Th2 Dominance of T Helper Cell Response to Preproinsulin in Individuals with Preclinical Type 1 Diabetes

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ABSTRACT: In human type 1 diabetes (T1D) autoantibodies to insulin precede clinical disease, while little is known about the contribution of insulin-specific T lymphocytes—in particular, T helper (Th) subsets. Here we have studied the *in vivo* primed cytokine response to preproinsulin in peripheral blood mononuclear cells (PBMCs) and two major Th cell subsets—CD45RO$^+$ memory cells and CD45RA$^+$ naive/resting cells—in 35 individuals with HLA-DRB1*04, DQB1*0302 diabetes risk marker: 12 patients with T1D, 12 autoantibody-positive (Ab$^+$) individuals, and 11 healthy controls. Cytokine secretion (TNF-α, IFN-γ, IL-2, IL-4, IL-5, and IL-10) was measured in the supernatants of the cultures stimulated with 21 overlapping preproinsulin peptides as well as proinsulin and insulin. In Ab$^+$ individuals our results reveal higher IL-4 levels in CD45RO$^+$ memory cells and higher IL-5 levels in CD45RA$^+$ naive/resting cells, while higher IL-2 production was found in PBMCs. In contrast, in PBMCs of T1D patients higher IFN-γ and IL-10 secretion was found. Our data delineate characteristic cytokine patterns in peripheral T lymphocytes from patients at different stages of the T1D development.

KEYWORDS: preproinsulin; PBMC; CD45RO$^+$ memory and CD45RA$^+$ naive/resting Th cells; cytokines

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INTRODUCTION

In type 1 diabetes (T1D), pancreatic beta-cells expressing preproinsulin and other autoantigens are targeted by T cell–mediated autoimmune destruction. However, little is known about the role of distinct T cell subsets and their cytokine secretion in T1D development. On the basis of their activation status, T helper (Th) cells could be divided in naive/resting cells expressing high molecular weight CD45RA marker and memory cells expressing low molecular weight CD45RO marker. Recently activated cells represent a transient stage and coexpress both markers $CD45RA^{+}RO^{+}$ (Peterson *et al*.[1] and references therein). Furthermore, Th cells exert their regulatory functions via the release of cytokines and are classified according to their cytokine patterns: Th1 cells predominantly secrete IFN-γ, TNF-α, and IL-2 and direct immune responses towards cell-mediated immunity, and Th2 cells preferentially produce IL-4, IL-5, and IL-10 and provide efficient help for B lymphocyte activation and induction of humoral immune responses. It has been proposed that Th1 cells contribute to the pathogenesis of organ-specific autoimmune diseases, while Th2 cells may prevent the disease.[2]

In order to evaluate the role of single Th cell subsets in T1D development, we stimulated peripheral blood mononuclear cells (PBMCs) and two Th subsets—memory $CD45RO^{+}$ and naive/resting $CD45RA^{+}$—with 21 overlapping peptides of autoantigen preproinsulin, and with proinsulin and insulin molecules. Cytokine secretion was quantified in the supernatants of the cultures and relative cytokine secretion per positive reaction calculated.

METHODS

*A total of 35 HLA-DRB1*04, DQB1*0302–positive individuals were analyzed*: 12 patients with T1D (median age 26 years, range 2–56 years; median duration of insulin treatment 6 months, range 1–12 months); 12 Ab^{+} school children without family history of T1D from the the Karlsburg type 1 diabetes risk study[3] (median age 20 years, range 8–24 years); and 11 healthy control subjects without family history of T1D (median 22 years, range 2–43 years).

Enrichment of $CD45RO^{+}$ memory and $CD45RA^{+}$ naive/resting Th cells from PBMC was performed in two steps using $CD4^{+}$ T cell isolation kit (Miltenyi Biotec, CA) and $CD45RA^{+}$ MicroBeads. Human proinsulin (kind gift of Lilly, Indianapolis, IN) and insulin (kind gift of Aventis, Frankfurt, Germany) were tested simultaneously in a modified proliferation assay described previously.[4,5] Proliferation assay has been modified for enriched Th cell subsets and is currently being evaluated in our laboratory as a part of the ongoing Second International Workshop for Standardization of T cell Assays.[6] Cells were stimulated with 21 overlapping preproinsulin peptides (16 amino acids long and 12 amino acids overlapping) and antigens (proinsulin, insulin) in 96-well microtiter plates for 5 days. IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10 were quantified in the supernatants of the cultures.

Cytokine measurements were performed using an antigen-capture ELISA from PharMingen (San Diego, CA). Spontaneous cytokine release—cells incubated under the same conditions but without the antigen—were subtracted from experimental values before they were included in the analysis. Relative cytokine release per posi-

a) PBMC

	TNFα	IFNγ	IL-2	IL-4	IL-5	IL-10
Ab+ individuals	++	++	+++$^{\circ\circ\circ\Delta\Delta}$	++++	+	nt
Patients with T1D	+++++$^{\Delta\Delta\Delta}$	+++++*	++	+++	+$^{\Delta\Delta\Delta***}$	+++++***
Controls	++++$^{\circ\circ}$	+	++	+++	+	++++

b) Memory Th cells (CD45RO)

	TNFα	IFNγ	IL-2	IL-4	IL-5	IL-10
Ab+ individuals	+	++	++	+++$^{\circ\circ\circ\Delta\Delta}$	+	+++
Patients with T1D	+++$^{\Delta}$	+	++	+++*	+$^{\Delta\Delta\Delta}$	++
Controls	+++++$^{***\circ}$	++++$^{***\circ\circ}$	+++++$^{***\circ\circ}$	+++	+$^{\circ\circ}$	++++$^{**\circ\circ}$

c) Naive/resting Th cells (CD45RA)

	TNFα	IFNγ	IL-2	IL-4	IL-5	IL-10
Ab+ individuals	+	++$^{\Delta\Delta\Delta}$	+	++	+++++$^{\circ\circ\circ\Delta}$	++
Patients with T1D	++++	++	++$^{\Delta\Delta}$	++	++***	++
Controls	+++++*	++++***	+++++$^{*\circ\circ}$	+++++*	+	++

FIGURE 1. Relative cytokine release per positive reaction. +++++ = maximal response (100%) for each cytokine in all three tested cell populations and in all three groups of individuals. Maximal cytokine response (224 pg/mL) per positive reaction for IFN-γ was found in PBMCs of the patients; for TNF-α in CD45RA cells of the controls, 66pg/mL; for IL-2 in CD45RO cells of the controls, 115 pg/mL; for IL-4 in CD45RA cells of the controls:, 64 pg/mL; for IL-5 in CD45RA cells of Ab^+ individuals, 570 pg/mL; and for IL-10 in PBMCs of the patients, 31 pg/mL. * = patients vs. controls; Δ = Ab^+ individuals vs. patients; o = Ab^+ individuals vs. controls. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; ****$P < 10^{-5}$; nt = not tested.

tive reaction was calculated by dividing cumulative response to 21 preproinsulin peptides and antigens for each cytokine by the number of positive reactions (≥1 pg/mL).

Statistical analyses were performed using the SPSS software package (SPSS GmbH Software, Munich, Germany). Differences in cytokine secretion were compared by the nonparametric Mann-Whitney U test for unpaired observations. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The major finding of the current study is Th2 dominance of Th cell response to preproinsulin in Ab^+ individuals: a higher IL-4 level was found in $CD45RO^+$ memory Th cells and a higher IL-5 level in $CD45RA^+$ naive/resting Th cells when com-

pared to both the patients and controls (FIG. 1). It is conceivable that IL-4- and IL-5-dominated responses of Th cells—in early prediabetes—are critical for the sustenance of antibody production. Interestingly, in PBMCs of Ab^+ individuals an increase in IL-2 response was present that was not found in memory and naive/resting cells of the same individuals. Dominance of the IL-2 response in PBMCs may aggravate proliferation of the autoreactive T cell pool in the prediabetic stage. Moreover, in the PBMCs of recently diagnosed T1D patients we found an increase in IFN-γ and IL-10 secretion. Taken together our results implicate specific roles for Th cell subsets in islet cell autoimmunity. They also implicate involvement of cell types other than Th cells (present in PBMCs) in T1D development. Indeed, in the NOD mouse model of T1D different types of immune cells, such as $CD8^+$ cells, NK cells, macrophages and B lymphocytes, are involved in the disease process.[7,8] Moreover, it has been shown that under certain conditions, "protective" Th2 responses may become destructive by activating typical Th1 effector mechanisms.[9] In conclusion, the cytokine patterns observed in peripheral circulation and in Th cell subpopulations of individuals with islet cell autoimmunity may prove useful in monitoring disease progression and in prevention trials.

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