High Frequency of Vα24$^+$ Vβ11$^+$ T-Cells Observed in Type 1 Diabetes

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OBJECTIVE — Natural killer T-cells (NKT cells) are believed to play an important role in the regulation of immune response, and a numerical and functional deficit of NKT cells has been reported to be associated with the pathogenesis of autoimmune diseases. Thus far, it has been shown that subjects with type 1 diabetes have a lower frequency of NKT cells than nondiabetic subjects. In this study, we measured the frequency of peripheral Vα24$^+$ Vβ11$^+$ T-cells, which include human NKT cells, in Japanese diabetic patients.

RESEARCH DESIGN AND METHODS — Peripheral blood samples were obtained from 164 Japanese diabetic patients and 67 healthy subjects. The diabetic patients were classified into four categories as follows: islet-associated autoantibody–positive (Ab$^+$) and –negative (Ab$^-$) classic type 1 diabetes, latent autoimmune diabetes in adults (LADA), and type 2 diabetes. We measured the frequency of peripheral Vα24$^+$ Vβ11$^+$ CD3$^+$ triple-positive cells.

RESULTS — Unexpectedly, a higher frequency of Vα24$^+$ Vβ11$^+$ T-cells was observed in Ab$^+$ and Ab$^-$ patients compared with LADA patients ($P = 0.0294$ and $P = 0.0021$), type 2 diabetic patients ($P < 0.0001$ and $P < 0.0001$), and healthy subjects ($P = 0.0046$ and $P = 0.0001$). Moreover, an inverse correlation between Vα24$^+$ Vβ11$^+$ T-cell frequency and disease duration was observed in Ab$^+$ ($\rho = -0.455$; $P = 0.0023$) and Ab$^-$ ($\rho = -0.432$; $P = 0.0162$) patients.

CONCLUSIONS — Our findings indicate that a high frequency of Vα24$^+$ Vβ11$^+$ T-cells is a unique finding in recent-onset classic type 1 diabetes, and measurement of Vα24$^+$ Vβ11$^+$ T-cell frequency may be useful to assess the disease activity of classic type 1 diabetes.

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Most type 1 diabetes is now considered to be a T-cell–mediated autoimmune disease. In the nonobese diabetic (NOD) mouse, which is an excellent model of type 1 diabetes, it was previously reported that a shift from a T-helper 2 (Th2)-dominant to a Th1-dominant T-cell response is correlated with the onset of type 1 diabetes (1). This shift might be associated with the failure of regulatory cells, possibly involving CD45RB^{low} CD4$^+$ cells (1), Th2 cells, and natural killer T-cells (NKT cells) (2).

NKT cells share characteristics of both conventional T-cells and NK cells. Some human NKT cells express an extremely restricted T-cell receptor (TCR) repertoire, including an invariant Vα24 chain preferentially paired with a Vβ11 chain, together with an NK cell receptor (NKR-P1A) (3–7). NKT cells specifically recognize the glycolipid α-galactosylceramide (α-GalCer), which is isolated as a natural product from marine sponges (8,9), and this recognition requires expression of major histocompatibility complex (MHC) class I–like molecule CD1d (10–12). Because of the capacity to produce large amounts of interferon-γ (IFN-γ) and interleukin (IL)-4 upon activation, these cells are believed to play an important role in the regulation of immune response (7,13,14).

A numerical and functional deficit of NKT cells (including Vα24$^+$ Vβ11$^+$ T-cells in humans) has been reported to be associated with the pathogenesis of autoimmune diseases in autoimmune-prone mice such as SJL mice (15) and MRL-lpr/lpr mice (16), and also in patients with systemic sclerosis (17,18), systemic lupus erythematosus (18,19), rheumatoid arthritis (18,20), and other autoimmune diseases (18). In NOD mice, a numerical and functional deficit of NKT cells is involved in the pathogenesis of diabetes through the breakdown of autoimmune regulatory systems (21–23), and the prevention of diabetes has been observed by adoptive transfer of CD4$^-$ CD8$^-$ TCR α/β$^+$ thymocytes, which are characteristic of mouse NKT cells (24). Type 1 diabetic patients were recently reported to have a lower frequency of NKT cells, which are deficient in IL-4 secretion (25,26). These findings indicate that the pathogenesis of type 1 diabetes, as well as other autoimmune diseases (18), is associated with a numerical and functional deficit of NKT cells.

In the present study, we measured the frequency of peripheral Vα24$^+$ Vβ11$^+$ T-cells, some of which are considered to be human NKT cells, in Japanese type 1 diabetic patients. Contrary to our expectations, a significantly higher frequency of Vα24$^+$ Vβ11$^+$ T-cells was observed in patients with classic type 1 diabetes compared with latent autoimmune diabetes in adults (LADA) (27), type 2 diabetes, and healthy subjects. Moreover, the frequency was inversely correlated with disease du-

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Abbreviations: α-GalCer, α-galactosylceramide; Ab$^+$, islet-associated autoantibody positive; Ab$^-$, islet-associated autoantibody negative; FITC, fluorescein isothiocyanate; GADA, glutamic acid decarboxylase antibody; IA2A, insulinoma-associated protein-2 antibody; IFN-γ, interferon-γ; IL, interleukin; IP-10, interferon-inducible protein-10; LADA, latent autoimmune diabetes in adults; MHC, major histocompatibility complex; NKT cells, natural killer T-cells; PC5, phycoerythrin-cyanine 5; PE, phycoerythrin; TCR, T-cell receptor; Th, T helper.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

ration in classic type 1 diabetes. These findings suggest that a higher frequency of peripheral $V\alpha24^+$ $V\beta11^+$ T-cells is a unique finding in recent-onset classic type 1 diabetes, but not in any other autoimmune diseases, and measurement of $V\alpha24^+$ $V\beta11^+$ T-cell frequency may be useful to assess the disease activity of classic type 1 diabetes.

RESEARCH DESIGN AND METHODS

Patients

Peripheral blood samples were obtained from 164 Japanese diabetic patients and 67 healthy subjects (39 men, 28 women; aged 40.5 ± 14.3 years) with informed consent for use in this study. The diabetic patients were classified into three categories: classic type 1 diabetes (n = 78; 41 men, 37 women; aged 40.6 ± 17.1 years; disease duration 4.0 ± 4.5 years; HbA_{1c} 8.4 ± 1.9%), LADA (27) (n = 26; 19 men, 7 women; aged 51.3 ± 12.1 years; disease duration 6.7 ± 5.6 years; HbA_{1c} 8.4 ± 1.9%), and type 2 diabetes (n = 60; 33 men, 27 women; aged 61.3 ± 10.1 years; disease duration 9.8 ± 6.7 years; HbA_{1c} 7.1 ± 1.4%). The diagnosis of classic type 1 diabetes was based on the criteria of the American Diabetes Association for type 1 diabetes, with pancreatic β-cell destruction as the primary cause of the disorder and a tendency toward ketoacidosis (28,29), irrespective of islet-associated autoantibody positivity. Briefly, type 1 diabetic patients were diagnosed on the basis of classic presentation of acute abrupt onset of symptoms (polyuria, polydipsia, body weight loss, and so on) with diabetic ketosis (or ketoacidosis) due to both sustained hyperglycemia and a defect of residual insulin secretion; in fact, serum C-peptide levels were 0.35 ± 0.07 ng/ml in classic type 1 diabetes near the time of diagnosis in this study. Moreover, we measured glutamic acid decarboxylase antibody (GADA) and/or insulinoma-associated protein-2 antibody (IA2A) as islet-associated autoantibodies, as previously described (30), and if either of the antibodies was detected, the patient was classified as islet-associated autoantibody positive. Therefore, classic type 1 diabetes consists of both islet-associated autoantibody–positive patients (Ab^+; n = 46) and –negative patients (Ab^-; n = 32). LADA patients were defined as adult diabetic patients who were positive for GADA and/or IA2A and who did not require insulin therapy for at least 12 months after the diagnosis of diabetes but who ultimately progressed to insulin dependence (27).

Immunostaining and flow cytometry

Heparinized peripheral blood (100 μl) was placed in 5-ml polystyrene round-bottom tubes (Becton Dickinson, Franklin Lakes, NJ). Each sample was incubated with 3 μl human phycoerythrin-cyanine 5 (PC5)-conjugated CD3 antibody (UCHT1, mouse IgG1; Immunotech, Marseille, France), 4 μl human fluorescein isothiocyanate (FITC)-conjugated Vα24 antibody (C15, mouse IgG1; Immunotech), and 4 μl human phycoerythrin (PE)-conjugated Vβ11 antibody (C21, mouse IgG2a; Immunotech) simultaneously on ice for 20 min. To exclude the possibility of nonspecific staining in this system, mouse IgG1-FITC (679.1Mc7; Immunotech) and mouse IgG2a-PE (U7.27; Immunotech) were used as isotype controls. After lysing erythrocytes, the samples were vortexed gently and incubated at room temperature for another 10 min. After centrifuging the tube at 450g for 5 min, the supernatant was removed, and the cells were washed with 0.1% BSA-PBS. The prepared cells were analyzed using a three-color flow cytometer, Epics Altra (Coulter). For enumeration of $V\alpha24^+$ $V\beta11^+$ $CD3^+$ triple-positive cells, at least 100,000 events were analyzed. After the lymphocytes were gated by forward scatter and side scatter, at least 25,000 counts of $CD3^+$ cells, which were identified by gating cells stained with human CD3 antibody-PC5, were analyzed. Then, we counted the number of $V\alpha24^+$ $V\beta11^+$ $CD3^+$ triple-positive cells. The frequency of $V\alpha24^+$ $V\beta11^+$ $CD3^+$ triple-positive cells was shown as a percentage of $CD3^+$ cells analyzed.

Statistical analysis

Results are presented as means ± SE. Differences in $V\alpha24^+$ $V\beta11^+$ T-cell frequency were analyzed using the Bonferroni/Dunn test or Mann-whitney U test for nonparametric unpaired observations. Differences in patient age were analyzed using the Kruskal-Wallis test for nonparametric unpaired observations. The correlation between $V\alpha24^+$ $V\beta11^+$ T-cell frequency and disease duration (or age) was analyzed by Spearman's rank-order correlation test. Multiple regression analysis was used to evaluate the association among $V\alpha24^+$ $V\beta11^+$ T-cell frequency, disease duration, and patient age.

RESULTS

High frequency of $V\alpha24^+$ $V\beta11^+$ T-cells observed in type 1 diabetes

NKT cells are now considered to be phenotypically diverse and complicated (5,31); therefore, it is difficult to accurately define the phenotype of NKT cells. Recently, some investigators reported that human NKT cells express a restricted TCR repertoire including an invariant Vα24 paired with Vβ11 (5,18,31–33), and they clonally expanded both in vivo (34) and in vitro in the presence of α-GalCer (35–37). Moreover, Poulton et al. (32) recently demonstrated that the combination of Vα24 antibody and Vβ11 antibody is an excellent surrogate NKT cell marker, comparable to CD1d tetramers loaded with α-GalCer. Thus, in this study, we measured the frequency of peripheral $V\alpha24^+$ $V\beta11^+$ $CD3^+$ triple-positive cells as a representative of human NKT cells. The frequency was expressed as percentage of $CD3^+$ cells analyzed.

Unlike the findings of several recent studies (21,22,25), it is intriguing that the frequency of $V\alpha24^+$ $V\beta11^+$ T-cells in the peripheral blood of Japanese type 1 diabetic patients overall (including classic type 1 diabetes and LADA, 0.112 ± 0.012%) was significantly higher than that in patients with type 2 diabetes (0.041 ± 0.006%; $P < 0.0001$) and healthy subjects (0.065 ± 0.009%; $P = 0.0019$). We then divided type 1 diabetic patients into the following two groups: classic type 1 diabetes (both Ab^+ and Ab^-) and LADA. There was a significantly higher frequency of $V\alpha24^+$ $V\beta11^+$ T-cells in classic type 1 diabetes (0.127 ± 0.015%) than in LADA patients (0.066 ± 0.010%; $P = 0.0043$), type 2 diabetic patients ($P < 0.0001$), and healthy subjects ($P < 0.0001$). However, there were no significant differences among LADA patients, type 2 diabetic patients, and healthy subjects. A difference in the frequency of $CD3^+$ cells among the groups was not observed (data not shown).

Moreover, we divided classic type 1 diabetic patients into the following two

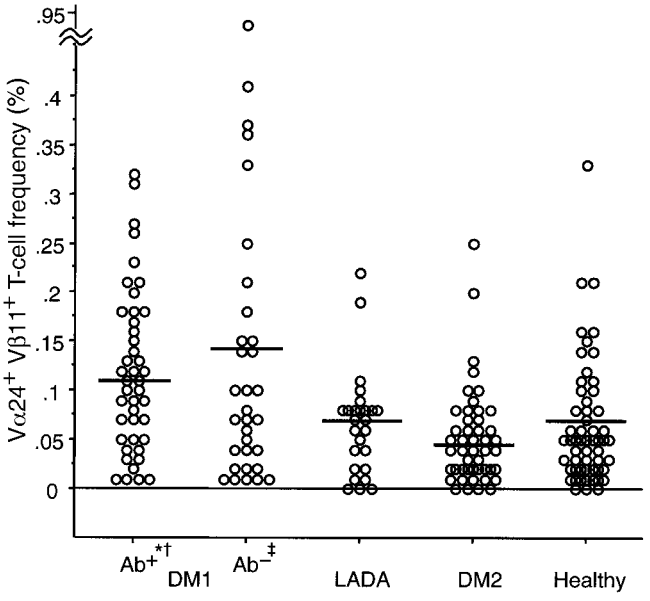

Figure 1—*Peripheral Vα24⁺ Vβ11⁺ T-cell frequency in classic type 1 diabetes (DM1), LADA, type 2 diabetes (DM2), and healthy subjects. Peripheral Vα24⁺ Vβ11⁺ T-cells were increased in Ab⁺ and Ab⁻ type 1 diabetic patients compared with LADA patients, type 2 diabetic patients, and healthy subjects. Vertical lines indicate mean of Vα24⁺ Vβ11⁺ T-cell frequency. *P < 0.005 (vs. type 2 diabetic patients and healthy subjects), †P < 0.03 (vs. LADA patients), ‡P < 0.003 (vs. LADA patients, type 2 diabetic patients, and healthy subjects).*

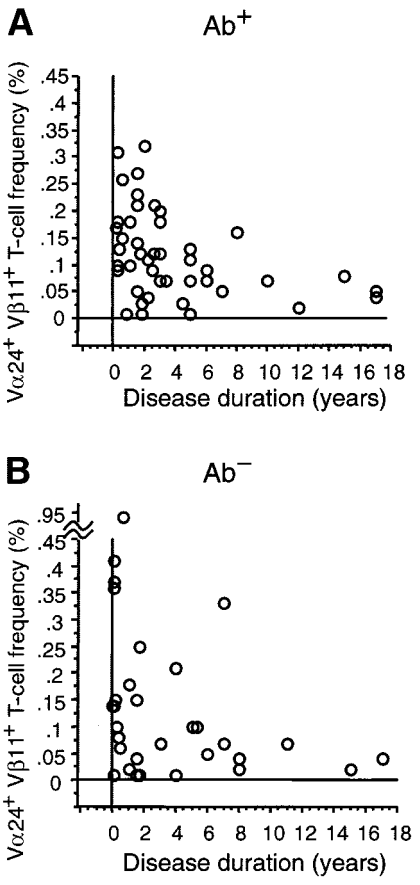

Figure 2—*Correlation between Vα24⁺ Vβ11⁺ T-cell frequency and disease duration in Ab⁺ and Ab⁻ classic type 1 diabetes. An inverse correlation between Vα24⁺ Vβ11⁺ T-cell frequency and disease duration was observed in each Ab⁺ (ρ = −0.455, P = 0.0023) and Ab⁻ (ρ = −0.432, P = 0.0162) classic type 1 diabetic patient.*

groups: islet-associated autoantibody (GADA and/or IA2A) positive (Ab⁺; n = 46) and negative (Ab⁻; n = 32). As shown in Fig. 1, a higher frequency of Vα24⁺ Vβ11⁺ T-cells was similarly observed in the Ab⁺ (0.116 ± 0.012%) and Ab⁻ (0.142 ± 0.033%) groups compared with LADA patients (P = 0.0294 and P = 0.0021, respectively), type 2 diabetic patients (P < 0.0001 and P < 0.0001, respectively), and healthy subjects (P = 0.0046 and P = 0.0001, respectively). However, there was no significant difference between Ab⁺ and Ab⁻ classic type 1 diabetic patients. Thus a higher frequency of peripheral Vα24⁺ Vβ11⁺ T-cells was observed in classic type 1 diabetes, irrespective of the positivity of islet-associated autoantibodies. To assess the reproducibility of our assay, the measurement of Vα24⁺ Vβ11⁺ T-cell frequency was repeated at an interval of 3–6 months in the same subjects and then was compared with the first result. As previously reported by Poulton et al. (32), there was no significant difference in the Vα24⁺ Vβ11⁺ T-cell frequency at such an interval, irrespective of metabolic controls (blood glucose level and glycohemoglobin) (data not shown).

Correlation between Vα24⁺ Vβ11⁺ T-cell frequency and disease duration in classic type 1 diabetes

It is generally considered that higher disease (insulitis) activity is expected in type 1 diabetes with shorter disease duration (38), because a severe β-cell mass reduction is suspected in cases of established (long-standing) type 1 diabetes. To assess whether the frequency of Vα24⁺ Vβ11⁺ T-cells in peripheral blood reflects disease activity in type 1 diabetes, we investigated the correlation between Vα24⁺ Vβ11⁺ T-cell frequency and disease duration. Classic type 1 diabetic patients were divided into the following four groups: recent-onset (disease duration <3 years) Ab⁺ (n = 26), recent-onset Ab⁻ (n = 19), established (disease duration ≥3 years) Ab⁺ (n = 20), and established Ab⁻ (n = 13). We previously reported that a significant difference of serum interferon-inducible protein-10 (IP-10) level, which presumably reflects the disease (insulitis) activity in type 1 diabetes, was observed between disease duration <3 years and ≥3 years in type 1 diabetes (38). As a result, recent-onset Ab⁺ patients showed a significantly higher Vα24⁺ Vβ11⁺ T-cell frequency than established Ab⁺ patients (0.143 ± 0.017% vs. 0.081 ± 0.013%; P = 0.0075), and recent-onset Ab⁻ patients also showed a significantly higher Vα24⁺ Vβ11⁺ T-cell frequency than established Ab⁻ patients (0.191 ± 0.050% vs. 0.072 ± 0.023%; P = 0.0474). Moreover, an inverse correlation between Vα24⁺ Vβ11⁺ T-cell frequency and disease duration was observed in Ab⁺ ($\rho = -0.455$; P = 0.0023) and Ab⁻ ($\rho = -0.432$; P = 0.0162) classic type 1 diabetes (Fig. 2), but not in LADA or type 2 diabetes.

When we combined the data of Vα24⁺ Vβ11⁺ T-cell frequency in Ab⁺ and Ab⁻ classic type 1 diabetic patients and similarly analyzed them, recent-onset (Ab⁺ and Ab⁻ combined) classic type 1 diabetic patients showed a significantly higher Vα24⁺ Vβ11⁺ T-cell frequency than those with established (Ab⁺ and Ab⁻ com-

Table 1—*Multiple regression analysis of relation among Vα24$^+$ Vβ11$^+$ T-cell frequency, disease duration, and age in classic type 1 diabetes*

Variable	Parameter	Standard coefficient	*t* value	Significance (*P*)	*r*
Vα24$^+$ Vβ11$^+$ T-cell frequency	Disease duration	−0.311	−2.799	0.0066	0.341 ($P = 0.0109$)
	Age	−0.103	−0.930	NS	

bined) classic type 1 diabetes (0.165 ± 0.022% vs. 0.069 ± 0.011%; $P < 0.0001$), LADA patients ($P < 0.0001$), type 2 diabetic patients ($P < 0.0001$), and healthy subjects ($P < 0.0001$). Similarly, an inverse correlation between Vα24$^+$ Vβ11$^+$ T-cell frequency and disease duration was observed in (Ab$^+$ and Ab$^-$ combined) classic type 1 diabetes ($\rho = -0.423$; $P = 0.0002$). Thus, irrespective of the positivity of islet-associated autoantibodies, a higher frequency of Vα24$^+$ Vβ11$^+$ T-cells was observed in recent-onset classic type 1 diabetes.

To assess whether Vα24$^+$ Vβ11$^+$ T-cell frequency was affected by patient age, the frequency was also compared with age. There was a significant difference in age among the four groups (classic type 1 diabetes, LADA, type 2 diabetes, and healthy subjects; $P < 0.0001$). Although there was a tendency for an inverse correlation between Vα24$^+$ Vβ11$^+$ T-cell frequency and patient age in Ab$^+$ and Ab$^-$ classic type 1 diabetes ($\rho = -0.294$, $P = 0.0108$), multiple linear regression analysis identified disease duration, rather than age, as a significant independent parameter determining the frequency (Table 1). Moreover, although there was a tendency for an inverse correlation between frequency and age in healthy subjects ($\rho = -0.286$), the correlation was very weak and was considered statistically meaningless. In another report (18), there was no correlation between Vα24$^+$ Vβ11$^+$ T-cell frequency and age. Accordingly, we believe that we can ignore the effect of age on Vα24$^+$ Vβ11$^+$ T-cell frequency in the present study. These results suggest that Vα24$^+$ Vβ11$^+$ T-cell frequency may be specifically associated with disease (insulitis) activity in classic type 1 diabetes.

CONCLUSIONS — It has been reported that a numerical and functional deficit of NKT cells (including Vα24$^+$ Vβ11$^+$ T-cells in humans) may be associated with the pathogenesis of type 1 diabetes (25,26). On the other hand, we demonstrated here the striking finding that peripheral Vα24$^+$ Vβ11$^+$ T-cells, some of which are considered to be human NKT cells, were significantly increased in Japanese type 1 diabetes, especially in recent-onset classic type 1 diabetes irrespective of islet-associated autoantibody positivity. It seems that our results conflict with the previous reports by Wilson et al. (25) and Kukreja et al. (26). Regarding the report by Wilson et al., a difference in onset age (child or adult) and/or disease duration, neither of which was described in their report (25), could account for the discrepancy between their report and ours, although we cannot exclude the possibility of a difference in genetic background and the definition of NKT cell markers between the two reports. Moreover, they focused on selected subjects, i.e., twin/triplet cases, and NKT cell frequencies in "usual" type 1 diabetic patients and the general population were not compared in their study.

Similarly, regarding the report by Kukreja et al. (26), there is the possibility that a difference in onset age and/or genetic background (Caucasian versus Japanese) could account for the discrepancy between their report and ours. Indeed, the newly diagnosed type 1 diabetic patients reported by Kukreja et al. were very young (9.4 ± 2.16 years old) compared with our newly diagnosed type 1 diabetic patients (35.2 ± 13.1 years old). Therefore, at least age should be matched; analysis of children should be done to address this question. Moreover, further assessment of the Vα24$^+$ Vβ11$^+$ T-cell population using other useful NKT cell-associated markers and cytokine profile of Vα24$^+$ Vβ11$^+$ T-cells are necessary to determine the reason for the discrepancies between their reports and ours.

Although we did not evaluate the cytokine profile of Vα24$^+$ Vβ11$^+$ T-cells in the present study, we speculate that increased NKT cells (probably including Vα24$^+$ Vβ11$^+$ T-cells), which would have an extreme Th1 phenotype in diabetic subjects as shown by Wilson et al. (25), may produce IFN-γ upon activation and stimulate diabetogenic lymphocytes to promote the destruction of pancreatic β-cells. On the other hand, considering that NKT cells were originally recognized as regulatory cells, we can also speculate that these cells may be secondarily increased to regulate the progression of recent-onset classic type 1 diabetes. Although measurement of Vα24$^+$ Vβ11$^+$ T-cell frequency is believed to be useful as a marker of cellular immunity in type 1 diabetes, further studies are necessary to confirm the significance of increased Vα24$^+$ Vβ11$^+$ T-cells in the development of type 1 diabetes.

Recently, we reported a correlation between levels of serum IP-10 (a chemokine that is considered to cause migration of activated Th1-type T-cells to the local lesion) and disease (insulitis) activity in type 1 diabetes (38). However, there has been no general consensus on a useful marker to assess disease activity of type 1 diabetes thus far. We showed here a high frequency of Vα24$^+$ Vβ11$^+$ T-cells in recent-onset classic type 1 diabetes, and the Vα24$^+$ Vβ11$^+$ T-cell frequency was inversely correlated with disease duration. These observations raise the possibility that the increased Vα24$^+$ Vβ11$^+$ T-cells may reflect active disease (or insulitis) status in classic type 1 diabetes. To confirm this possibility, it is necessary to assess the relationship between the severity of insulitis in the human pancreas and Vα24$^+$ Vβ11$^+$ T-cell frequency in the peripheral blood of type 1 diabetic patients in a future study. Interestingly, we found no direct correlation between Vα24$^+$ Vβ11$^+$ T-cell frequency and serum IP-10 level (data not shown), suggesting that the two factors can be used as independent markers of cellular immunity in type 1 diabetes.

Although we attempted to assess the relationship between Vα24$^+$ Vβ11$^+$ T-cell frequency and serum C-peptide, there was no correlation between the two (data not shown). Considering that type 1 diabetes progresses as an inflammatory β-cell dysfunction without actual β-cell destruction until late in the disease process (1), a low level of C-peptide does not necessarily reflect β-cell destruction (or disease [insulitis] activity), and at times may reflect β-cell dysfunction. Therefore, although no correlation between Vα24$^+$ Vβ11$^+$ T-cell frequency and C-peptide was observed in the present study, this

negative finding does not refute our reports on the relationship between $V\alpha24^+$ $V\beta11^+$ T-cell frequency and disease (insulitis) activity, and rather $V\alpha24^+$ $V\beta11^+$ T-cell frequency may be a useful disease activity marker independent of C-peptide value.

Because there is no report of an increase in $V\alpha24^+$ $V\beta11^+$ T-cells in other autoimmune diseases in humans thus far, we would like to emphasize that a high $V\alpha24^+$ $V\beta11^+$ T-cell (partially including human NKT cell) frequency in peripheral blood is a unique finding in recent-onset classic type 1 diabetes and is not seen in any other autoimmune diseases (18). We observed no increase in the frequency of this subset in patients with other autoimmune diseases such as rheumatoid arthritis or Grave's disease (data not shown). These findings will contribute greatly to understanding the roles of $V\alpha24^+$ $V\beta11^+$ T-cells in autoimmune diseases and to diagnosing and evaluating disease activity in type 1 diabetes.

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