; IL-2, interleukin-2; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PPI, preproinsulin; TT, tetanus toxoid.



Type 1 diabetes is the result of a progressive T-cell–mediated autoimmune destruction of insulin-producing β-cells in the pancreatic islets of Langerhans (1). Type 1 diabetes is a model disease for studying the progression of autoimmunity because various clinical stages can be clearly defined by a combination of autoantibody measurements and metabolic testing, as was recently shown in two prospective studies (the Diabetes Prevention Trial–Type 1 Diabetes and the European Nicotinamide Diabetes Intervention Trial) (2–4). These trials illustrated the power of present predictive tools to identify at-risk individuals. However, although autoantibodies are predictive parameters that mark progressive β-cell destruction, they have failed to directly mirror T-cell activation and T-cell–mediated β-cell destruction (5). It is therefore highly unlikely that autoantibodies can be used as surrogate biomarkers to monitor the effects of interventional immune therapies (5).

In humans, β-cell destruction is dependent on autoimmune T-cells whose antigen-specific receptors recognize β-cell–derived peptides that bind to risk-associated HLA class II molecules (1,6–10). Evidence has been provided that T-cells modulate the autoimmune process and that autoreactive T-cells can transfer disease (11). It is therefore possible that successful immune intervention, applied during the prodromal phase or at the onset of type 1 diabetes, will result in changes in the autoreactive T-cell repertoire. Currently it is possible to identify β-cell–specific T-cells using standard in vitro proliferation assays, but autoantigen-reactive T-cells are also detected in healthy individuals (12,13). More sophisticated technologies measuring cytokine expression of autoreactive T-cells have failed to unambiguously separate type 1 diabetic patients from HLA-matched control subjects (14). Therefore, the identification and enumeration of disease-associated T-cells is of major importance in tracking disease progression in pre-diabetic individuals and developing diagnostic tools for monitoring immune interventions in people with pre–type 1 diabetes and type 1 diabetes (10,15).

We speculated that an activation-associated phenotype of T-cells, reflecting ongoing disease, might provide a clue in specifically identifying disease-associated T-cells. Our interest was primarily directed toward the identification of disease-associated $CD4^+$ T-cells. To define autoreactive T-cells, we used a two-step approach. First, we established GAD65- and proinsulin/insulin–specific $CD4^+$ T-cell lines from type 1 diabetic patients and HLA-matched healthy control subjects and compared their surface expression

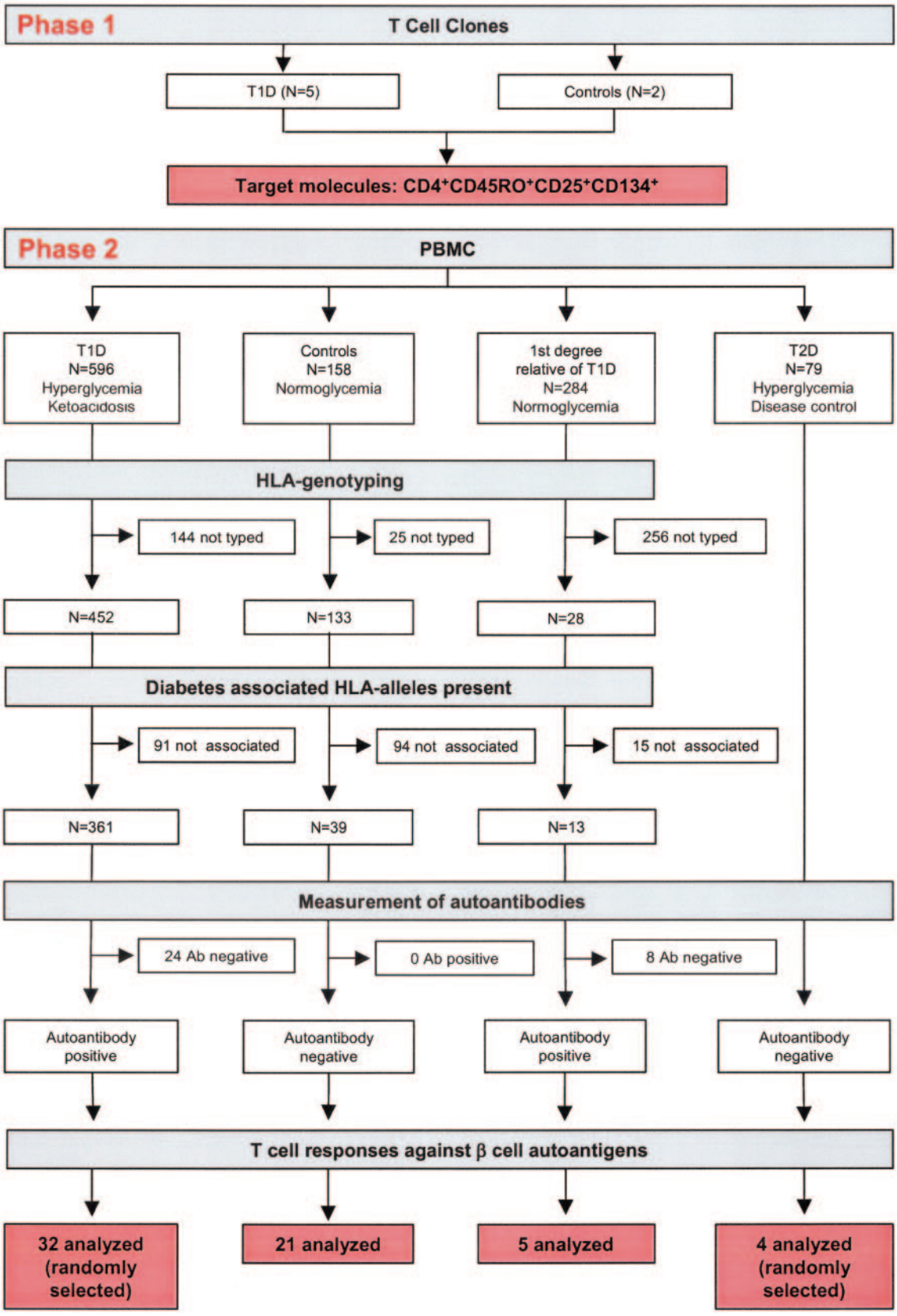


**FIG. 1. Trial protocol. T1D, type 1 diabetes; T2D, type 2 diabetes.**

patterns. Having defined a specific T-cell phenotype, we then used a cross-sectional approach to measure the presence of circulating peripheral $CD3^+CD45RA^-$ memory T-cells with a similar phenotype in type 1 diabetic patients, pre-diabetic probands, and healthy control subjects. Our studies revealed that dual expression of CD25

TABLE 1
Baseline characteristics of the examined probands and patients

| Proband | Sex | HLA alleles | | Autoantibodies | | | |
|---|---|---|---|---|---|---|---|
| | | DRB1* | DQB1* | GADA | IA-2A | IAA | ICA |
| T1D-1 | F | 0401,0801 | 0301,0302 | + | + | − | + |
| T1D-2 | M | ND | ND | + | + | ND | + |
| T1D-3 | F | 1101,15 | 0301,0602 | − | − | − | − |
| T1D-4 | M | 0301,06 | 0201/− | + | + | ND | − |
| T1D-5 | F | 0401,06 | 0302/− | ND | ND | ND | ND |
| T1D-6 | F | 0405,06 | 0302/− | + | + | ND | + |
| T1D-7 | M | 0401,0401 | 0301,0302 | + | + | + | + |
| T1D-8 | F | 0701,0801 | ND | ND | ND | ND | ND |
| T1D-9 | M | 0401,1301 | ND | ND | ND | ND | ND |
| T1D-10 | M | 0401,0402 | 0302,0302 | + | + | − | − |
| T1D-11 | M | ND | ND | + | + | + | − |
| T1D-12 | M | 0404,1001 | 0302,0501 | + | − | + | − |
| T1D-13 | F | 0401,0401 | 0301,0302 | ND | ND | ND | ND |
| T1D-14 | F | 0801,1601 | 0302,0502 | ND | ND | ND | ND |
| T1D-15 | M | 0301,0401 | 0201,0302 | − | − | − | − |
| T1D-16 | F | 0401,0401 | 0302,0302 | ND | ND | ND | ND |
| T1D-17 | F | 0301,0401 | 0201,0302 | + | + | + | + |
| T1D-18 | M | 0301,0401 | 0201,0302 | + | − | − | + |
| T1D-19 | M | 0301,0401 | 0201,0302 | + | − | + | − |
| T1D-20 | M | 0401,0401 | 0302,0301 | + | + | + | + |
| T1D-21 | F | 0301,0401 | 0201,0302 | + | + | − | + |
| T1D-22 | M | 0301,0301 | 0201,0201 | + | − | − | + |
| T1D-23 | M | 0401,0401 | 0302,0302 | + | + | + | − |
| T1D-24 | F | ND | ND | − | − | − | − |
| T1D-25 | F | 0401,0701 | 02,0302 | + | − | + | − |
| T1D-26 | M | 0401,0402 | 0302,0302 | + | + | − | − |
| T1D-27 | M | ND | ND | + | + | + | − |
| T1D-28 | M | 0401,0404 | 0302,0302 | + | − | + | − |
| T1D-29 | F | ND | ND | + | − | + | − |
| T1D-30 | M | ND | ND | + | − | + | − |
| T1D-31 | F | 0301,0401 | ND | − | − | − | − |
| T1D-32 | F | 0301,0401 | 0201,0302 | + | − | − | − |
| Pre–T1D-1 | M | 0401,1501 | 0302,0602 | + | − | ND | + |
| Pre–T1D-2 | M | 0301,1601 | 0201,0502 | + | − | ND | + |
| Pre–T1D-3 | M | 0402,1301 | 0302,0603 | + | + | + | + |
| Pre–T1D-4 | M | 0401,0401 | 0302,0302 | + | + | + | + |
| Pre–T1D-5 | F | 0301,1601 | ND | − | − | + | − |
| CP-1 | M | 0404,1001 | 0302,0501 | − | − | − | − |
| CP-2 | M | 0301,0401 | 0201,0302 | − | − | − | − |
| CP-3 | M | 0401,0401 | 0301,0302 | ND | ND | ND | ND |
| CP-4 | F | 0301,0401 | 0501,0301 | ND | ND | ND | ND |
| CP-5 | F | 0401,1601 | 0302,0502 | − | − | − | − |
| CP-6 | F | 0401,1301 | 0302,0603 | ND | ND | ND | ND |
| CP-7 | F | 0401,1501 | 0302,0602 | − | − | − | − |
| CP-8 | F | 0301,13 | 0201,0603 | ND | ND | ND | ND |
| CP-9 | M | 0301,11 | 0201/− | ND | ND | ND | ND |
| CP-10 | F | 0301,0401 | 0201,0302 | − | − | − | − |
| CP-11 | F | 0301,0408 | 0201,0301 | ND | ND | ND | ND |
| CP-12 | F | 0408,0701 | 0302,0303 | − | − | − | − |
| CP-13 | F | 0401,1601 | 0502,0302 | ND | ND | ND | ND |
| CP-14 | M | 1501,04 | 0302,0602 | − | − | − | − |
| CP-15 | M | 0401,1001 | ND | − | − | − | − |
| CP-16 | F | 0401,1501 | 0301,0602 | ND | ND | ND | ND |
| CP-17 | F | 1501,04 | 0302,0602 | − | − | − | − |
| CP-18 | F | 0301,0701 | 0201,0201 | − | − | − | − |
| CP-19 | F | 0401,1301 | 0302,0603 | − | − | − | − |
| CP-20 | F | 0301,0401 | 0201,0302 | − | − | − | − |
| CP-21 | M | 0401,0301 | 0302,0201 | − | − | − | − |
| T2D-1 | F | 1001,15 | 0501,0602 | − | − | − | − |
| T2D-2 | F | 0801,1301 | 0402,0603 | − | − | + | − |
| T2D-3 | F | 0701,1201 | 0201,0603 | − | − | − | − |
| T2D-4 | M | ND | ND | − | − | − | − |

Normal range of autoantibodies: anti-GAD65 autoantibody (GADA) <3.9 Karlsburg units (KU)/l, anti–IA-2 autoantibody (IA-2A) <1.85 KU/l, anti-insulin autoantibody (IAA) <281.48 μU/l, and anti-ICA <20 JDF units. ND, not determined.

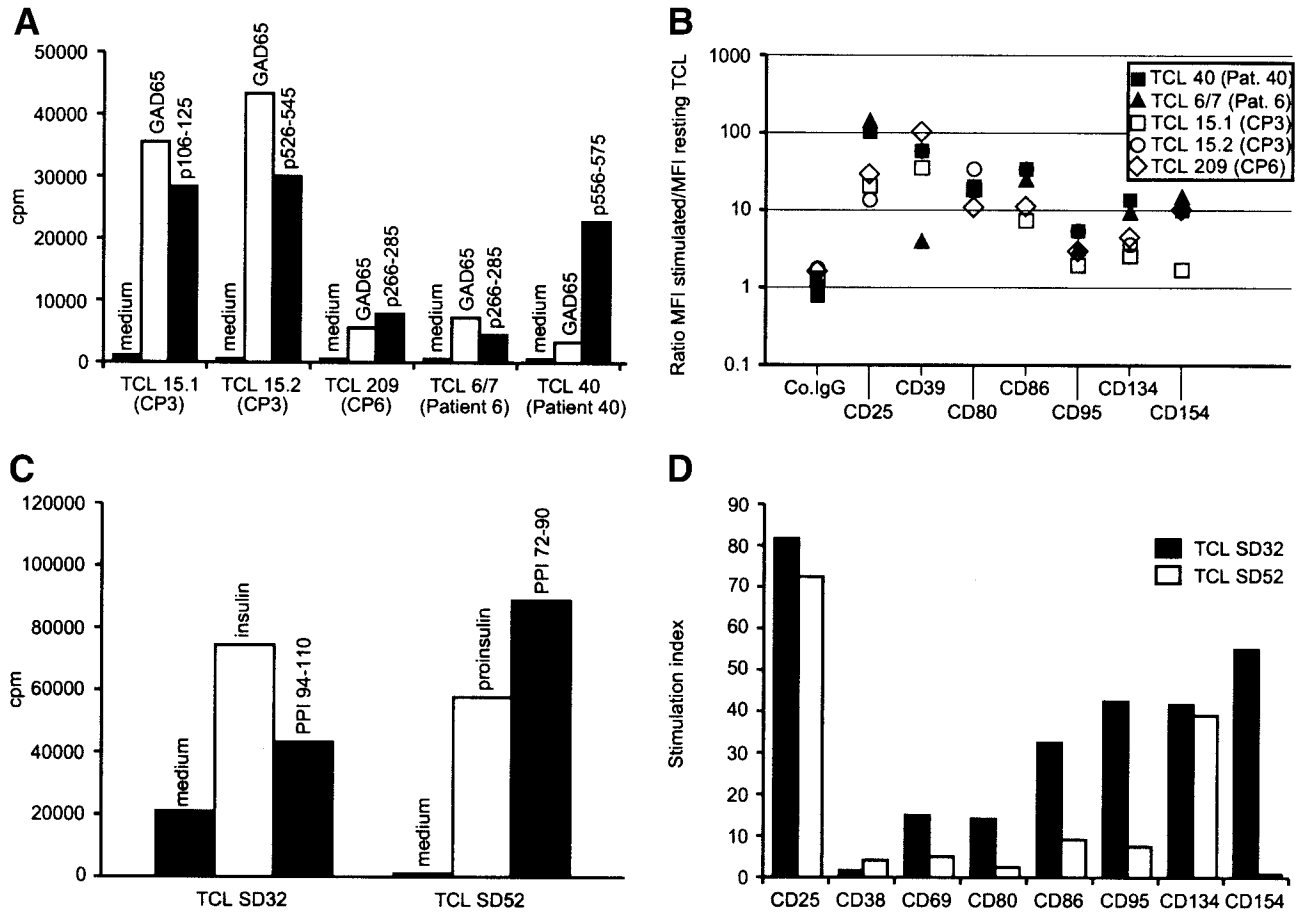


**FIG. 2. Characterization of long-term T-cell lines. *A*: GAD65 responses of T-cell lines derived from HLA-DRB1*0401–positive control subjects (CP) and type 1 diabetic patients. *B*: FACS analysis of surface marker expression of GAD65-specific T-cell line (TCL) after being stimulated with corresponding peptides. *C*: Insulin- and proinsulin-specific responses of two T-cell lines from an HLA-DRB1*0401–positive type 1 diabetic patient. *D*: Surface phenotype of proinsulin-specific T-cell line after being stimulated with corresponding peptides. The incorporation of radioactive thymidine is shown in *A* and *C*. The ratio of mean fluorescence intensities of T-cell blasts versus those of resting T-cell cluster are indicated in *B*. *D*: The percentage of T-cells expressing the indicated activation markers after being stimulated with corresponding peptides divided by the percentage of activation marker–positive T-cells after being stimulated with medium alone is given. MFI, mean fluorescence intensity.**

and CD134 delineated disease-associated T-cells. We also found that T-cells with this double-positive phenotype were detected in pre-diabetic probands but not in type 2 diabetic patients. This ability to unambiguously delineate type 1 diabetes–associated T-cells using multiparameter fluorescence-activated cell sorter (FACS) technology is a major breakthrough, enabling disease progression to be monitored and providing a useful tool to evaluate ongoing immune intervention studies.

## RESEARCH DESIGN AND METHODS

The trial protocol is outlined in Fig. 1. The autoantibody profiles and HLA genotypes of the study subjects are given in Table 1. Type 1 and type 2 diabetic patients were distinguished using World Health Organization and American Diabetes Association criteria. Pre-diabetic individuals ($n = 5$) were identified by the presence of an HLA-risk haplotype (high-risk genotypes known to be present in Caucasians) and positivity for islet cell autoantibodies (ICAs); four of the five probands had a family history of type 1 diabetes. Their blood glucose and $HbA_{1c}$ levels were determined to be within the normal range. Individuals were included as healthy control subjects if they had a diabetes-related HLA genotype, lacked a family history of type 1 diabetes, and had no evidence of any other autoimmune disease, including type 1 diabetes.

HLA class II genotyping was performed with sequence-specific oligonucleotides after DNA amplification with HLA-DRB1–, HLA-DQA1–, and HLA-DQB1–specific primers (16). The study was approved by the ethics board of the Medical Faculty of the University of Ulm (September 1998).

The autoantibody assays used have been previously described in detail (17,18). Briefly, GAD65 and insulinoma-associated protein 2 (IA-2) autoantibodies were measured by fluid-phase $^{125}$I-antigen–binding assays using recombinant human GAD65 (Diamyd Diagnostics, Stockholm, Sweden) and recombinant human IA-2ic proteins (BRAHMS Diagnostica, Berlin, Germany). The anti-GAD65 assay has a diagnostic sensitivity and specificity of 88 and 96%, respectively. The anti–IA-2 assay achieved 58% diagnostic sensitivity and 100% specificity in the first Diabetes Antibody Standardization Program proficiency evaluation of the Immunology of Diabetes Society and the Centers for Disease Control and Prevention in 2001. Insulin-specific autoantibodies were measured by an established microassay using a protein A method (17,18). The 99th percentile, used as the cutoff point, was calculated from 991 healthy schoolchildren. Serum concentrations of GAD65 and IA-2 autoantibodies, calculated using an in-house standard serum, are given as Karlsburg units per liter, and insulin autoantibodies, analyzed by microassay, are given in microunits per liter. ICAs were measured by indirect immunofluorescence on cryosections of human pancreas after being incubated overnight at 4°C. The detection limit was 5 JDF units. ICA levels ≥20 JDF units were considered to be positive. The assay achieved an analytical sensitivity and specificity of 100% in the 13th ICA Workshop in 1998.

**Generation of T-cell lines specific for GAD65 and proinsulin.** Peripheral blood mononuclear cells (PBMCs) obtained from type 1 diabetic patients with recent onset and normal subjects were isolated from heparinized venous blood by density gradient sedimentation. All cell cultures were set up in RPMI medium containing 5% human serum (pooled from at least six male donors). T-cell lines were generated from PBMCs of newly diagnosed type 1 diabetic patients essentially as previously described (19). The antigens used for stimulation were GAD65 (10 μg/ml), purchased from Diamyd Diagnostics (endotoxin content <0.3 units/ml; Limulus lysate assay), and highly purified GAD65 peptides (5 μg/ml), derived from a library of 20-mer oligopeptides with a 10-mer overlapping region (19). Individual GAD65 peptides are indicated by the starting amino acid position (e.g., p106 spans GAD65 from amino acid 106

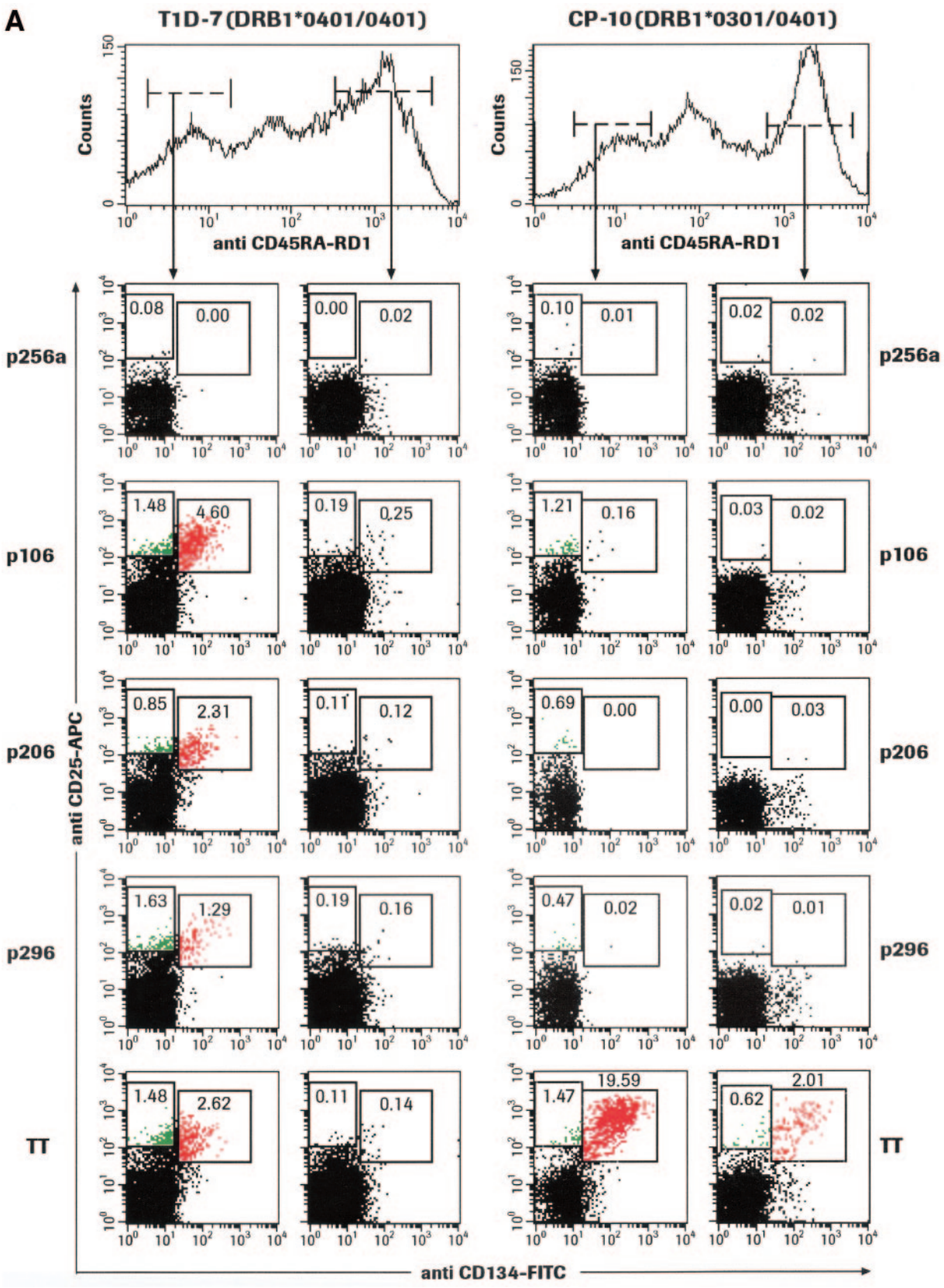


**FIG. 3. Surface marker analysis of stimulated PBMCs. *A*: Marker expression of type 1 diabetic patient T1D-7 and healthy control subject CP-10 after the PBMCs were stimulated with GAD65 peptides. Lymphocytes pregated for CD3 expression were further analyzed for the expression of CD45RA and the activation markers CD25 and CD134. Shown is the percentage of memory ($CD45RA^-$) and naïve ($CD45RA^+$) T-cells expressing CD25 alone (green dots) or in combination with CD134 (red dots). Peptide-reactive T-cells of the type 1 diabetic patient were derived mainly from the $CD45RA^-$ memory T-cell cluster and were of a predominant $CD25^+CD134^+$ double-positive phenotype. T-cells of the healthy control subject upregulated CD25 only. Stimulation with the recall antigen TT induced double-positive T-cells in both the patient and the control subject. *B*: Activation marker analysis of a twin pair discordant for type 1 diabetes. Depicted are the activation marker–positive memory T-cells against control antisense peptide p256a and GAD65 peptide p266.**

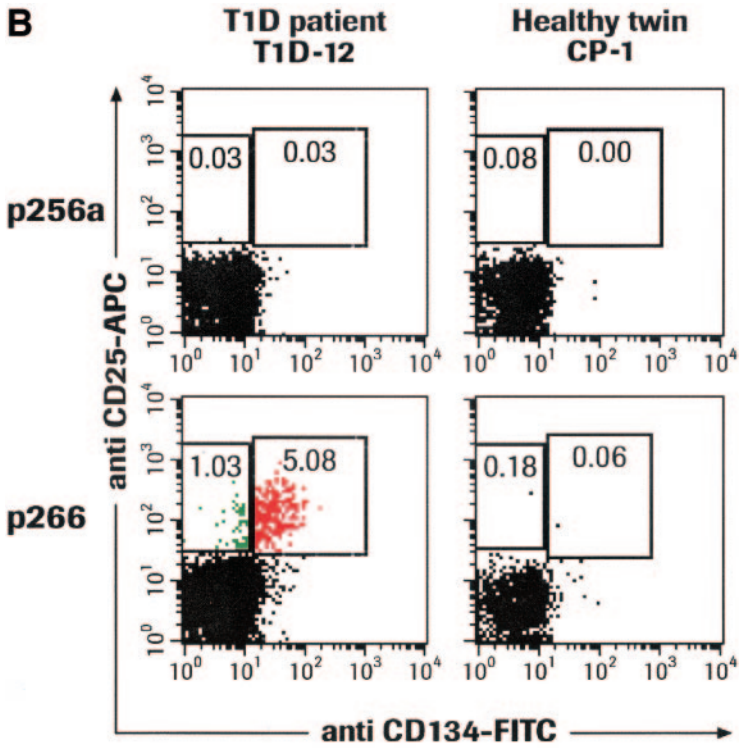


**FIG. 3—Continued.**

to 125). Human insulin/proinsulin (kindly provided by Eli Lilly, Greenfield, CT) was used in a concentration of 10 μg/ml; preproinsulin (PPI) peptides were 17–20 mers, with an overlapping region of 10–12 residues, and were used in a concentration of 10 μg/ml. The synthesis and purification of insulin β-chain and PPI peptides have been described elsewhere (20,21). For primary stimulation, ~200 microcultures, each containing 1.0–1.5 × $10^5$ PBMCs per well, were established for each subject. After a stimulation period of 6–7 days with antigen alone, interleukin-2 (IL-2; recombinant; Roche Diagnostics, Penzberg, Germany) was added at a final concentration of 15 units/ml. After at least three rounds of restimulation with autologous antigen-presenting cells (APCs), PBMCs from HLA class II fully matched healthy control donors were used as APCs for further restimulation of the T-cell lines with native antigen.

**Stimulation assays using T-cell lines.** Stimulation assays using long-term T-cell lines, recombinant GAD65, proinsulin proteins, or GAD65- and PPI-derived peptides were performed as previously described (19). Briefly, T-cell lines were restimulated with HLA-matched PBMCs (irradiated with 40 Gy) pulsed with antigens or peptides and analyzed after 72 h for radioactive thymidine incorporation as a measure of proliferation and for surface marker expression by flow cytometry.

**Surface marker phenotyping.** All monoclonal antibodies (mAbs) used were obtained from BD Biosciences Pharmingen, unless otherwise indicated. The following fluorescence-labeled antibodies were used for screening surface markers on T-cell lines after peptide stimulation: anti–CD25-APC (clone M-A251; antibody recognizes epitope region B), anti–CD39-PE (Tü66), anti–CD80-FITC (BB1), anti–CD86-FITC (2331[FUN-1]), anti–CD95-FITC (DX2), anti–CD134-FITC (ACT35), and anti–CD154-PE (TRAP1). Appropriate isotype control reagents were used to delineate positive and negative populations. After incubating the peptide-activated T-cell lines for 30 min at 4°C with the appropriate mAbs, cells were washed in PBS/0.5% BSA and directly analyzed by flow cytometry (FACSCalibur and CellQuest software).

**Short-term antigen stimulation assay using PBMCs.** Assays were set up in 96-well, flat-bottom microtiter plates using 3 × $10^5$ PBMCs/well at a final volume of 200 μl. The PBMCs were preincubated in RPMI/5% human serum without antigen overnight at 37°C with 7% $CO_2$; this preincubation step allowed the APCs to adhere and was found to increase responses. Antigens and peptides were added the next day. The final concentrations used were 10 μg/ml for GAD65/proinsulin– and GAD65/PPI–derived peptides and 5 μg/ml for tetanus toxoid (TT; Behring, Marburg, Germany). Media alone and with antisense GAD65 peptide p256a were used as negative controls. The samples were incubated for 96–120 h at 37°C with 7% $CO_2$. Supernatants were aspirated and cells were detached by cold 0.2% EDTA/PBS and transferred by repetitive pipetting into 5-ml Falcon tubes (Falcon no. 352058). The cells were sedimented by centrifugation and resuspended in a mixture of fluorescence-labeled antibodies in PBS/0.5% BSA: anti–CD3-PerCP, anti–CD25-APC, anti–CD134-FITC, and anti–CD45RA-RD1 (Beckmann Coulter). The tubes were incubated at 4°C for 20 min and washed twice with 2.5 ml PBS/0.5% BSA. Supernatants were aspirated and cells were resuspended with 250 μl PBS/0.5% BSA. Cells were analyzed with the FACSCalibur flow cytometer. In most cases, 50,000–100,000 events were acquired, gated on CD3 expression and a light scatter gate to include only viable lymphocytes. Cells were further pregated on CD45RA positive/negative clusters and finally analyzed for CD25 and CD134 expression. We used responses observed against medium or a control antisense GAD65 peptide (p256–275 synthesized in antisense denoted p256a) for setting the gates to delineate positive and negative populations. These control responses were set up for each individual tested. Gates were set so that stimulation with these control subjects resulted in 0.01–0.1% activation marker–positive memory T-cells ($CD45RA^-$). The responses to GAD65-derived peptides were then determined with set gates and defined as positive provided that the frequency of $CD25^+$ or $CD25^+/CD134^+$ cells was >0.3% and at least three times higher than the responses observed with the control subjects. To induce CD134 expression in control T-cells, PBMCs were preincubated overnight without antigen and then stimulated with peptides for 48 h. After this period, a low level of IL-2 (15 units/ml) was added to the microcultures, and the samples were incubated for an additional 48 h. Flow cytometry was performed as described above. A multiparameter FACS assay revealed an interassay variation <7%.

**Statistical analysis.** Categorical data were subjected to $\chi^2$-test analysis. $P < 0.05$ was considered significant.

## RESULTS

**Generation of autoreactive T-cell lines from type 1 diabetic patients and control subjects.** Disease-associated T-cell lines were derived from three type 1 diabetic patients (GAD65-specific T-cell lines from patients 6 and 40; proinsulin-specific lines from patient T1D-20) and two control subjects (only GAD65-specific T-cell line) carrying the high-risk HLA haplotype DRB1*0401/DQB1*0302 using a culture technique previously described (19). Microcultures showing a stimulation index of 3–20 after being restimulated with GAD65, proinsulin, or insulin proteins were further propagated. T-cell lines were probed in proliferation assays using complete peptide libraries of GAD65 or PPI peptides. In all, five GAD65-specific and two

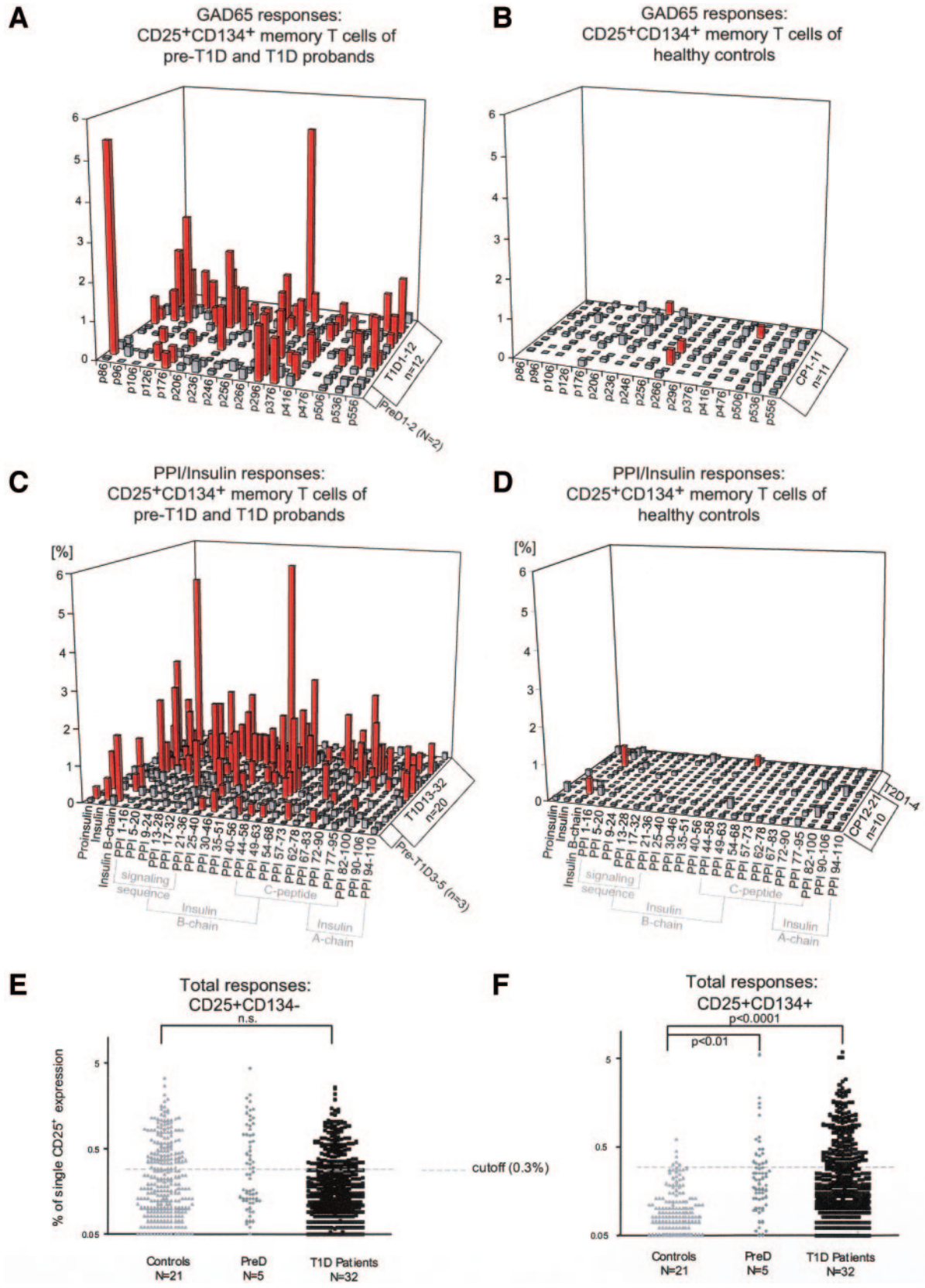


**FIG. 4. Prevalence of CD25$^+$CD134$^+$ double-positive memory T-cells in PBMCs of type 1 diabetic (T1D) patients and healthy control subjects. CD25$^+$CD134$^+$ memory T-cell responses against GAD65 peptides in pre–type 1 diabetic and type 1 diabetic probands (*A*) and healthy control subjects (*B*) and against preproinsulin/insulin proteins and peptides in pre–type 1 diabetic and type 1 diabetic probands (*C*) and type 2 diabetic patients and healthy control subjects (*D*). Responses reaching the cutoff level ≥0.3% are shown in red (see Fig. 3). In terms of GAD65 peptide stimulation, two pre-diabetic individuals and 9 of 12 type 1 diabetic patients responded with dual-activation marker expression, recognizing several GAD65 peptides ($P < 0.01$ vs. control subjects). After PBMCs were stimulated with PPI antigen/peptides, one of three pre-diabetic individuals and 18 of 20 type 1 diabetic patients showed coexpression of CD25$^+$CD134$^+$ ($P < 0.0001$ for patients vs. control subjects). Also shown is the frequency of CD25$^+$ (*E*) and combined CD25$^+$CD134$^+$ (*F*) memory T-cells of all study subjects against that of peptide libraries derived from**

PPI peptide–specific T-cell lines were found. The characteristics of the GAD65-specific T-cell lines derived from patients 6 and 40 have been previously described (19). All T-cell lines recognized autoantigen-derived peptides in the context of diabetes-associated HLA molecules. Two T-cell lines isolated from healthy control subject CP3 (15_1 and 15_2) responded to the GAD65-derived peptides p106–125 and p526–545, respectively, whereas the T-cell line isolated from control subject CP6 (209) was specific for a peptide located in the middle region of GAD65 (p266–285) (Fig. 2*A*). The same peptide was recognized by both T-cell lines of patient 6 (20), whereas the T-cell line of patient 40 recognized another HLA-DR4–restricted epitope, p556–575 (Fig. 2*A*). The PPI peptide–specific T-cell lines of the third patient recognized epitopes present in the A (PPI 94–110) and C (PPI 72–90) protein chains, respectively (Fig. 2*B*).

**Upregulation of activation markers in the T-cell lines.** We next determined the phenotype of the T-cell lines using a variety of mAbs. All T-cell lines were composed of $CD4^+$ memory ($CD45RA^-$) T-cells (data not shown). The expression of various activation-associated cell surface molecules was studied after the T-cell lines were stimulated for 72 h with their specific peptides. We found that the levels of CD25, CD86, and CD134 were increased in the GAD65-specific T-cell lines of type 1 diabetic patients compared with control subjects (Fig. 2*A*). Likewise, the two PPI peptide–specific T-cell lines isolated from a type 1 diabetic patient showed concomitant upregulation of CD25 and CD134 (Fig. 2*B*). These results revealed a component of the high-affinity lymphocyte receptor for IL-2, CD25, and the OX40 member of the tumor necrosis factor receptor family, CD134, as candidates for identifying disease-associated memory T-cells after autoantigen stimulation.

**$CD25^+CD134^+$ expression on autoantigen-reactive peripheral T-cells.** To determine whether $CD25^+CD134^+$ coexpression could also be detected on antigen-reactive T-cells circulating in the peripheral blood, we stimulated PBMCs of a type 1 diabetic patient and a partial HLA-matched control donor with GAD65 peptides known to bind to their type 1 diabetes risk–associated HLA class II molecules (22,23). The PBMCs were stimulated for 96 h with peptides in the absence of exogenous IL-2 and then analyzed directly by multiparameter flow cytometry. The initial lymphocyte gates were set to include only viable $CD3^+$ T-cells. To demonstrate that the responses were derived from memory T-cells, an analysis was performed in parallel in the $CD45RA^-$ (memory) and $CD45RA^+$ (naïve) T-cell clusters. Figure 3*A* displays a typical example of one type 1 diabetic patient responding to the GAD65 peptide p106, whereby the majority of responsive cells were found to reside in the $CD45RA^-$ memory T-cell population (4.6 vs. 0.25%). Double-positive T-cells were also found in response to GAD65 peptides p206 and p296 in the patient. In contrast, double-positive cells were not detected in stimulated PBMCs of the control subject. Although several GAD65 peptides led to weak responses, only CD25 expression was increased on T-cells in the healthy control subject.

A striking example of the capacity of the $CD25^+CD134^+$ double-positive phenotype to distinguish disease-associated T-cells of a patient from autoreactive T-cells of a healthy control subject was found in the analysis of an identical twin pair discordant for type 1 diabetes (Fig. 3*B*). Memory T-cells of the diabetic proband responded to GAD65 peptide p266, with strong upregulation of both CD25 and CD134, whereas double-positive T-cells were not detected in the healthy sibling. The finding that T-cells responded to only one GAD65 peptide in this diabetic proband is due to the fact that the class II molecules encoded by his risk-associated HLA-DRB1*0404 allele could bind fewer peptides of the test panel. Follow-up of this pair over a >5-year period did not reveal the appearance of autoantibodies or any evidence of disease in the healthy twin.

**Autoreactive T-cell responses in study cohorts.** Having confirmed that our assay could identify short-term in vitro reactivated $CD25^+CD134^+$ positive T-cells in PBMCs of type 1 diabetic patients, we extended our analyses to a study cohort composed of pre-diabetic probands, type 1 diabetic patients, and healthy control subjects selected as carrying risk-associated HLA alleles (see Table 1). PBMCs were stimulated with a panel of 17 GAD65 peptides, 21 PPI peptides, or both, which were selected for their capacity to bind to diabetes-associated HLA class II molecules (22,23). A TT protein and an antisense GAD65 peptide, in addition to medium alone, were included as positive and negative controls. All samples were subsequently analyzed by FACS. High numbers of double-positive cells were detected in pre-diabetic subjects and type 1 diabetic patients but not in the healthy donors (Fig. 4*A–D*). This distinction was not apparent when $CD25^+CD134^-$ single-positive T-cells were compared in patients and control subjects (Fig. 4*E* and *F*). Screening also revealed that $CD25^+CD134^+$ double-positive memory T-cells of type 1 diabetic patients, but not of healthy subjects, recognized a variety of different GAD65 and PPI peptides, consistent with active ongoing autoimmunity and epitope spreading (Fig. 4*A* and *C*). Double-positive T-cells responding to several peptides were also found in pre-diabetic individuals; such probands are known to have active autoimmune T-cells. Furthermore, significant distinctions could also be made between pre-diabetic probands and healthy control subjects based on the frequency of double-positive T-cells (Fig. 4*E* and *F*).

Because insulin is routinely used as a therapeutic agent, resulting in daily vaccination, we studied insulin-treated type 2 diabetic patients as disease control subjects. Memory T-cells of type 2 diabetic patients responding to PPI peptides showed no CD134 upregulation, demonstrating that autoreactive $CD25^+CD134^+$ double-positive memory T-cells were characteristic of type 1 diabetes only (Fig. 4*D*). This contention was strengthened by the fact that GAD65- and PPI peptide–specific T-cells of some pre-diabetic subjects who were not exposed to exogenous insulin showed significant upregulation of CD25 and CD134.

**Induction of $CD25^+CD134^+$ coexpression in control T-cells.** Overall, the phenotype of the GAD65-responsive T-cells of the control subjects ($CD25^+CD134^-$) suggested that these cells might have originated from incompletely activated T-cells (24,25). We tested several conditions in an attempt to convert CD25 single-positive control T-cells to a double-positive phenotype. Pretreatment of PBMCs

**both autoantigens (17 GAD65 and/or 24 proinsulin stimuli). Using the cutoff value, the number of cultures with CD25 single-positive cells did not differ significantly between type 1 diabetic patients and control subjects, whereas the frequency of $CD25^+CD134^+$ T-cells differed significantly between cultures testing pre-diabetic and control PBMCs ($P < 0.01$) and PBMCs of type 1 diabetic patients and control subjects ($P < 0.001$).**

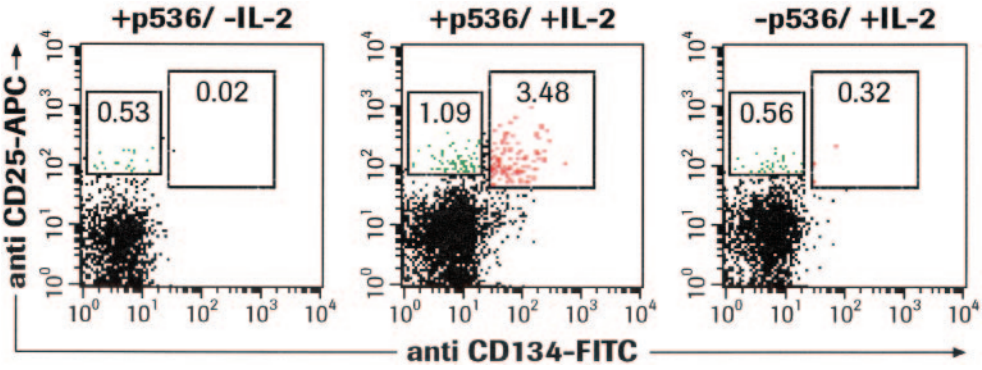


**FIG. 5. GAD65-specific peptide in combination with IL-2 induced CD134 upregulation in T-cells of a healthy control subject. In the absence of IL-2, GAD65 peptide induced the upregulation of CD25, whereas when IL-2 was added, activated T-cells coexpressed both CD25 and CD134. This was not observed when PBMCs were stimulated with IL-2 in the absence of peptide.**

from control subjects ($n = 4$) with either of the two danger signals (lipopolysaccharide or tumor necrosis factor-α) before peptide stimulation did not upregulate CD134 expression. In contrast, the addition of exogenous IL-2 during the final 48 h of the culture induced a strong neo-expression of CD134, provided that GAD65 peptides were also present (Fig. 5). This indicated that GAD65-reactive T-cells of healthy donors were unable to secrete amounts of IL-2 sufficient to sustain complete activation and proliferation, but this checkpoint could be overcome with exogenous IL-2.

**$CD25^+CD134^+$ coexpression on TT-reactive T-cells.** Vaccination with exogenous antigens should lead to a complete activation of T-cells and the generation of competent memory T-cells. Because vaccination with the TT protein is known to result in long-lasting T-cell memory, we studied responses to this model antigen to address two questions: *1*) Was the double-positive $CD25^+CD134^+$ phenotype of memory T-cells a unique feature of type 1 diabetic patients? and *2*) Was the overall phenotype similar for T-cells responding to foreign antigen applied with costimulatory agents (i.e., adjuvants) versus T-cell responding in an autoimmune setting? When we assessed memory T-cell responses to the recall TT protein, we found no significant differences in the number of double-positive T-cells between type 1 diabetic patients and healthy control subjects ($P = 0.98$). With one exception (TID-29), all investigated pre–type 1 diabetic probands and healthy control subjects responded specifically to stimulation with TT by coexpressing $CD25^+CD134^+$. Co-expression of CD25 and CD134 was upregulated on T-cells, showing a high median of ~5.5% in both groups (range 0.14–41.78 in type 1 diabetic patients and 1.03–22.18 in control subjects) (Table 2).

## DISCUSSION

Identifying disease-associated T-cells is of major importance in understanding the development of type 1 diabetes, especially when disease progression or immune intervention needs to be monitored. To identify a unique phenotype characteristic of autoreactive T-cells in type 1 diabetic patients, we established GAD65- and proinsulin-specific T-cells from patients and HLA-matched control subjects. We determined that coexpression of CD25 and CD134 (Ox40) delineated autoreactive T-cell lines derived from patients. We then compared the PBMCs of patients and control subjects and found that memory T-cells of type 1 diabetic patients responding to GAD65 and proinsulin could be distinguished from those of healthy control subjects by their coexpression of CD25 and CD134. Co-expression of CD25 and CD134 was also found on autoantigen-reactive T-cells of pre-diabetic probands. Both patients and pre-diabetic subjects had double-positive T-cells that recognized several GAD65 or PPI peptides, which indicated epitope spreading.

Viglietta et al. (26) recently showed that T-cells from type 1 diabetic patients could be distinguished from those of control subjects using antibody inhibition of CD80/CD86 (B7) costimulatory molecules in proliferation assays of PBMCs and GAD65 antigens; in that study, the inhibition of B7 costimulation did not affect responses of patient T-cells but did strongly reduce those of control subjects. These observations also support the contention that autoreactive T-cells in patients represent robust memory cells. Although such a blocking assay could be used to monitor autoimmune responses in patients, there are many advantages to being able to visualize ongoing responses. Therefore, we established an approach using multiparameter flow cytometry. Application of this method showed that *1*) autoantigen-reactive T-cells in pre-diabetic probands, patients, and HLA-matched healthy control subjects displayed a $CD45RA^-$ memory T-cell phenotype, indicating that they had been primed previously in vivo; *2*) all patients responded to multiple epitopes; and *3*) reactivated memory T-cells of type 1 diabetic patients could be distinguished from those of healthy control subjects by their coexpression of CD25 and CD134.

CD25 is a component of the high-affinity receptor for IL-2 and is upregulated as an early activation marker after T-cell receptor engagement. CD134 (Ox40) is an inducible co-receptor molecule thought to be critical for the survival and expansion of inflammation-mediating T-cells (27–29). Therefore, the coexpression of CD25 and CD134 by memory T-cells derived from patients and pre-diabetic probands appears to reflect a unique imprinting on T-cells that occurred during their priming in vivo (30–32). Only activated APCs express CD134L and can participate in CD134L-to-CD134 cross-talk with T-cells (27,30).

Based on animal models, we propose that the in vivo exposure of T-cells from control donors to β-cell–specific peptides did not induce complete T-cell activation, thereby enabling them to be distinguished from the β-cell–specific responses of patients by their failure to express CD134. This may have been the consequence of an initial activation of naïve T-cells by immature or nonprofessional APCs in a noninflammatory environment. Insufficient activation

TABLE 2
Response to TT as measured by frequency of TT-reactive memory T-cells

| Proband | Frequency | |
|---|---|---|
| | $CD25^+CD134^-$ | $CD25^+CD134^+$ |
| T1D-1 | 0.33 | 8.00 |
| T1D-2 | 1.09 | 2.67 |
| T1D-3 | ND | ND |
| T1D-4 | 1.23 | 33.00 |
| T1D-5 | 0.15 | 3.00 |
| T1D-6 | 2.17 | 6.42 |
| T1D-7 | 1.48 | 2.62 |
| T1D-8 | 0.55 | 2.07 |
| T1D-9 | 0.93 | 7.45 |
| T1D-10 | 1.40 | 62.08 |
| T1D-11 | 0.96 | 74.52 |
| T1D-12 | ND | ND |
| T1D-13 | 2.64 | 6.63 |
| T1D-14 | 3.64 | 28.80 |
| T1D-15 | 0.85 | 6.78 |
| T1D-16 | 0.90 | 11.01 |
| T1D-17 | 0.00 | 1.80 |
| T1D-18 | 3.84 | 41.78 |
| T1D-19 | 2.07 | 11.30 |
| T1D-20 | 0.83 | 1.20 |
| T1D-21 | 1.28 | 22.52 |
| T1D-22 | 7.41 | 6.08 |
| T1D-23 | 0.56 | 20.74 |
| T1D-24 | 0.34 | 9.34 |
| T1D-25 | 0.64 | 1.01 |
| T1D-26 | 1.71 | 13.32 |
| T1D-27 | 11.86 | 6.26 |
| T1D-28 | 0.66 | 14.03 |
| T1D-29 | 1.77 | 0.14 |
| T1D-30 | 0.16 | 1.71 |
| T1D-31 | 1.16 | 1.61 |
| T1D-32 | 0.38 | 1.75 |
| Pre–T1D-1 | 0.85 | 7.91 |
| Pre–T1D-2 | 5.28 | 41.90 |
| Pre–T1D-3 | 1.67 | 3.32 |
| Pre–T1D-4 | 0.49 | 4.27 |
| Pre–T1D-5 | 0.30 | 1.70 |
| CP-1 | ND | ND |
| CP-2 | 1.18 | 6.51 |
| CP-3 | 1.21 | 4.12 |
| CP-4 | ND | ND |
| CP-5 | 4.82 | 3.15 |
| CP-6 | 0.87 | 2.85 |
| CP-7 | 1.52 | 25.23 |
| CP-8 | 1.83 | 10.26 |
| CP-9 | 0.89 | 14.98 |
| CP-10 | 1.21 | 22.89 |
| CP-11 | 5.98 | 5.84 |
| CP-12 | 5.05 | 1.57 |
| CP-13 | 0.81 | 10.20 |
| CP-14 | 6.52 | 12.09 |
| CP-15 | 4.23 | 1.05 |
| CP-16 | 2.70 | 1.03 |
| CP-17 | 1.73 | 2.39 |
| CP-18 | 10.12 | 17.63 |
| CP-19 | 0.72 | 6.90 |
| CP-20 | 3.75 | 22.18 |
| CP-21 | 4.98 | 1.51 |
| T2D-1 | 0.28 | 0.00 |
| T2D-2 | 0.63 | 0.07 |
| T2D-3 | 2.95 | 5.80 |
| T2D-4 | 15.40 | 10.60 |

Data are percent. Frequency of ≥0.3% was chosen as the cutoff point.

of T-cells in type 2 diabetic patients and control subjects may have led to their upregulation of IL-2 receptor expression and loss of CD45RA expression but not to their sustained proliferation and differentiation into effector cells, as indicated by their failure to express the late activation/costimulation marker CD134. Thus this pool of potentially autoreactive T-cells is probably self-limiting and nondestructive and may have regulatory functions (33). A fraction of these insufficiently activated cells may re-enter the circulation, thereby becoming detectable in PBMCs after being reactivated in vitro. As an alternative explanation, it could be speculated that only the APCs of patients expressed CD134L and that this led to an upregulation of CD134 on their T-cells after they were restimulated in vitro. However, this does not appear to be the case because many TT-reactive cells in healthy control subjects and type 2 diabetic patients were found to coexpress CD25 and CD134. Furthermore, the lack of CD134 expression on peptide-specific T-cells of normal control subjects could be overcome by exposure to exogenous IL-2. The milieu experienced by autoreactive T-cells in type 1 diabetic patients may also be different to that of type 2 diabetic patients and healthy control subjects. Because both type 1 diabetic patients and control subjects produced double-positive memory T-cells after standard vaccination with TT, there was no apparent broad immune deviation in either group.

A limitation of this study was its cross-sectional design; our findings need to be confirmed by prospective studies. Such studies are now feasible because we recently showed that well-standardized freezing procedures preserve the functional capacity of T-cells and allow their subsequent analysis (34). In addition, our FACS analysis was found to be very robust, with an interassay variability <7%.

The ability to track disease-associated, autoreactive T-cells over time will now allow the monitoring of disease progression in pre-diabetic individuals. Furthermore, our data provide evidence for an important role of costimulatory signaling in the development of autoimmune diabetes, thus allowing timely therapeutic interventions to halt β-cell destruction to be designed (35–37).

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